Heterokaryon-Based Reprogramming of Human B Lymphocytes for Pluripotency Requires Oct4 but Not Sox2

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Abstract

Differentiated cells can be reprogrammed through the formation of heterokaryons and hybrid cells when fused with embryonic stem (ES) cells. Here, we provide evidence that conversion of human B-lymphocytes towards a multipotent state is initiated much more rapidly than previously thought, occurring in transient heterokaryons before nuclear fusion and cell division. Interestingly, reprogramming of human lymphocytes by mouse ES cells elicits the expression of a human ES-specific gene profile, in which markers of human ES cells are expressed (hSSEA4, hFGF receptors and ligands), but markers that are specific to mouse ES cells are not (e.g., Bmp4 and LIF receptor). Using genetically engineered mouse ES cells, we demonstrate that successful reprogramming of human lymphocytes is independent of Sox2, a factor thought to be required for induced pluripotent stem (iPS) cells. In contrast, there is a distinct requirement for Oct4 in the establishment but not the maintenance of the reprogrammed state. Experimental heterokaryons, therefore, offer a powerful approach to trace the contribution of individual factors to the reprogramming of human somatic cells towards a multipotent state.

Introduction

Reprogramming somatic cells to become ES-like is an important goal in cell replacement therapy since it affords the opportunity to generate and use patient-specific ES derived cells as grafts. Epigenetic reprogramming can be achieved in different ways including nuclear transfer [1–4] or the forced expression of one or more transcription factors [5,6]. Retroviral-mediated expression of four transcriptional regulators, Oct4, Sox2, c-Myc and Klf4, was shown to drive mouse fibroblasts into an ES-like (iPS) state, albeit at low frequency [7,8]. Reprogramming of human fibroblasts has also recently been achieved in a parallel approach using Oct4, Sox2 and either Nanog plus Lin28 [9] or Klf4 plus c-Myc [10]. These pioneering studies have illustrated the importance of several factors for iPS, but also suggested that additional ones may be needed for efficient conversion to pluripotency. Reprogramming can also be achieved by cellular fusion, a process that occurs spontaneously in vitro [11], in vivo [12] and experimentally using specific agents [13]. For example, fusion of differentiated cells with pluripotent ES cells, embryonic carcinoma (EC) or embryonic germ (EG) cells, induces the expression of pluripotency-associated markers in the hybrid cells [14–18] and chromatin remodelling at specific sites in the somatic cell genome [14,15,18–21]. While these data show that reprogramming occurs through the epigenetic resetting of gene expression programs in the differentiated cell, it has been unclear whether nuclear fusion and genome duplication are absolutely required for successful conversion [22]. Here we investigated the requirements for, and the stability of, dominant reprogramming of human B cells by fusion with mouse ES cells. We show that reprogramming is surprisingly rapid and occurs within heterokaryons in which lymphocyte and ES cell nuclei remain spatially discrete. Furthermore, our data show that while Oct4 is critical for successful reprogramming of human lymphocytes to an ES-like state, Sox2 is not required. Thus our data outline an alternative strategy for defining the factors that are required for inducing a pluripotent state in human somatic cells.

Results

Reprogramming of Gene Expression Is Initiated in ES Cell Heterokaryons Prior to Nuclear Fusion

Human B cells were fused with mouse ES cells using polyethylene glycol (PEG). The nuclear events in fused cells were monitored by fluorescence microscopy and quantitative RT-PCR to analyse gene expression (Figure 1). To facilitate the identification of fused cells, E14tg2a mouse ES cells were pre-labelled with DiD and human B cells with DiI and dual-stained cells were purified by FACS (typically 10–15% of cells, Figure S1A). Human (B cell-derived) and mouse (ES cell-derived) nuclei were distin-
guished on the basis of DAPI and human-specific Lamin A/C labelling, and the proportion of cells containing two discrete (heterokaryons) or conjoined nuclei (hybrids) was assessed over time (Figure 1B). Up to 2 days following cell fusion 95–99% of dual labelled cells were identified as heterokaryons in which a single human and a single mouse nucleus were evident (illustrated in Figure 1B, central image). The kinetics of nuclear fusion were also confirmed by fluorescence in situ hybridization (FISH) analysis in which probes specific for mouse chromosomes (γ-satellite, red) or human chromosomes (α-satellite, green) were used to detect interspecies chromosome mixing indicative of hybrid formation (Figure S1B and Text S1).

The expression of pluripotency-associated genes and lymphocyte-associated genes by human B cell-derived nuclei was assessed by qRT-PCR, using primers that selectively amplify the human transcripts. Expression of human Oct4, Nanog, Cripto, Dnmt3b and Tle1 was detected in cells as early as 1 day after fusion and human Rex1 after 2 days (Figure 1C and Figure S1C). Although the levels were low in heterokaryons (<1% of that detected in human ES cells, cell line NCL1), these increased over time and were undetectable in non-fused (or self-fused, not shown) human B cells or control mouse ES cells. Expression of hTert was detected from day 4 onwards (Figure S1D), while hHprt expression was equivalent at all stages, as anticipated (Figure 1D). Mouse lymphocyte-specific gene transcripts (mCD19, mCD37 and mCD45) were not detected throughout the analysis (not shown), confirming the dominance of ES cells in conversion [15,18].

