Klf4 reverts developmentally programmed restriction of ground state pluripotency

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Mouse embryonic stem (ES) cells derived from pluripotent early epiblast contribute functionally differentiated progeny to all foetal lineages of chimaeras. By contrast, epistem cell (EpiSC) lines from post-implantation epithelialised epiblast are unable to colonise the embryo even though they express the core pluripotency genes *Oct4*, *Sox2* and *Nanog*. We examined interconversion between these two cell types. ES cells can readily become EpiSCs in response to growth factor cues. By contrast, EpiSCs do not change into ES cells. We exploited *PiggyBac* transposition to introduce a single reprogramming factor, Klf4, into EpiSCs. No effect was apparent in EpiSC culture conditions, but in ground state ES cell conditions a fraction of cells formed undifferentiated colonies. These EpiSC-derived induced pluripotent stem (Epi-iPS) cells activated expression of ES cell-specific transcripts including endogenous *Klf4*, and downregulated markers of lineage specification. X chromosome silencing in female cells, a feature of the EpiSC state, was erased in Epi-iPS cells. They produced high-contribution chimaeras that yielded germline transmission. These properties were maintained after Cre-mediated deletion of the *Klf4* transgene, formally demonstrating complete and stable reprogramming of developmental phenotype. Thus, re-expression of Klf4 in an appropriate environment can regenerate the naïve ground state from EpiSCs. Reprogramming is dependent on suppression of extrinsic growth factor stimuli and proceeds to completion in less than 1% of cells. This substantiates the argument that EpiSCs are developmentally, epigenetically and functionally differentiated from ES cells. However, because a single transgene is the minimum requirement to attain the ground state, EpiSCs offer an attractive opportunity for screening for unknown components of the reprogramming process.

KEY WORDS: Induced pluripotent stem (iPS) cell, Chimaera, Leukaemia inhibitory factor (Lif), Reprogramming, Mitogen-activated protein kinase (Erk) kinase (Mek/Mkk), Embryonic stem (ES) cell

INTRODUCTION

In the mouse, pluripotent stem cell lines can be established from two distinct phases of early development (Rossant, 2008). Embryonic stem (ES) cells are obtained from naïve epiblast in pre-implantation blastocysts (Batlle-Morera et al., 2008; Brook and Gardner, 1997; Evans and Kaufman, 1981; Martin, 1981). Epistem cells (EpiSCs) are derived from columnar epithelial epiblast of the early post-implantation embryo (Brons et al., 2007; Tesar et al., 2007). ES cells retain the character of early epiblast and can be incorporated into the host embryo when injected into blastocysts (Gardner and Rossant, 1979). They subsequently contribute to all lineages of the developing and adult mouse (Beddington and Robertson, 1989; Bradley et al., 1984; Gardner and Rossant, 1979; Nagy et al., 1993). By contrast, neither freshly isolated post-implantation epiblast cells nor EpiSCs are capable of functional colonisation of a host blastocyst (Rossant et al., 1978; Tesar et al., 2007).

Both ES cells and EpiSCs are capable of multilineage differentiation in vitro and can form teratomas when grafted into adult mice (Brons et al., 2007; Tesar et al., 2007). Both cell types express the three transcriptional regulators, Oct4 (Pou5f1 – Mouse Genome Informatics), Sox2 and Nanog, that are generally considered to constitute the core pluripotency network (Boyer et al., 2005; Loh et al., 2006; Wang et al., 2006). However, there are significant differences in gene expression between ES cells and EpiSCs (Tesar et al., 2007). Furthermore, the culture conditions for maintaining the two cell types are quite distinct. ES cells self-renew in response to the cytokine leukaemia inhibitory factor (Lif) (Smith et al., 1988; Williams et al., 1988) and either serum, bone morphogenetic protein, or the inhibition of Mek/Erk signalling (Burdon et al., 1999; Ying et al., 2003; Ying et al., 2008). They are driven into differentiation by FGF/Erk signalling (Kunath et al., 2007; Stavridis et al., 2007). EpiSCs, by contrast, are maintained by FGF and activin (Brons et al., 2007).

The molecular basis for restriction of egg cylinder epiblast and EpiSCs as compared with naïve epiblast and ES cells is presently unclear. This issue acquires added significance in light of evidence that human embryo-derived stem cells are more akin to EpiSCs than to ground state ES cells (Brons et al., 2007; Rossant, 2008; Tesar et al., 2007). Here, we report on requirements for the interconversion of mouse ES cells and EpiSCs.

MATERIALS AND METHODS

EpiSC derivation and culture

EpiSCs were derived from E5.75 mouse embryos using activin A (20 ng/ml) and Fgf2 (12 ng/ml) essentially as described (Brons et al., 2007), except that we employed N2B27 medium (Ying and Smith, 2003). OE cell lines were derived from F1 embryos carrying an Oct4GiP (eGFPiresPuro) transgene (Ying et al., 2002). EpiSCs were also derived from non-transgenic strain 129 embryos. Cells were used between 10 and 25 passages. Differentiated cells could be eliminated as required from OE cultures by puromycin (1 μg/ml) selection for expression of the Oct4GiP transgene.

Embryonic stem cell and induced pluripotent stem (iPS) cell culture

2i/Lif comprises the Mek inhibitor PD0325901 (1 μM), the Gsk3 inhibitor CHIR99021 (3 μM), and leukaemia inhibitory factor (Lif, 100 U/ml) in N2B27 medium (Ying et al., 2008). Cultured cells were expanded by dissociation with trypsin and replating every 3 days.

