DNA Synthesis Is Required for Reprogramming Mediated by Stem Cell Fusion

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SUMMARY

Embryonic stem cells (ESCs) can instruct the conversion of differentiated cells toward pluripotency following cell-to-cell fusion by a mechanism that is rapid but poorly understood. Here, we used centrifugal elutriation to enrich for mouse ESCs at sequential stages of the cell cycle and showed that ESCs in S/G2 phases have an enhanced capacity to dominantly reprogram lymphocytes and fibroblasts in heterokaryons and hybrid assays. Reprogramming success was associated with an ability to induce precocious nucleotide incorporation within the somatic partner nuclei in heterokaryons. BrdU pulse-labeling experiments revealed that virtually all somatic nuclei acquired the capacity to perform DNA synthesis within 24 hr of fusion with ESCs. This was essential for successful reprogramming because drugs that inhibited DNA polymerase activity effectively blocked pluripotent conversion. These data indicate that nucleotide incorporation is an early and critical event in the epigenetic reprogramming of somatic cells in experimental ESC-heterokaryons.

INTRODUCTION

Epigenetic reprogramming is a feature of normal embryonic development (Feng et al., 2010) that can also be induced experimentally using a range of strategies (Gurdon and Melton, 2008; Yamanaka and Blau, 2010). For example, differentiated somatic nuclei can regain pluripotency upon injection into oocytes (nuclear transfer) or through the forced expression of specific combination of transcription factors that induce a pluripotent stem (iPS) cell state (Gurdon, 1960; Takahashi and Yamanaka, 2006). Conversion of somatic cells toward pluripotency is associated with distinctive changes in the chromatin and DNA methylation status of the somatic genome (Deng et al., 2009; Simonsson and Gurdon, 2004) thought to be important for stable re-expression of core pluripotency factors such as Oct4, Sox2, and Nanog (reviewed by Papp and Plath, 2011). A third strategy for reprogramming somatic cells is by cell-cell fusion. There is an accumulating literature describing fusions between embryonic stem cells, embryonic carcinoma (EC) and embryonic germ (EG) cell lines with somatic cell partners such as thymocytes, lymphocytes, fibroblasts, or hepatocytes derived from the same or a different species (Miller and Ruddle, 1976; reviewed by Soza-Ried and Fisher, 2012). Collectively, these experiments have shown that somatic nuclei can be reprogrammed to acquire the epigenetic and developmental properties of their pluripotent partner (Ambrosi et al., 2007; Cowan et al., 2005; Do et al., 2007; Foshay et al., 2012; Matveeva et al., 1998; Pereira et al., 2008; Tada et al., 1997, 2001; Tat et al., 2011). Although the molecular mechanisms that determine the success and direction (or dominance) of this conversion are not fully understood, complete reprogramming is achieved 5–7 days after fusion with ESC, EG, and EC cells and is thought to occur in two steps. First, transient heterokaryons are formed in which both parental nuclei remain spatially discrete but share a common cytoplasm. Low levels of pluripotent gene expression from the somatic partner are initiated in a proportion of heterokaryons and increase over a 3–4 day period before the parental nuclei fuse to generate hybrids (Pereira et al., 2008). This second step has been proposed to stabilize or “fix” newly acquired gene expression profiles, enabling the resulting tetraploid cells to generate pluripotent colonies (reviewed by Serov et al., 2011). Because the first step occurs in the absence of cell division, it has been generally assumed that DNA synthesis is not required to initiate reprogramming.

Although some evidence supports this view (Bhutani et al., 2010), other studies have suggested that DNA synthesis may be required to reverse cis-mediated silencing of genes such as Oct4 and Nanog (Foshay et al., 2012) or have suggested that somatic genome reprogramming occurs during the first cell cycle (Han et al., 2008). In this regard, classic cell fusion experiments performed more than 40 years ago using HeLa cells (Rao and Johnson, 1970) had shown that early (or precocious) DNA synthesis is induced in G1-phase cells upon fusion with...
cells at later stages of the cell cycle (in S or G2 phases). As DNA synthesis provides an unrivaled opportunity for chromatin and nucleosome remodeling as well as changes to DNA methylation, it is important to establish whether there is any involvement of DNA synthesis in heterokaryon-mediated reprogramming in order to understand the mechanisms behind this conversion.