Increased expression of human pluripotency-associated genes over this 8-day period was mirrored by a reduction in expression of several human lymphocyte-associated genes within the second (hCD45, hCD37 and hCD19) or third day (hCD20 and hFuc3) of heterokaryon formation (Figure 1D). Collectively these data show that upon dominant reprogramming, activation and silencing of tissue-specific gene programs begins ahead of, and therefore does not require, nuclear fusion and cell division. In addition, since these results examine gene expression at the population level, it is possible that gene expression varied between individual heterokaryons and hybrid cells.

As the reprogramming of somatic cells has been previously shown to result in altered DNA methylation at specific loci [15,18,21,23], we examined changes in the methylation status of the human Oct4 gene promoter [24] and as a control, the Igf2/H19 imprinting control region (ICR) [25]. As illustrated in Figure 1E, human B cells prior to fusion showed high levels of DNA methylation throughout the hOct4 promoter and across a single Igf2/H19 allele. Following cell fusion, DNA methylation of hOct4 in reprogrammed B cells declined, consistent with a trend towards a hypomethylated state as seen in the human ES cell line H1. Demethylation of the hOct4 promoter was detected prior to nuclear fusion and cell division, a result that is consistent with active chromatin remodelling of the locus prior to expression. No changes in DNA methylation at Igf2/H19 ICR were detected over this period, consistent with its imprinted status [25].

Induction of a Human ES-Specific Gene Expression Profile
A comparison of the relative abundance of gene-specific transcripts in reprogrammed human B cells (Figure 2A, right-hand column), showed a strong similarity with the gene expression profiles of several human ES cell lines (NCI1 [26], HI, H7, H9 [27]; Figure 2A, left-hand column). For example, while Oct4 was abundantly expressed in all human and mouse ES cell lines, Nanog and Cripto expression was consistently much lower than Oct4 (100–1000 fold) for each of the mouse ES cell lines analysed (OS25, CCE, E14, ZHBTc4; Figure 2A, middle panel). In human ES cell lines however, Oct4, Nanog and Cripto transcripts were similarly abundant, consistent with that seen in reprogrammed human B cells. Expression of some pluripotency-associated genes, for example Sox2, was variable and often required extended periods of time (>8 days) for detection (not shown). This could reflect the fact that genes such as Sox2 are subject to multiple layers of repressive epigenetic modifications in B cells including DNA and histone methylation [28,29] and late replication [30], or that they require a higher threshold of activators for overt expression.

Similarities between gene expression profiles of human ES cell lines and hB x mES fused cells prompted us to examine additional markers that are expressed solely by either human or mouse ES cells [31–34]. These included fibroblast growth factor receptors (Fgf1 and Fgf2) and Fgf2 (expressed by human ES cells), Bmp4 and leukaemia inhibitory factor (Li)f receptor (expressed by mouse ES cells) and SSEA4, a surface glycoprotein selectively expressed by human ES cells [27] (Figure S2B). This analysis revealed that reprogrammed cells expressed increasing amounts hFgf1, hFgf2 and hFgf2 but did not express hBmp4 or hLi f or upregulate the downstream kinase hJak3 (Figure 2B). Thus, these data show that while dominant conversion is driven by mouse ES cells (that express Bmp4 and Lif prior to fusion, Figure S2A), reprogrammed heterokaryons and hybrid cells show a remarkably different expression profile resembling human, rather than mouse ES cell lines. Consistent with this, fusion of mouse ES cells and human B cells resulted in SSEA4 expression by 13–16% of the cells (days 2–8 as shown in Figure 2C). Isolation of SSEA4-positive cells confirmed that this subset contained successfully reprogrammed cells that express hOct4, hNanog and hCripto (Figure S2C), while SSEA4-negative cells were not reprogrammed. The observation that only a proportion of heterokaryons are successfully reprogrammed, as judged by hOct4 DNA demethylation and SSEA4 expression, might partly explain why the levels of transcripts encoding pluripotency factors are lower in reprogrammed cultures than established hES cell lines.

To ask whether the reprogramming of human B cells by mouse ES cells reverts multipotency potential, hB x mES cultures were treated with retinoic acid (RA) 6–8 days after cell fusion in order to induce differentiation (Figure 3). Prior to RA treatment most cells in hB x mES colonies showed alkaline phosphatase (AP) activity (Figure 3A), and expressed human AP transcripts (not shown). Hybrid colonies also expressed several pluripotency-associated...
Reprogramming Lymphocytes for Pluripotency

A

Human B-lymphocytes (hB) → Mouse embryonic stem cells (mES) → PEG induced cell fusion → Heterokaryon (hB x mES) → Hybrid cell

B

| Time / days after fusion | % heterokaryons | % hybrids |
|-------------------------|----------------|----------|
| 0                       | 11             | <1       |
| 1                       | >99            | <1       |
| 2                       | 98             | 2        |
| 3                       | 62             | 38       |