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ES cells overexpressing Klf4 were generated by electroporation of Oct4βgeo reporter cells (IOUD2) (Burdon et al., 1999) with a pPyCAGKlf4iP construct followed by puromycin selection (1 μg/ml). For ES cell to EpiSC differentiation, cells were plated at a density of $1\text{-}2\times10^4$ per cm^2 in fibronectin-coated plates. Twenty-four hours after plating, the medium was changed to N2B27 containing activin A and Fgf2. Thereafter, cells were maintained in EpiSC culture conditions and passaged every 2-3 days. For colony formation, 1000 cells were plated per well in fibronectin-coated 6-well plates in activin A/Fgf2, or in laminin-coated plates in 2i/Lif. After 6 days, colonies were fixed and stained for alkaline phosphatase. Colonies were scored using ImageJ software.

PiggyBac vector transfection

To establish PiggyBac (PB) transgenic EpiSC lines, 1×10^6 cells were co-transfected using Lipofectamine 2000 (Invitrogen) with 1 μg of pGG137Klf4 or control pGG131 vector plus 2-3 μg of the PBase-expressing vector pCAGPBase (Wang et al., 2008). Transfection efficiency was evaluated by flow cytometry for DsRed expression. To select for stable transfectants, hygromycin (200 μg/ml) was applied for at least 5 days. To delete transgenes, 1×10^5 cells were transfected with 1 μg of Cre expression plasmid using Lipofectamine 2000. Five days after transfection, DsRed-negative cells were purified and individually deposited into a 96-well plate using a MoFlo high-performance cell sorter (DakoCytomation). After expansion, genomic PCR was employed to identify revertants lacking the *Klf4* transgene. RT-PCR was used to confirm the lack of *Klf4* transgene and of DsRed expression.

iPS cell induction and propagation

EpiSCs, either stable transfectants isolated after hygromycin selection or cells immediately after transfection, were plated at a density of 1×10^4, 5×10^4 and 1×10^5 cells per well of 6-well tissue culture plates in EpiSC culture condition. After 24 hours, medium was replaced with that containing 2i/Lif and subsequently refreshed every other day. The number of Oct4-GFP-positive clones was manually counted using fluorescence microscopy. ES cell-like clones were picked after 14 days in 2i/Lif and subsequently expanded by Accutase (PAA Laboratories) dissociation and replating every 3-4 days.

RT-PCR

Total RNA was prepared using the RNeasy Mini Kit (Qiagen) with DNaseI treatment. First-strand cDNA was synthesised using Superscript III reverse transcriptase (Invitrogen). Unless specified otherwise, real-time PCR was performed using Taqman Gene Expression Assays (Applied Biosystems). Gene expression was determined relative to *Gapdh* using the ΔCt method. Expression of the *Klf4* transgene and of *DsRed* was determined by standard curve calibration. All quantitative PCR (qPCR) reactions were performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems).

Taqman probes

Oct4, Mm00658129_gH; *Klf4*, Mm00516104_m1; *Klf2*, Mm01244979_g1; *Klf5*, Mm00456521_m1; *Nanog*, Mm02384862_g1; *Rex1*, Mm03053975_g; *Fgf5*, Mm00438615_m1; *Lefty*, Mm00438615_m1; brachyury (*T*), Mm01318252_m1; *Nr0b1*, Mm00431729_m1; *Stella* (*Dppa3*), Mm00836373_g1; *Gapdh*, 4352339E; β-actin (*Actb*), 4352341E.

Chimaera production

Term chimaeras were produced by microinjection into C57BL/6 blastocysts. Selected female chimaeras were mated with C57BL/6J black males. Germline transmission from cultured cell-derived oocytes manifests in agouti offspring.

RESULTS AND DISCUSSION

We derived EpiSCs from E5.75 mouse embryos carrying the Oct4GiP transgene (Ying et al., 2002). Cell lines were established and maintained without feeders in serum-free N2B27 medium (Ying and Smith, 2003) supplemented with activin A and Fgf2 (bFGF) (Brons et al., 2007). They grew as monolayers of closely apposed cells on a fibronectin substrate (Fig. 1A). The majority of cells

expressed the Oct4-GFP reporter (Fig. 1A). Consistent with the original descriptions (Brons et al., 2007; Tesar et al., 2007), the EpiSCs we derived expressed the pluripotency markers *Oct4* and *Nanog*, but not the early epiblast marker *Stella* (*Dppa3*) (Fig. 1B). EpiSCs also differ from ES cells by upregulation of the post-implantation markers *Fgf5*, *T* (brachyury) and *Lefty* (see Fig. S1 in the supplementary material). We established both male and female EpiSC lines. Immunofluorescence revealed a prominent body of nuclear staining for the repressive histone modification trimethylated H3 lysine 27 (me3H3K27) in the female line (Fig. 1C). This is diagnostic of a silent X chromosome (Silva et al., 2003). Thus, an emphatic epigenetic distinction between early and late epiblast is conserved in ES cells and EpiSCs, respectively. This is reflected in a differential ability to colonise chimaeric embryos (Tesar et al., 2007). We found that after morula aggregation, Oct4GiP EpiSCs could mix with inner cell mass (ICM) cells in blastocysts, but that they quickly downregulated GFP. Consistent with this, no contribution was detectable in egg cylinders after embryo transfer (see Fig. S6 in the supplementary material).