Embryonic stem cells and the pluripotent cells of the epiblast from which they arise, have a very unusual cell-cycle structure characterized by a short cell-cycle time, truncated G1 phase, and a large proportion of cells in DNA synthesis (S) phase (Fluckiger et al., 2006; White and Dalton, 2005). Pluripotent cells in the mouse epiblast devote more than 50% of cell-cycle time to S phase and a similarly high proportion of mouse ESC, EG, and EC cells (35%–50%) are reported to be in S phase (Savatier and Afanasieff, 2002; Stead et al., 2002). This unusual profile is associated with high levels of Cdk activity and anaphase-promoting complex/cyclosome (APC/C) substrates present throughout the cell cycle (Fujii-Yamamoto et al., 2005; Koledova et al., 2010b; Yang et al., 2011). A recent report has suggested that Cdk activity in ESCs may oscillate in a manner that is muted as compared with differentiated or somatic cells (Ballabeni et al., 2011). Although the biological consequences of this unusual cell cycle are not known, evidence that ESCs loose this profile upon differentiation (Bar-On et al., 2010; Calder et al., 2013; Koledova et al., 2010a; Orford and Scadden, 2008) and conversely, that somatic cells regain it when reprogrammed (Ghule et al., 2011; Ruiz et al., 2011; Singh and Dalton, 2009), have suggested that it may be important for rapid self-renewal of pluripotent cells.

One of the consequences of ESCs having impaired or muted cell-cycle checkpoints is that many of the drugs that have been traditionally used to synchronize or block somatic cells at specific stages of the cell cycle are often either ineffective or promote differentiation in ESCs, rendering cell-cycle studies in undifferentiated ESCs problematic (Calegari and Huttner, 2003; Han et al., 2008; Neganova and Lako, 2008; Ruiz et al., 2011). To circumvent this, we have optimized a biophysical cell separation method to enrich for ESCs in discrete phases of the cell cycle. Using this methodology we asked whether the ability of ESCs to dominantly reprogram differentiated cells was influenced by their cell-cycle stage. Our results show that ESCs in late S/G2 phases of the cell cycle have a markedly enhanced ability to reprogram somatic cells and provide evidence that this is because they induce the somatic nucleus to undergo a round of precocious DNA synthesis shortly after fusion.

RESULTS

Cell-Cycle Synchronization of ESCs by Centrifugal Elutriation

Counterflow centrifugal elutriation allows the separation of heterogeneous cell populations into fractions of uniform size and density (Banfalvi, 2008). As size and density reflect cell-cycle stage, we used this approach to isolate mouse ESCs at sequential stages of the cell cycle from undifferentiated cultures. Briefly, single cell suspensions of E14 ESCs were loaded into an elutriation chamber and centrifuged at constant speed. Fractions were collected at increasing flow rates (6–17 ml/min, F8 to F16) and evaluated for their DNA content by staining with propidium iodide (PI) followed by fluorescence-activated cell sorting (FACS) analysis. Typical DNA content profiles of unsynchronized ESCs and of sequential elutriated fractions are shown in Figure 1A (top and bottom panels, respectively) where the gates used to define G1, G2, and S phase are marked. Fractions F8 and F9 contained predominantly G1-phase ESCs (>80%), and fraction 16 was enriched for cells in G2 (>70%), whereas S-phase cells centered around fraction F12. The consistency of this separation approach was confirmed in five independent experiments (Figure S1A available online). To quantify cells undergoing DNA synthesis within each fraction we also subjected samples to a 45 min pulse of BrdU (100 μM) after elutriation and then identified and scored BrdU incorporating cells using immunofluorescence microscopy, as illustrated in Figure 1B (anti-BrdU, green). Among undifferentiated asynchronous ESC cultures BrdU label was routinely detected in ~35% of cells and the pattern of BrdU distribution within nuclei was similar to the patterns previously reported for S-phase stages in somatic cells (Azuara et al., 2003; McNairn and Gilbert, 2003), as shown here for human B cells (hB) (Figure 1B and legend). In particular, early S phase (I, II) and late S phase (IV, V) patterns were detected in 42% and 20% of BrdU-labeled mouse ESCs and characteristically marked DNA replication at either diffuse euchromatic sites or within blocks of heterochromatin, respectively. Mid-S-phase cells (III) accounted for the remaining 38% of BrdU-labeled cells in which DNA synthesis was focused at either perinuclear domains (a), perinucleolar sites (b) or within dispersed sites of DAPI-intense heterochromatin (c). Using this BrdU pulse-labeling strategy we enumerated S-phase cells within elutriated ESC samples (Figure 1C). Fractions F11 to F12 were enriched for S-phase cells (>60% of cells incorporated BrdU), consistent with previous PI staining profiles. Fractions F8 and F9 contained relatively few S-phase cells (6%–14%) with labeling patterns indicative of early S phase (I and II). Fraction F16 typically contained 20%–30% BrdU positive cells and these cells showed a typical late S-phase distribution pattern (IV and V).