C

| Gene | Relative expression (mGAPDH) | Time after fusion / days |
|------|-----------------------------|--------------------------|
| hOct4|                             |                          |
| hNanog |                            |                          |
| hCripto |                            |                          |
| hDnmt3b |                            |                          |
| hTle1  |                            |                          |
| hRex1  |                            |                          |

D

| Gene   | Relative expression (mGAPDH) | Time after fusion / days |
|--------|------------------------------|--------------------------|
| hCD19  |                             |                          |
| hCD37  |                             |                          |
| hCD20  |                             |                          |
| hCD45  |                             |                          |
| hPax5  |                             |                          |
| hHprt  |                             |                          |

E

| Gene | hOct4 | hIgf2/H19 ICR |
|------|-------|---------------|
| hB (d0) |       |               |
| d2     |       |               |
| d4     |       |               |
| d8     |       |               |
| hES (H1) |   |               |
Markers, including hNanog protein (detected using a human Nanog-specific antibody) and the human embryonic-specific antigens SSEA4, TRA-1-60 and TRA-1-81 [27] (Figure S3).

Following treatment with RA, AP activity and expression of hOct4, hNanog and hRex1 was reduced (Figure 3C), while morphological heterogeneity within colonies increased. RA treatment induced the expression of genes associated with extra-embryonic (hCd22, hHaud1 and hGata6), endoderm (hSox2, hHnf4 and hCollagenIVβ1), mesoderm (hMsi1, hEbf3 and hMyod) and ectoderm (hNestin, Figure 3B) differentiation in hB x mES, but not in control hB cells (Figure 3C, blue and black lines respectively). Differentiation also resulted in increased DNA methylation of the hOct4 promoter (Figure 3D) to levels similar with that seen in differentiated human cells (Figure 1E). Taken together, these results show that reprogramming of human B cells by mouse ES cells resets gene expression and multi-lineage potential.

Interspecies Reprogramming of Human B Cells Requires mOct4 but Not mSox2

Oct4 is part of the core regulatory circuitry in ES cells [35] and it is essential for pluripotency and self-renewal [36]. To assess the potential role of mouse-derived Oct4 as a dominant ‘trans’ acting factor within inter-species heterokaryons we generated ES cells expressing Flag-tagged mouse Oct4 protein (Figure 4A) and fused these with human B lymphocytes (Figure 4B). Flag-tagged Oct4 (derived from mouse ES cells) was seen to accumulate within human nuclei 3 to 6 hours after cell fusion (Figure 4B, complete kinetic analysis shown in Figure S4A). In addition, Oct4 protein was present in heterokaryon nuclei (at 3 hours) before transcription of hOct4 was initiated (at 24 hours). Thus, translocation of the ES-derived Oct4 into human lymphocyte nuclei precedes reprogramming. Conversion of human fibroblasts to ES-like cells has been shown to require the activation of at least four factors including Oct4, Sox2 and either Nanog plus Lin28 [9] or Klf4 plus c-Myc [10]. Recently it was shown that mouse ES cells lacking Sox2, a factor thought to be vital for preventing extra-embryonic differentiation, can remain pluripotent provided with elevated Oct4 levels [37]. To investigate the relative importance of Oct4 and Sox2 in reprogramming, mouse ES cells that are inducible null (Tet-off) for mOct4 (ZHBTC4 [36]) or for mSox2 (2TS22C [37]) were used as fusion partners with human B cells. These inducible null ES cell lines were constructed and characterised previously [36,37] and display a rapid (within 24 hours) and complete elimination of Oct4 or Sox2 gene/protein expression upon doxycycline (+Dox) treatment. In our hands, pre-treatment of ZHBTC4 cells with Dox for 6 and 12 hours, resulted in a progressive decrease in mOct4 gene expression (Figure 4C), without significantly affecting the expression of other pluripotency-associated genes in these cells or the efficiency which they fuse with human B cells (Figure 4B).

Successful reprogramming, as judged by induction of several human genes (Oct4, Nanog, Gropo, Dnmt3b, Sox2, Tie1, Ret and Rex1) was however reduced (+6 hours) or eliminated (+12 hours) by pre-treatment of ZHBTC4 cells with Dox (Figure 4D, a complete kinetic analysis is provided in Figure S4C). Likewise, knocking down mOct4 using short interference RNA (siRNA) in E14tg2a mES cells (Figure S5A and Text S1) also abolished reprogramming activity (Figure S5B). These results confirm that mOct4 expression is critically important for initiating successful reprogramming, in keeping with previous reports [7–10,38]. The extinction of human lymphocyte-specific genes was however not impaired by Oct4 removal (Figure S4C), a result that may support previous findings that the activation and silencing of gene expression programs in heterokaryons are mechanistically distinct processes [13]. Eliminating mSox2 expression in the mouse ES cell (Figure 5A, 2TS22C had, in contrast, a relatively mild effect on reprogramming efficiency (Figure 5B, compare values at 0, 12 and 24 hours of Dox treatment). Furthermore, reprogramming was fully restored in fusions using 201 cells, a Sox2-deficient mES cell line in which mOct4 expression is up-regulated [37] (Figure 5A, B values shown in red and complete kinetics shown in Figure S6). These data show that Oct4, but not Sox2, is critical for the dominant reprogramming activity of mouse ES cells. Interestingly, using 201 cells we observed the enhanced induction of hSox2 (Figure 5B, red arrow), a result that suggests that mouse-derived Oct4 levels may be important for initiating hSox2 expression in somatic nuclei.