EpiSCs also lose expression of Oct4 and differentiate when transferred to conventional mouse ES cell culture conditions (Brons et al., 2007). Recently, however, it has been established that small molecules that selectively inhibit the Mek/Erk MAP kinase signalling cascade and glycogen synthase kinase 3 (Gsk3) provide, in combination with Lif, an optimal environment for derivation and propagation of ES cells from different rodent backgrounds in serum-free medium (Buehr et al., 2008; Ying et al., 2008) (J.N., unpublished). The combination of two inhibitors with Lif (2i/Lif) also promotes the generation of iPS cells (Silva et al., 2008). We therefore tested whether EpiSCs cultured in 2i/Lif might acquire features of ground state pluripotency. However, after transfer into 2i/Lif, EpiSCs underwent massive differentiation and death such that Oct4-GFP-expressing cells were entirely eliminated by 3 days (Fig. 1D). Some differentiated cells persisted, but in multiple platings of 1×10^7 EpiSCs not a single Oct4-GFP-expressing colony was obtained. Since genetic background has a strong influence on the derivation and propagation of ES cells and on iPS cell generation (Batlle-Morera et al., 2008; Silva et al., 2008), we also examined EpiSCs from the permissive 129 strain. These EpiSCs also failed to survive in 2i/Lif (data not shown). We conclude that the EpiSC represents a stable cell state that does not naturally revert to naïve pluripotent status.

The origin of ES cells and EpiSCs from early and late epiblast, respectively, suggests that ES cells might be capable of becoming EpiSCs. Indeed, ES cells transferred into EpiSC culture conditions continued to proliferate. After passaging, cultures became relatively homogenous and EpiSC-like. Thereafter, they displayed the marker profile of EpiSCs rather than of ES cells, with maintained Oct4, reduced Nanog and downregulated *Rex1* (*Zfp42* – Mouse Genome Informatics), *Nr0b1* and *Klf4* (Fig. 1E; see Fig. S2 in the supplementary material). Furthermore, EpiSCs derived from female ES cells showed a coincidence of Oct4 expression and X chromosome inactivation (Fig. 1F). This signature distinguishes EpiSCs from ES cells and differentiated somatic cell types. To confirm that this ES cell-derived EpiSC state was truly differentiated, we transferred cells back into 2i/Lif. Occasional ES cell-like colonies could initially be recovered, but not after four or more passages in activin plus Fgf2 (data not shown). We conclude that ES cells differentiate into EpiSCs, although a minority of undifferentiated cells persists for a while, as is commonly observed in other in vitro ES cell differentiation schema (Lowell et al., 2006; Smith, 2001).

One of the genes prominently downregulated during differentiation of ES cells into EpiSCs is *Klf4*. Klf4 has been implicated in ES cell self-renewal (Jiang et al., 2008; Li et al., 2005). *Klf4* is induced by Lif/Stat3 signalling in ES cells, but not in EpiSCs (Fig. 2A). To test whether Klf4 might regulate the ES cell to EpiSC transition, we stably transfected ES cells with a Klf4 expression plasmid. These cells show greatly reduced dependency on Lif for self-renewal, as previously reported (Li et al., 2005). On transfer to EpiSC culture conditions, however, they responded similarly to parental ES cells, growing as a monolayer and downregulating ES cell-specific marker expression while maintaining Oct4 (Fig. 2B). This indicates that forced expression of Klf4 does not prevent

conversion into EpiSCs. However, even after ten passages in activin and Fgf2, ES cell colonies were obtained at low frequency upon transfer to 2i/Lif (Fig. 2C). Therefore, constitutive Klf4 either allows long-term persistence of a small fraction of undifferentiated ES cells, or enables a fraction of EpiSCs to dedifferentiate and regain the ground state.

To distinguish between these possibilities, we investigated whether forced expression of Klf4 in embryo-derived EpiSCs could induce ground state pluripotency. We used *PiggyBac* (*PB*) vector-chromosome transposition to achieve high efficiency stable transfection (Wang et al., 2008). The *PB* vector contains independent *CAG* promoter units directing expression of the *Klf4*
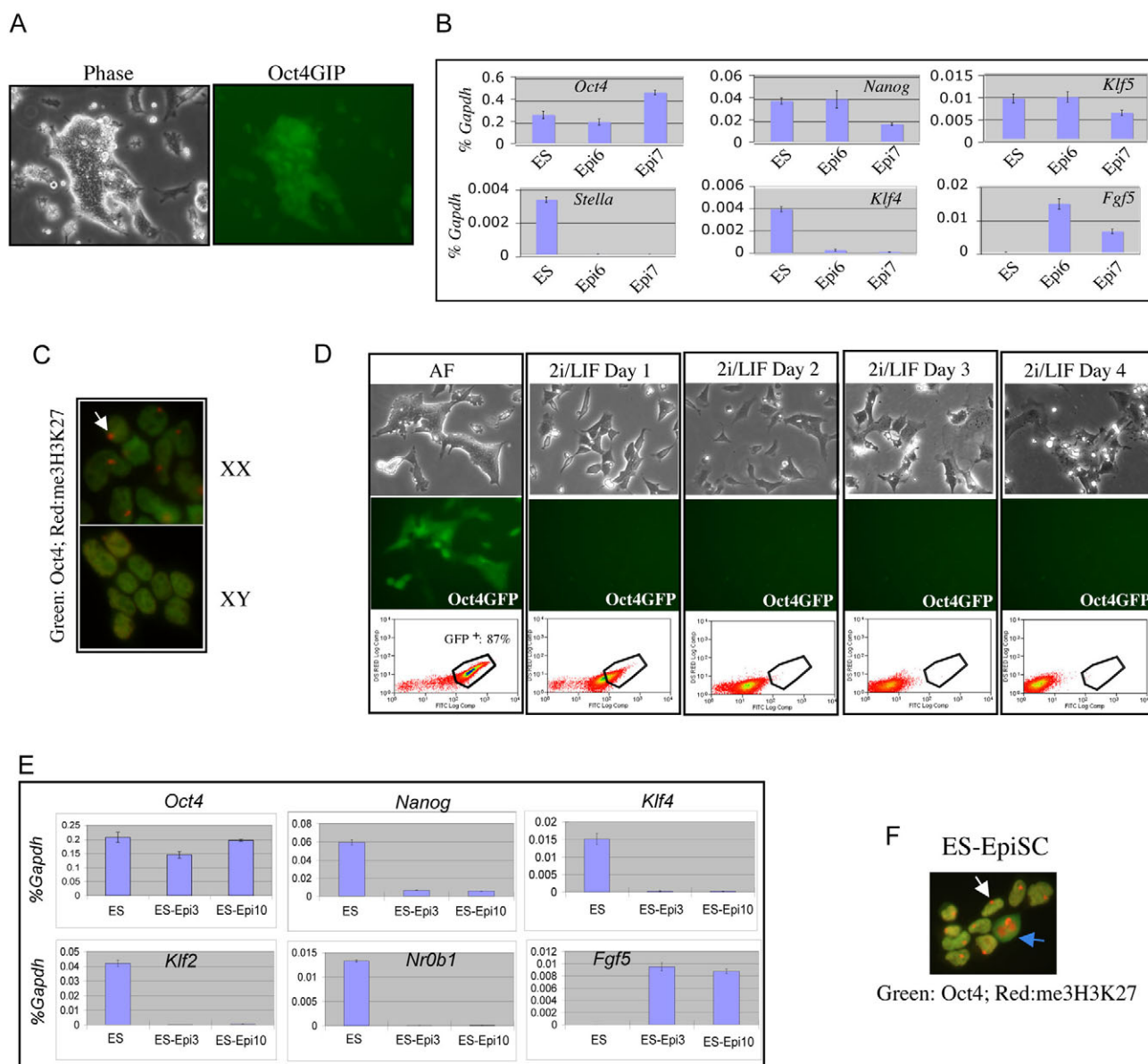
Fig. 1. EpiSCs are distinct from, and do not spontaneously convert to, ES cells. (**A**) Phase contrast and fluorescence images of established EpiSC line. (**B**) qRT-PCR analysis of marker gene expression in ES cells and EpiSCs. ES, ES cells in 2i/Lif. Epi6 and Epi7 are two independent EpiSC lines. *y*-axis, relative expression normalised to *Gapdh*. (**C**) Immunostaining of male and female EpiSCs for me3H3K27 and Oct4. White arrow indicates focus of staining diagnostic of an inactive X chromosome. (**D**) EpiSCs lose Oct4 expression and differentiate or die in 2i/Lif. AF, EpiSC cultured in activin A plus Fgf2. (**E**) qRT-PCR analysis of ES cell differentiation into EpiSCs upon culture in Fgf2 and activin. Epi3 and Epi10 indicate cells cultured in Fgf2 and activin for three and ten passages, respectively. *y*-axis, relative expression normalised to *Gapdh*. (**F**) Oct4 and me3H3K27 immunostaining of female ES cell-derived EpiSCs. EpiSCs both express Oct4 and exhibit a nuclear body indicative of the inactive X (white arrow). Blue arrow indicates a dividing cell.