ES-Derived mOct4 Is Dispensable for Maintaining the Reprogrammed Status of Somatic Cells

To assess whether gene expression by the reprogrammed cell is stable (self sustaining) or requires the continuous supply of factors provided by the mouse ES cell, we generated hybrid cells between lymphocytes and ES cells in which Oct4 expression could be conditionally withdrawn (ZHBTC4, experimental outline depicted in Figure 6A). In these experiments fusions were performed between mouse lymphocytes carrying a silent, Oct4-driven GFP transgene (GOF18APE) and mouse ZHBTC4 ES cells, to allow successfully reprogrammed hybrid cells to be identified on the basis of GFP re-expression by day 10 (Figure 6A). Hybrid clones contained a rearranged IgH locus, consistent with their derivation from mouse B cells (Figure S7A and Text S1), displayed twice the
DNA content of diploid cells (4n, Figure 6B) and were karyotypically stable over the study period (not shown). As anticipated, hybrid cells expressed ZBTB4- and derived Oct4geo transcripts and several pluripotency-associated genes, but did not express B cell markers such as CD19, Pax5 and Ly108 (Figure S7B). Two hybrid clones were selected for study (hybrid 1 and 2) and were treated with Dox to selectively ablate expression of ZBTB4- derived Oct4geo (Figure 6C; Figure S7C shows the strategy used to selectively detect Oct4geo transgene expression). Withdrawal of ZBTB4-derived Oct4 did not alter the expression of mNanog and mSox2 in reprogrammed cells (Figure 6C), and did not precipitate differentiation towards trophoblast or the up-regulation of mCdks2 and mHand1 expression [36] (Figure 6D and Figure S7D); events that are induced by the removal of Oct4 from the parental ZHTB4- line (Figure 6D right hand panel and Figure 6E). Thus, our data show that reprogramming of lymphocytes by mouse ES cells induces an epigenetically stable (and heritable) resetting of gene expression in the lymphocyte nucleus.

Discussion

In this study we show that reprogramming human lymphocytes can be achieved using mouse ES cells as a cell fusion partner, a process that induces the re-expression of endogenous human genes normally associated with human blastocyst development and human ES cell lines. Successful interspecies reprogramming is initiated in heterokaryons prior to chromosome intermixing, and generates cells that express human FGF signalling pathway components and human ES-specific surface molecules such as SSEA4, TRA-1-60 and TRA1-81. We show that this reprogramming is critically dependent upon Oct4, since Oct4 deletion abolishes the reprogramming capacity of mES cells. Conversion of human B cells into ES-like cells results in the re-modelling of the somatic genome with loss of DNA methylation at the hOct4 locus. Importantly, once reprogramming is initiated by factors produced by the dominant (ES) nucleus, we show that withdrawal of mOct4 does not compromise the phenotype of hybrid cells. This result implies that the reprogrammed state, once initiated, is both self-sustaining and heritable.

One surprising aspect of the reprogramming data shown here is the rapidity of gene conversion and DNA demethylation that occurs within heterokaryons. As successful reprogramming is only achieved in a proportion of heterokaryons (<15%), it is likely that partial DNA replication (or repair) is required for lymphocyte conversion. Previous studies have shown that reprogramming in experimental heterokaryons using adult cells from different lineages [13,39], can be initiated before genome duplication and cell division. Here we show that conversion of unipotent lymphocytes towards multipotency is achieved in transient heterokaryons prior to cell division. Re-activation of human Oct4 and Nanog by human nuclei, has been shown to occur rapidly upon DNA de-methylation and Tdt1 activation induced by Xenopus oocytes [21,40]. The rapid re-activation of endogenous pluripotency-associated genes seen in inter-species heterokaryons is consistent with transient re-activation studies that have reported hOCT4 expression by MEFs [41] or NSCs [22] fused with mouse ES or EC cells. Collectively these results may have an impact for generating human ES-like cells. Proof that mouse ES cells can dominantly reset the multi-lineage potential in human somatic cells, together with evidence that this process begins prior to nuclear fusion, suggests that improved methods for removing mouse chromosomes from heterokaryons [42] may be applicable for generating human stem cell lines. Alternatively, using conditionally targeted mouse ES cells to dissect the roles of individual proteins thought to be critical for multipotent reprogramming, may provide a rationale for using distinct protein cocktails to directly re-set lineage potential.