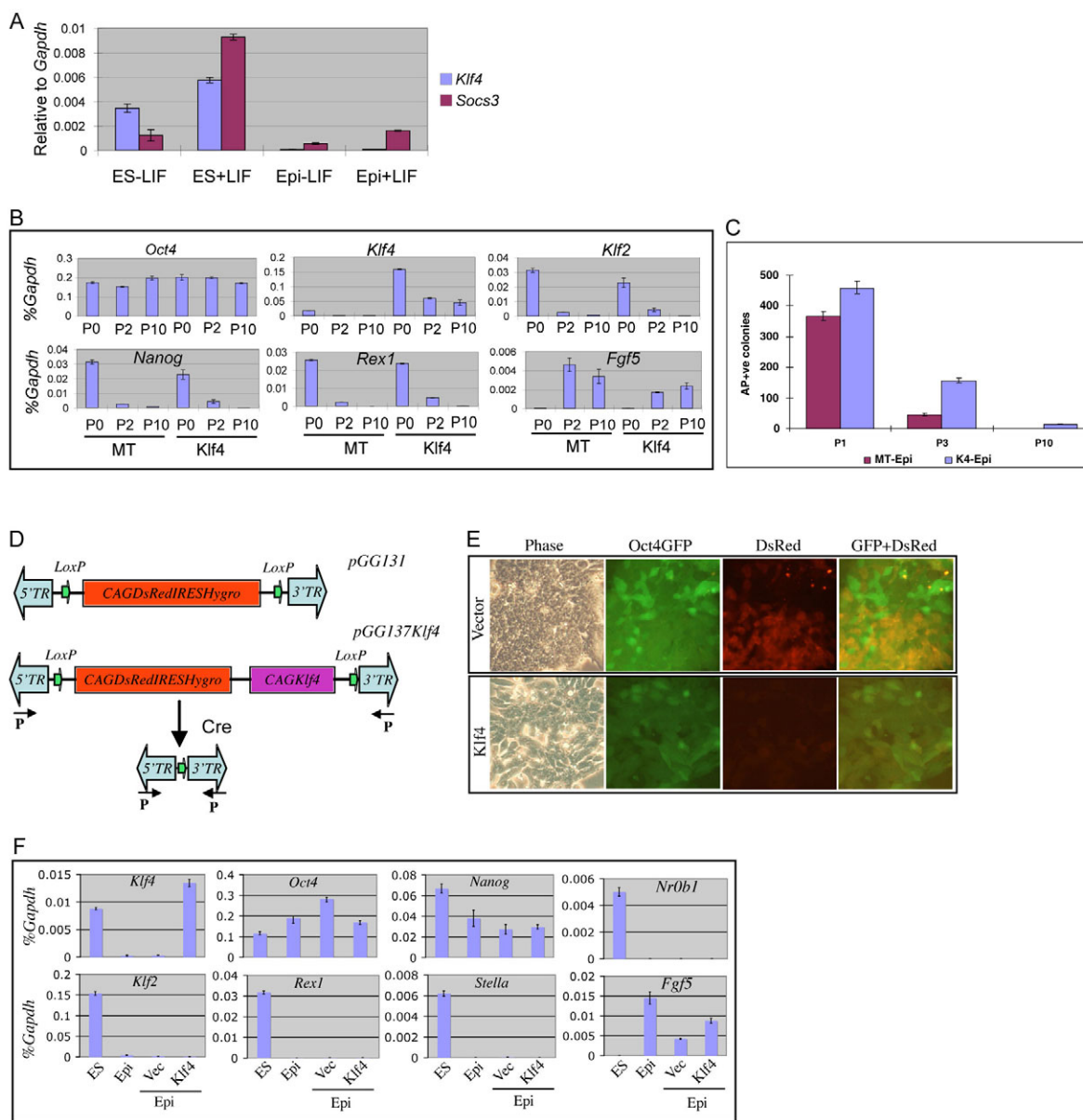
Fig. 2. Klf4 neither prevents ES cell differentiation into EpiSCs nor converts an EpiSC population into ES cells in the presence of activin and FGF. (**A**) qRT-PCR analysis of Lif induction of *Klf4* in ES cells but not in EpiSCs. Cells were stimulated with Lif (+LIF) for 1 hour. (**B**) ES cells constitutively expressing *Klf4* acquire an EpiSC marker profile in Fgf2 plus activin A. MT, empty vector transfectants. P0, P2 and P10 indicate passage numbers in Fgf2/activin. (**C**) Constitutive Klf4 expression permits continued recovery of ES cell colonies after culture in activin and Fgf2. One thousand cells were plated for each sample in triplicate at the indicated passage (P) number. MT, empty vector transfectants; K4, Klf4 transfectants. (**D**) *PiggyBac* vector for expression of *Klf4* (*pGG137Klf4*), and control *PiggyBac* vector (*pGG131*). Arrows (P) indicate PCR primers used to amplify the *PB LTR* fragment after Cre-mediated recombination. (**E**) Hygromycin-selected *Klf4* and control vector-transfected EpiSCs. (**F**) qRT-PCR analysis showing that forced *Klf4* expression does not induce ES cell marker gene expression in EpiSC culture. ES, ES cells; Epi, EpiSCs; Vec, EpiSC transfected with control vector *pGG131*; Klf4, EpiSCs transfected with *pGG137Klf4*. *y*-axis, relative expression normalised to *Gapdh*.

open reading frame and of a *DsRed* reporter with a linked hygromycin resistance gene (Fig. 2D). LoxP sites adjacent to the *PB* terminal repeats allow for excision of both expression units. Fewer DsRed-expressing cells were obtained after *Klf4* transfection than following control vector transfection (data not shown) and the level of DsRed expression was reduced. Overexpression of *Klf4* might therefore be toxic to EpiSCs. Nonetheless, *Klf4/DsRed*-expressing EpiSCs were isolated following hygromycin selection (Fig. 2E). In activin and Fgf2 they did not upregulate ES cell-specific genes (Fig. 2F) and female cells maintained an inactive X chromosome as

judged by me3H3K27 staining (see Fig. S3 in the supplementary material). We conclude that the expression of *Klf4* at a similar RNA level to that present in ES cells is not alone sufficient to reset EpiSCs and instate full pluripotency in cells maintained in activin and Fgf2.

We then examined the effect of Klf4 transfection in 2i/Lif conditions, which have been shown to promote the final stages of iPS cell generation from neural stem cells and fibroblasts (Silva et al., 2008). Klf4-transfected EpiSCs were transferred into 2i/Lif 48 or 72 hours after transfection. They exhibited a wave of differentiation and cell death, similar to non-transfected EpiSCs.
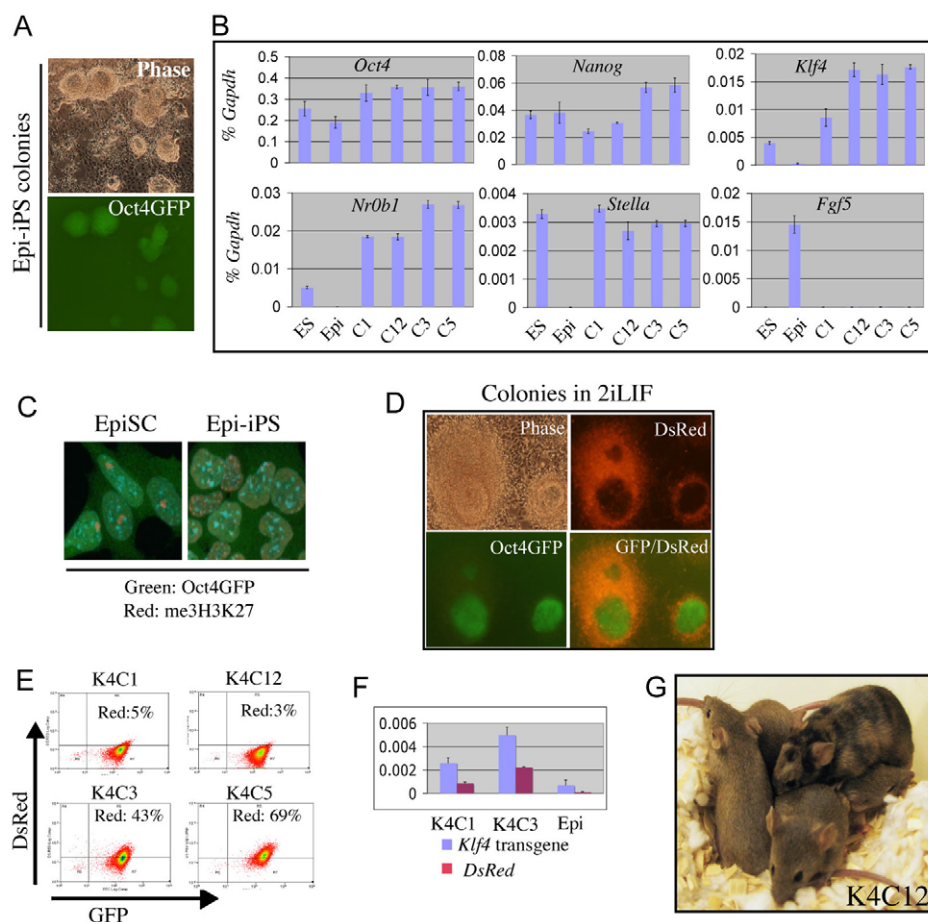
Fig. 3. EpiSCs transfected with Klf4 can convert to ground state pluripotency. (**A**) Oct4-positive colonies obtained by transfection with Klf4 and transfer to 2i/Lif after 72 hours. Images were taken after 9 days in 2i/Lif. (**B**) qRT-PCR analysis of marker gene expression in ES cells, EpiSCs and derivative Epi-iPS cells isolated in 2i/Lif. y-axis, relative expression normalised to *Gapdh*. (**C**) me3H3K27 staining of female EpiSCs and derivative Epi-iPS cells. (**D**) Images of Epi-iPS colonies after 10 days in 2i/Lif, showing mutually exclusive expression of DsRed and Oct4-GFP. (**E**) Flow cytometry analysis of four expanded Epi-iPS clones. Two clones retain weak but detectable red fluorescence. (**F**) qRT-PCR analysis of *Klf4* transgene and *DsRed* expression in Epi-iPS cell clones and parental EpiSC line. y-axis, relative expression normalised to *Gapdh*. (**G**) Chimeric mouse produced from the K4C12 Epi-iPS clone and agouti germline offspring.

After 4 days in 2i/Lif, however, multiple Oct4-GFP-positive colonies emerged (Fig. 3A). These colonies had the tightly packed three-dimensional aggregate form typical of ES cells in 2i. They arose at a frequency of 0.1-0.2% of cells surviving transfection (21 colonies from 1×10^4 cells in one typical experiment). GFP-positive colonies were never obtained in 2i/Lif from multiple control vector transfections. We then tested whether stably transfected EpiSCs propagated in activin and Fgf2 could convert in 2i/Lif. Klf4 transfectants generated colonies in 2i/Lif with similar kinetics to cells transferred directly after transfection (see Fig. S4 in the supplementary material). The yield was higher, at ~1%. This could point to some element of reprogramming proceeding in the stable transfectants, but could also be explained by elimination of non-transfectants and cells with toxic overexpression of Klf4. Significantly, the frequency did not noticeably increase with passaging of the stable transfectants, indicating that if there is any partial reprogramming this does not accumulate in the cultures.

Ten out of 12 colonies picked from a transfection with transfer to 2i/Lif after 72 hours expanded with undifferentiated morphology and stable expression of Oct4-GFP. qRT-PCR analysis showed the marker profile of ES cells, with upregulation of *Stella* and *Klf2*. Conversely, *Fgf5* and brachyury mRNAs were lost (Fig. 3B; see Fig.