In the experiments presented here we have shown that reprogrammed human cells express a profile of transcripts, signalling molecules and surface antigens that are similar to those seen in human ES cells, and different from mouse ES cells. This suggests that an early human embryonic “program” of gene expression is initiated in human nuclei by trans-acting (mouse) factors. Differences between the expression profiles of mouse ES cells and human reprogrammed nuclei probably reflect discrepancies in cis-acting regions between the mouse and human genomes. In agreement with this idea, a study in which the entire hTert gene was introduced into mice, showed expression of the transgene was similar to endogenous hTert in humans, rather than mouse endogenous mTert [43]. It is interesting to speculate that some of the well-publicised differences between human and mouse ES cells may indeed reflect intrinsic species dissimilarities, rather than temporal differences in stem cells isolation [44,45]. We show that after fusion of human lymphocytes to mouse ES cells (that are LIF and Bmp dependent), human ES-like cells are generated that express FGF signalling components (and are not dependent of LIF/ Bmp). Thus, our data suggest that differences between human and mouse ES cells may reflect distinct signalling and transcriptional networks, rather than necessarily when or where they were isolated during embryogenesis.

We show here that Sox2, in contrast to Oct4, is not required to convert human lymphocytes into a multi-potent state. This observation contrasts with results obtained previously using iPS strategies to reprogram mouse and human fibroblasts [7–10,38], mouse hepatocytes and stomach cells [46] and mouse B-lymphocytes [47]. Whether this is because of differences relating to the overexpression of transcription factor cocktails used in iPS, or that reprogramming occurs over an extended time period (pluripotency-associated genes such as Oct4, Nanog and Sox2 are reactivated after 2 weeks of transduction [48,49]), is not known. However, as Sox2 was recently shown to be dispensable for the activation of Oct–Sox enhancers in mouse ES cells [37], it is also possible that additional Sox family members such as Sox4, Sox11 and Sox15, may have redundant functions with Sox2 in reprogramming. Interestingly, by enhancing Oct4 levels in Sox2-
deficient ES cells (ES-2O1) we show elevated expression of hSox2 by reprogrammed human B cells. Recent genome-wide studies have shown that Sox2 is a direct target of Oct4 in both human [35] and mouse [50] ES cells, a fact that could explain why hSox2 is efficiently reprogrammed using ES cells that overexpress mouse Oct4. In our hands, overexpression of exogenous Oct4 in lymphocytes did not induce pluripotent conversion (Pereira & Terranova, unpublished results), a finding that argues that additional chromatin remodelling factors, perhaps including those known to interact with Oct4 [51,52] or associated with the process of DNA demethylation, may be critical for successful reprogramming. Collectively, our data show that interspecies heterokaryons can...
Figure 4. Oct4 is required for successful reprogramming. (A) Mouse ES cells expressing a tagged Oct4 protein (Flag-mOct4) were generated by insertion of Flag-tagged mouse Oct4 cDNA in E14tg2a ES cells (parental cell line). Western blotting with anti-Oct4 and anti-Flag antibodies confirmed the presence of Flag-tagged Oct4 protein by transduced cells. Equivalent protein loading is shown with Lamin B detection. (B) Immunofluorescence analysis of cultured heterokaryons 6 hours after cell fusion showed the presence of ES cell-derived Oct4 (Flag-Oct4, green) in a human nucleus (arrowed). Human nuclei were distinguished from mouse nuclei on basis of diffuse versus punctuate DAPI staining (blue).
respectively. Actin labelling (red) delineates the cell membrane. Images are confocal sections of heterokaryons containing a single mouse (with DAPI intense foci) and a single human nucleus. Scale bar, 10 μm. (C) In ZHBTc4 ES cells endogenous Oct4 was replaced by an inducible transgene (Oct4geo) which can be downregulated by addition of doxycycline (Dox) [36]. Quantitative RT-PCR analysis showed that 6 hours (+6) and 12 hours (+12) after Dox treatment, mOct4 was progressively downregulated, while expression of other pluripotency-associated genes (mNanog, mCripto, mRex1 and mSox2) was largely unaffected. (D) ES cells expressing normal levels of Oct4 (–), partially reduced (Dox+6) or lacking Oct4 expression (Dox+12) were fused to hB-lymphocytes. Successful reprogramming was assessed by quantifying the abundance of human ES-associated transcripts two days after fusion by qRT-PCR. Activation of pluripotency genes in hB-lymphocytes was reduced or impaired when Oct4 was ablated. Data were normalised to Gapdh expression. Error bars indicate the s.d. of 2–3 independent experiments.

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Figure 5. Sox2 is dispensable for reprogramming. (A) In 2TS22C ES cells endogenous Sox2 is replaced by an inducible transgene (Sox2Zeo) which can be downregulated by addition of doxycycline (Dox) [37]. Quantitative RT-PCR analysis showed that 12 hours (+12) and 24 hours (+24) after Dox treatment, mSox2 was downregulated while expression of other pluripotency-associated genes (mNanog, mCripto, mRex1) and mOct4 continued to be expressed. 2O1 ES cells are Sox2-deficient mES cells (asterisk) in which mOct4 expression is up-regulated (red bars). (B) ES cells expressing Sox2 (–), Sox2 depleted cells (Dox+12, Dox+24) and 2O1 cells were fused to hB-lymphocytes. Successful reprogramming was assessed by quantifying the abundance of human ES-associated transcripts two days after fusion by qRT-PCR. Activation of pluripotency genes in hB-lymphocytes occurs in the absence of mSox2. An elevated induction of hSox2 using 2O1 cells as a fusion partner is highlighted by an arrow (red). All data were normalised to Gapdh expression and error bars indicate the s.d. of 2–3 independent experiments.