S5 in the supplementary material). We examined me3H3K27 immunostaining and found that the nuclear body corresponding to the inactive X chromosome was lost in Oct4-GFP-positive cells after transfer to 2i/Lif (Fig. 3C). A potential disadvantage of using the *PB* vector and *CAG* promoter is that transgenes might not be silenced. However, in each of the GFP-positive clones we observed partial or complete loss of visible DsRed expression (Fig. 3D,E), although qRT-PCR analysis revealed that the transgenes were not completely silenced (Fig. 3F).

We injected cells without visible DsRed expression into C57BL/6 blastocysts. Healthy chimaeras were obtained with extensive agouti coat colour contributions (Fig. 3G). Female chimaeras mated with C57BL/6 males produced agouti offspring, indicating transmission of the cultured cell genome. This confirms that the developmental capacity has been fully derestricted and the authentic pluripotent state established. These cells should therefore be considered as EpiSC-derived iPS cells, or Epi-iPS cells.

We examined the copy number of *PB* integrations by genomic PCR analysis of ten Epi-iPS cell clones. Each Epi-iPS cell line carried 1-3 *PB* insertions (Fig. 4A). To determine whether the low, but still detectable, *Klf4* transgene expression might play a role in maintaining the induced phenotype we excised the transgene
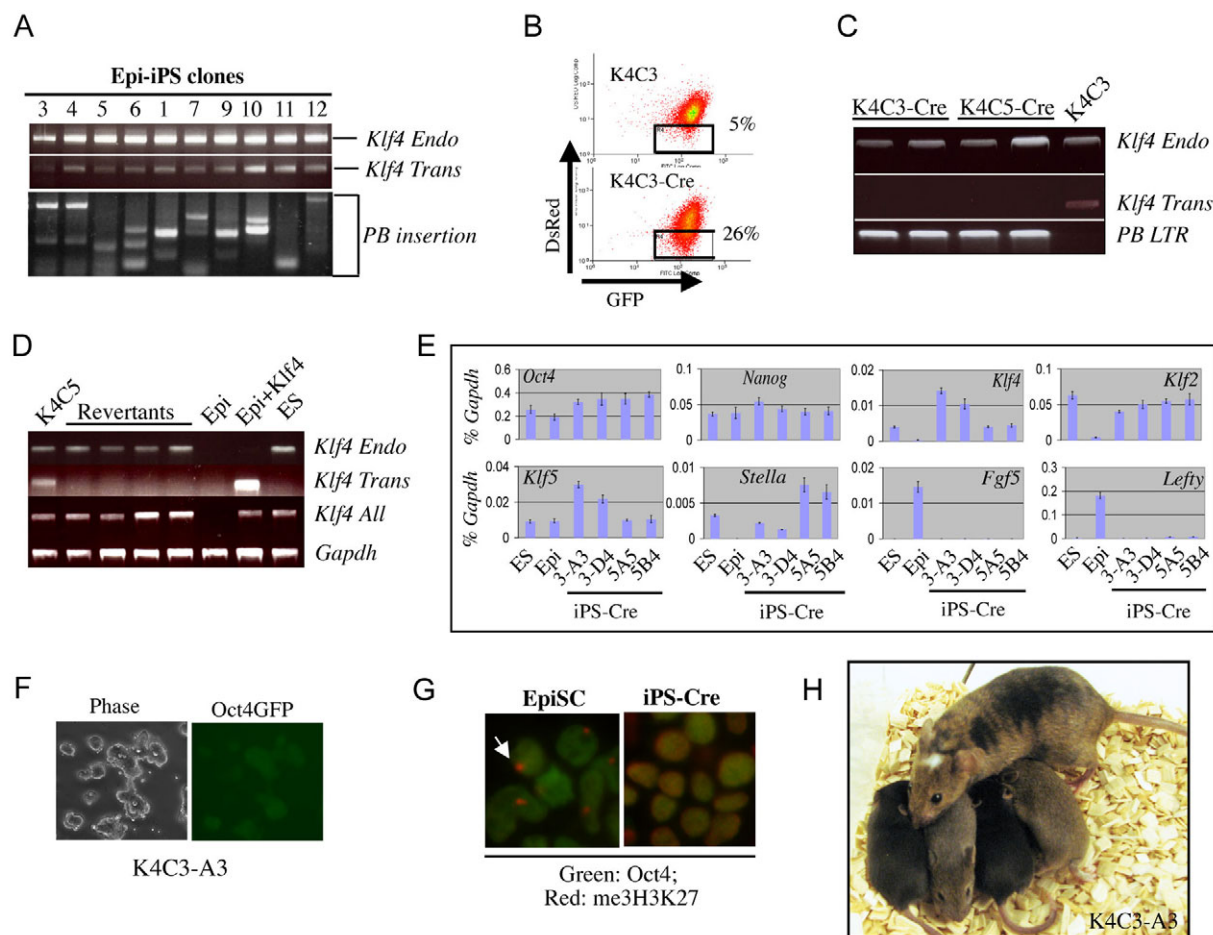
Fig. 4. Retention of ground state pluripotency after transgene excision. (**A**) Splinkerette-PCR reveals 1-3 *PB* insertions in each iPS clone. (**B**) Flow cytometry showing the DsRed-negative population in the K4C3 line before and after Cre transfection. (**C**) Genomic PCR showing loss of the *Klf4* transgene and gain of the *PB-LTR* fragment in two revertant clones. (**D**) RT-PCR analysis showing the lack of *Klf4* transgene and *DsRed* expression in expanded Cre-reverted cells. (**E**) Marker gene expression in Cre-reverted Epi-iPS cells (iPS-Cre) compared with ES cells and EpiSCs. Data are for two independent Cre-revertant clones derived from each of Epi-iPS cell lines K4C3 and K4C5. *y*-axis, relative expression normalised to *Gapdh*. (**F**) Maintained morphology and Oct4-GFP expression in a Cre-reverted Epi-iPS cell line. (**G**) me3H3K27 staining of *Klf4* transgene-deleted iPS cells as compared with parental EpiSCs. (**H**) Chimeric mouse made with revertant K4C3-A3 cells, and agouti offspring denoting germline transmission.

copies. We chose two DsRed-positive clones and transfected each with a Cre expression plasmid. After 5 days, cells that no longer expressed DsRed were isolated using flow cytometry with single-cell deposition into 96-well plates (Fig. 4B). Resulting clones were screened by genomic PCR for absence of the *Klf4* transgene and presence of a reverted *PB* fragment (Fig. 4C). Two thirds of the expanded clones retained only the *PB* terminal repeats. RT-PCR analysis failed to detect expression of the *Klf4* transgene or *DsRed* from these revertants (Fig. 4D). They retained ES cell morphology, Oct4-GFP expression and ES cell marker profile (Fig. 4E,F). The X chromosome silencing mark, me3H3K27, was undetectable (Fig. 4G). These cells incorporated efficiently into the ICM and subsequently the egg cylinder after morula aggregation (see Fig. S6 in the supplementary material). We injected transgene-deleted cells into blastocysts and obtained viable high-contribution chimaeras (see Table S1 in the supplementary material). Female chimaeras from two out of three clones produced agouti offspring in their first litter (Fig. 4H), indicative of transmission of iPS cell-derived oocytes. Therefore, complete removal of the *Klf4* transgene does not destabilise the

induced ground state. This establishes that reprogramming has been finalised and does not depend upon ongoing transgene expression or insertional mutagenesis.

The restricted potency of EpiSCs as compared with ES cells appears to mirror the developmental progression from naïve pre-implantation epiblast to epithelialised egg cylinder (Rossant, 2008). ES cells in vitro recapitulate this conversion with stable alterations in gene expression, growth factor dependence and epigenetic status. These differentiation changes may be completely reversed by re-expression of a single gene that is normally downregulated in EpiSCs. Klf4 in combination with culture in 2i/Lif reverts EpiSCs to the naïve ground state. This is mediated by transcriptional resetting accompanied by activation of contrasting epigenetic processes that restrict expression of exogenous DNA sequences and erase X chromosome inactivation. Interestingly, although colonies with features of iPS cells are detectable within 7 days of Klf4 transfection, all clones recovered showed stable integration of at least one copy of the transgene, suggesting a requirement for sustained expression to effect reprogramming. However, the combination of *PB*-mediated

low copy number integration and Cre-mediated excision facilitated formal proof that once attained, the Epi-iPS cell state does not require the continuous presence of introduced DNA elements (Okita et al., 2008; Stadtfeld et al., 2008).

It is apparent that the exogenous transcription factor requirements for inducing pluripotency vary with the starting cell type as do the efficiency and kinetics of molecular reprogramming (Aoi et al., 2008; Kim et al., 2008; Silva et al., 2008). It is striking, however, that even though other core components of the pluripotent network are already present, only ~1% of Klf4-expressing EpiSCs become iPS cells. This emphasises that even though there are transcriptional similarities, EpiSCs are truly differentiated from ground state ES cells. Their reprogramming efficiency is limited in an analogous manner to that of somatic cells by currently unknown parameters. However, because a single transgene is sufficient, we suggest that EpiSCs might provide an attractive system in which to screen for new components of the reprogramming process.

Finally, continuous expression of Klf4 does not prevent ES cell differentiation into EpiSCs when exposed to inductive extrinsic factors. Nonetheless, downregulation of Klf4 may help to ensure developmental restriction of epithelialised epiblast in the embryo and safeguard against dedifferentiation to a naïve and teratogenic condition. We suggest that the creation of iPS cells might be intimately related, mechanistically, to the molecular transitions through which ground state pluripotency is generated and then restricted in the early phases of mammalian embryogenesis.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/7/1063/DC1

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Table S1. Chimaera generation from *Klf4* Epi-iPS clones

Clone	Blastocysts injected	Number born	Number of chimaeras	Coat chimaerism			Germline transmission
				High	Medium	Low	
K4C1	18	1	1 (died)		1		N/A
K4C12	40	15	11	3	6	2	Two females, both 100%
K4C3Cre A3	30	6	4	4			One female, 50%; second female lost litter
K4C3Cre D4	30	5	2 (1 died)		1	1	Not tested
K4C5Cre A5	46	7 (4 died)	1		1		One female, 100%

EpiSCs are derived from hybrid 129×MF1 (Swiss albino) embryos. Chimaeras were generated in C57BL/6 blastocysts and bred with C57BL/6 males. Resultant non-black pups indicate germline transmission. The cell lines are female and therefore only female chimaeras were mated. K4C1 and K4C12 are Epi-iPS clones with the *Klf4* transgene; K4C3Cre and K4C5Cre clones are revertants of K4C3 and K4C5, respectively, after *Klf4* transgene deletion by Cre-mediated recombination. The percentages in the germline transmission column are of agouti pups (Epi-iPS cell-derived) in the first litters.