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Figure 6. Reprogramming is self-sustaining and can be maintained in the absence of ES-derived Oct4. (A) To address whether reprogramming is stable or subject to reversion, we ablated Oct4 expression after hybrid formation. ZHBTc4 ES cells [mES with endogenous Oct4 replaced by an inducible transgene (Oct4βgeo) which can be downregulated by addition of doxycycline (Dox)] were fused with mouse B-lymphocytes (mB) carrying a GFP transgene under the control of Oct4 promoter (GOF18ΔPE). Reprogramming of mB results in the re-activation of GFP in hybrid colonies (d10, lower panels). Kinetic analysis of single cells (upper panels) showed that transgene re-activation occurs in heterokaryons (day 2, 2 arrows), and hybrid cells (day3, arrowhead). mB cells are shown as negative controls. Nuclei were visualised with DAPI staining (blue). Scale bars, 10 μm. (B) Hybrid clones (mES x mB, 4n) that re-expressed GFP were isolated and analysed by FACS. mES, mB and mES x mB hybrid cells...
provide an interesting and complimentary approach to iPS, allowing the factors that are required to directly induce pluripotency to be defined individually and in combination.

**Materials and Methods**

**Cell Culture**

EBV-transformed hB clones were maintained in RPMI supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine and antibiotics (10 μg/ml Penicillin and Streptomycin). The Abelson transformed Oct4-GFP B-cell line was derived from the Oct4-GFP transgenic mice (GOF18#AP) [53] bone marrow, cloned and grown in RPMI supplemented with 20% FCS, non-essential amino acids, L-glutamine, 50 μM 2-mercaptoethanol, antibiotics and IL-7 (5 ng/ml; R&D Systems, Minneapolis, MN). Mouse ES cells were grown and maintained undifferentiated either on irradiated SNL feeder layers (E14Tg2a, Roche Diagnostics, Mannheim, Germany). Briefly, ES cells and lymphocytes using 50% polyethylene glycol, pH7.4 (PEG 1500; Invitrogen) was applied 48 hrs after and resistant clones were manually picked and screened by Western blot. Human ES cell lines H1, H7 and H9 cells [27] were cultured in medium differentiated mouse ES cells at 0.5×10^6 cells/cm^2. To eliminate unfused hB cells, Ouabain (10^{-2} M; Sigma) was added to the medium 4 hours after cell fusion. When OS25, ZHBTc4 and 2TS22C cell lines were used, proliferating ES cells were eliminated by the addition of 10^{-2} M Ara-C (Cytosine β-D arabinofuranoside; Sigma) 4–6 hours after fusion and then removed after 16 hours. When E14tg2a ES cells or derivatives were used, HAT (20 μM hypoxanthine, 0.08 μM aminopterin and 3.2 μM thymidine; Sigma) was added to the medium 24 hours after fusion.

**Quantitative RT-PCR Analysis**

RNA extraction was performed using RNA-BEE reagent (Tel-Test Inc., Friendswood, TX) and residual DNA was eliminated using the DNA-free kit (Ambion, Austin, TX). 3 μg of total RNA was then reverse transcribed using Superscript First-Strand Synthesis system (Qiagen) with oligo (dT)_{12-18} (Invitrogen). cDNAs of interest were then quantified using real-time qPCR amplification. Real-time PCR analysis was carried out on a Opticon DNA engine using Opticon Monitor software (MJ Research Inc., Waltham, MA), running the following program: 95°C for 15 min, then 40 cycles of 94°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec, followed by plate-read. PCR reactions included 2× Sybr-Green PCR Mastermix (Qiagen), 300 nM primers and 2 μl of template in a 35 μl reaction volume. Each measurement was performed in triplicate and data normalized according to Gapdh expression. Primers were designed with Primer Express software (Applied Biosystems) and tested for the specific detection of human transcripts (and not mouse). Standard curves were calculated on serial dilutions of positive control cDNA. Primer sequences used for this analysis are indicated in Table S1.

**Bisulfite Genomic Sequencing**

Bisulfite modification of DNA was carried out using the EZDNA methylation kit (Zymogenetics Inc., Orange, CA) according to manufacturer’s recommendations. PCR primers that recognise bisulfite-converted human DNA only are listed in Table S1. Amplified products were cloned into pCR2 (Invitrogen) and ten clones were randomly picked and sequenced.

**Antibodies, Imaging, and FACS Analysis**

For immunofluorescence and FACS analysis, the following antibodies and dilutions were used: mouse monoclonal anti-human Lamin A/C (VP-L550; Vector Laboratories Inc., Burlin-
game, CA) at 1:100 dilution; rabbit polyclonal anti-GFP (A11222; Molecular Probes) at 1:200 dilution; mouse monoclonal anti-human SSEA4 (MC-913-70; Developmental Hybridoma Studies Bank, Iowa City, IA) at 1:3 dilution; mouse monoclonal anti-human TRA-1-60 and TRA-1-81 (MAB4360 and MAB4391; Chemicon International, Temecula, CA) at 1:12 and 1:20 dilutions, respectively; rabbit polyclonal anti-human Nanog and Nestin (ab21624 and ab20944; Abcam Ltd., Cambridge, UK) at 1:100 dilution; mouse monoclonal anti-Flag (F3165, Sigma) at 1:1000 dilution; secondary antibodies conjugated with fluorochromes were purchased from Molecular Probes and used at 1:400 dilution. Immunofluorescence was performed as previously described [13]. Mouse and human nuclei were distinguished in the resulting heterokaryons by counterstaining with 4,6-diamidino-2-phenylindole (DAPI) and human Lamin A/C staining. Individual cells were delineated by F-actin staining (Phalloidin; A12380, Molecular Probes). For alkaline phosphatase assays, hybrid colonies 8 days after cell fusion were stained with alkaline phosphatase assay kit (Sigma). All slides were analyzed on a Leica TCS SP5 confocal microscope and processed with Leica software and Adobe Photoshop. Images of live GFP fluorescent hybrid colonies and alkaline phosphatase staining were collected using a Leica DM IRE2 microscope running Metamorph software. For FACS analysis a FACS caliber (BD Biosciences) with CellQuest software was used. FACS purification was performed using a FACS aria cell sorter. Western blot analysis was performed as previously described [55] using a goat anti-Oct3/4 polyclonal antibody (sc-6216; Santa Cruz Biotechnology Inc.). Each lane contained 20 μg total protein.

Supporting Information

Figure S1 Characterisation of heterokaryon reprogramming of fused hB x mES cells. (A) Human B-lymphocytes (hB) and mouse embryonic stem cells (mES) were respectively labelled with the cell membrane dyes DiI and DiD and fused in the presence of polyethylene glycol (PEG). Fused cells, identified by double-labelling (upper right quadrant), were sorted by FACS and cultured. (B) Mouse and human nuclei were distinguished by FISH using probes specific for mouse γ-satellite DNA (red) or human α-satellite DNA (green), and DAPI counterstained (blue). Confocal sections of human B cells (hB) and mouse ES cells (mES) before and after cell fusion (hB x mES) are shown. Heterokaryons (cells in which parental nuclei share the same cytoplasm but remain discrete, day 1 and 2) were identified up to 2 days after fusion, but by day 3 hybrid formation (where genomes are mixed in the same nucleus, day 3) was detected. Scale bar, 10 μm. (C) Expression of human ES-specific (hOct4, hNanog) and human lymphocyte-specific (hCD20, hCD45) transcripts detected by RT-PCR using human-specific primers. Prior to fusion, hB cells expressed hGata6, hCD20 and hCD45 but not embryonic stem cell-specific genes. Following heterokaryon formation (hB x mES d2), human pluripotency-associated genes hOct4 and hNanog were expressed (upper panel) and hCD20 and hCD45 were extinguished (lower panel). mES-, RT- and H2O2 were used as negative controls and human embryonic stem cells (hES) as a positive control. hGata6 was used to standardise input. (D) Expression of human hTet2 transcripts detected by qRT-PCR 0 to 8 days after cell fusion using human-specific primers. Positive (hES-NCL1, black bars) and negative (hB) controls for this analysis were included. Data were normalised to hGata6 expression. Error bars indicate the s.d. of 3 independent experiments.

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Figure S2 Differences between human and mouse ES cells and the identification of SSEA4 positive reprogrammed cells. (A) Expression of Fgf1, Fgf2, Fgf5, Bmp4, Bmp7, and Jak3 was assessed by qRT-PCR in human ES cells (hES, NCL1), mouse ES cells (mES) and human B-lymphocytes (hB). Fgf1, Fgf2, and Fgf5 were uniquely expressed by human ES cells. (B) FACS analysis showed that >90% of hES cells (H1 cell line) expressed SSEA4, while hB and mES do not (2.1% and 1.5% respectively). A proportion of heterokaryons showed SSEA4 expression (15.8%) 8 days after cell fusion (hB x mES d8). (C) FACS sorting of SSEA4 positive cells purifies reprogrammed cells that express hOct4, hNanog, and hCRIPTO, as assessed by qRT-PCR. Data were normalised to Gapdh expression.

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Figure S3 Expression of human-specific embryonic antigens in hybrid cells. Human B cells (hB) and mouse ES cells (mES) were fused and the resulting colonies (hB x mES d8) expressed hNanog protein (red) and the human ES-specific antigens SSEA4, TRA-1-81 and TRA-1-60 (green) as assessed by immunofluorescence. Control hB cells did not express any of the markers. DAPI staining is shown in blue. Images are single confocal sections. Scale bar, 50 μm.

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Figure S4 Kinetic analysis of Oct4 protein distribution in heterokaryons and the importance of Oct4 for successful reprogramming. (A) Flag-mOct4 ES cells were fused to hB cells and Oct4 protein detected by immunofluorescence at 0, 1, 3, 6, 9, and 12 hours with Oct4 or Flag antibodies (green). Heterokaryons were scored according to the following Oct4 distribution: Oct4 protein not detected (Negative), stronger staining in mES-derived nucleus than hB nucleus (mES>hB), nuclei equally labelled (mES=hB), stronger in the human nucleus (mES<hB). Confocal sections of representative heterokaryons from each of the categories are shown (upper panels). Human nuclei were distinguished from mouse nuclei on basis of diffuse versus punctuate DAPI staining (blue), respectively. Actin labelling (red) delineates the cell membrane. Scale bar, 10 μm. n = 100. (B) The ability of mouse ES cells to fuse to human B cells is unaffected by doxycycline (Dox) treatment. ZHBTc4 and hB cells were labelled (with DiD and DiI, respectively) and PEG-fused. Fusion efficiencies were obtained by FACS, as a percentage of double-labelled cells. (C) ZHBTc4 ES cells expressing Oct4 (black bars), or in which Oct4 expression has been partially or completely ablated (grey and white bars, respectively) were fused to hB-lymphocytes. The activation of human ES-specific genes (hOct4, hNanog, hCRIPTO, hDmnt3b, hSox2, hTet2, hTet1, and hRet) and silencing of lymphocyte-specific genes (hCD19, hCD45, and hCd37) were quantified by qRT-PCR over the period of 3 days after cell fusion. hHprt was added as a control gene. Data were normalised to hGata6 expression. Error bars indicate the s.d. of 2–3 independent experiments.

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Figure S5 siRNA-mediated knock-down of mOct4 abolishes reprogramming. (A) E14tg2a ES cells were transfected with either mOct4-siRNA or target-less-siRNA (a negative control siRNA designed to have no expected targets in human and mouse cells) vectors. 48 hours later, transfected cells (GFP+) were FACS sorted and analysed by quantitative RT-PCR analysis. mOct4-siRNA targeted cells showed >90% reduction in Oct4 transcript levels as compared to cells transfected with target-less-siRNA (control). (B) E14tg2a ES cells expressing mOct4-siRNA or control-siRNA
were fused to hB-lymphocytes, and successful reprogramming was assessed by quantifying the abundance of human ES-associated transcripts (h\text{Nanog} and h\text{CRIPTO}) two days after fusion by qRT-PCR. Successful reprogramming judge by the activation of human pluripotency-associated transcripts was abolished by pre-treatment of mES cells with Oct4-siRNAs. Data were normalised to \text{GAPDH} expression. Error bars indicate the s.d. of 2 independent experiments.

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**Figure S6** Kinetic of human lymphocyte reprogramming by mES cells after Sox2 ablation. 2TS22C (black bars), Sox2 depleted mES cells after Sox2 ablation. 2TS22C (black bars), Sox2 depleted mES cells after Sox2 ablation. Data were normalised to h\text{Nanog} and h\text{CRIPTO} expression. Error bars indicate the s.d. of 2 independent experiments. mES expression. Error bars indicate the s.d. of 2 independent experiments.

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**Figure S7** Characterisation of mouse embryonic hybrid cells. (A) Contribution of the lymphocyte genome within hybrid cells was confirmed by detection of a rearranged IgH locus (D-J region). IgH rearrangement was seen in B-lymphocytes (mB), hybrid cells (mES x mB) but not in mES cells. The rearranged DNA can be detected by PCR amplification and visualized on the gel as a 750 bp band. (B) Lymphocyte-specific genes (m\text{CD19}, m\text{Pax5}, and m\text{IgI108}) were not detected in hybrid cells although ES-specific genes (m\text{Oct4}, m\text{Nanog}, m\text{Sox2}, m\text{Rex1}, and m\text{GCLy}) remain detectable by RT-PCR. (C) Specific detection of Oct4 transgene (Oct4\text{betageo}) by RT-PCR with primers within \text{betageo} cassette, which specifically amplify ZHBTc4-derived Oct4 but not endogenous m\text{Oct4}. mES and mB cells were included as controls. m\text{GAPDH} was used to standardise input. (D) Doxycycline (Dox) treatment of ZHBTc4-mES cells results in morphological changes characteristic of trophectoderm differentiation (upper panel). These were not observed in hybrid clones 4 and 12 under the same conditions. GFP protein (Oct4 promoter-driven) remains detectable in hybrid cells throughout the experiment, as assessed by immunofluorescence.

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**Table S1** Primers used in this study.

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**Text S1** Supplementary methods.

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**Author Contributions**

Conceived and designed the experiments: CIF RT TM AGF. Performed the experiments: CIF NK JR KJM. Analyzed the data: CIF AGF. Contributed reagents/materials/analysis tools: WC. Wrote the paper: CIF MM AGF.
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