As drug-based treatments that arrest or delay ESC cell-cycle progression have been reported to promote differentiation and cell death (Burdon et al., 2002; Calegari and Huttner, 2003; Neganova and Lako, 2008; Orford and Scadden, 2008; Ruiz et al., 2011) we asked whether elutriated ESC samples successfully resumed cell cycle upon reculture. Fractions F8/F9, that were relatively homogeneous and enriched for G1-phase ESCs, were monitored for cell-cycle progression using PI staining at successive culture times (Figure 1D). G1-enriched ESC samples (F9, day 0) progressed through to S (day 1) and G2 (day 2) phases of the cell cycle, and subsequently showed a PI profile that resembled nonsynchronous ESC cultures (day 3). No increase in cell differentiation or significant loss of cell viability was detected in these cultures throughout the time course. Furthermore, a direct comparison of cell survival in nonsynchronous, G1-, S-, and G2/M-enriched ESCs cultured immediately after elutriation showed a similar viability between fractions (Figure S1B).
ESC Reprogramming Capacity Varies with Cell-Cycle Stage

A previous study of hybrids generated between mouse thymocytes or fibroblasts fused with mouse ESCs that had been grown to different degrees of confluence (Sullivan et al., 2006) had suggested that reprogramming could be optimized using stem cells enriched for G2/M phases of the cell cycle. We used counterflow centrifugal elutriation to isolate cell-cycle stages from other variables that may occur in cultures grown to different densities. The ability of elutriated ESC samples to stably reprogram mouse B lymphocytes was compared using puromycin resistant mouse B cell targets that carried a silent Oct4–GFP transgene (GOF18PE) (Yeom et al., 1996). These cells were fused in a 1:1 ratio with unsynchronized or elutriated mouse ESC fractions and the resulting cells were plated at limiting dilution in drug-containing media for 12 days as described previously (Pereira et al., 2010) (Figure 2A). Puromycin-resistant hybrid colonies expressing alkaline phosphatase (AP+, Puro−) were enumerated and compared to values obtained with non-synchronized ESCs (Figure 2B). In parallel, some colonies were

Figure 1. Separation of Mouse ESCs According to Cell-Cycle Stage Using Counterflow Centrifugal Elutriation

(A) Unsynchronized mouse E14tg2A ESCs cells were subjected to counterflow centrifugal elutriation and sequential fractions (F8, F9, F12, F13, F16, denoting flow rates) were stained with propidium iodide (PI) to assess DNA content by FACS. Gates used to define cells in G1, S, or G2/M are indicated in the top panel. (B) BrdU labeling patterns that characterize successive stages of S phase in human B (hB) and mouse ESCs (mESC) are shown. Early S (I) phase is distinguished by a fine diffuse labeling of multiple euchromatic sites that gradually increase in number and intensity by stage II. Mid S phase (III) shows BrdU incorporation at the nuclear periphery (IIIa) and outlining nucleoli (IIIb), and in ESCs a pronounced increase in the overall number of foci (IIIc). In later stages (IV and V) large constitutive heterochromatin domains are evident. Scale bars, 5 μm. (C) The abundance of S-phase cells in each elutriated fraction was determined by BrdU pulse labeling (45 min, 100 μM) and anti-BrdU immunostaining. (D) Mouse ESCs enriched for G1 using counterflow centrifugal elutriation (F9) were cultured for 1–3 days to monitor progression through the cell cycle following elutriation. Unsynchronized ES cells are provided for comparison (in gray), and additional information is given in Figure S1.

References

Sullivan et al., 2006
Yeom et al., 1996
Pereira et al., 2010
expanded to evaluate Oct4-GFP expression, DNA content, karyotype, and the potential of the resulting hybrid cells to differentiate. This analysis indicated that S/G2-enriched fractions of mouse ESCs (F13) generated at least 5-fold more pluripotent hybrid colonies than G1-enriched fractions (F8) and supported the idea that ESCs at later stages of cell cycle have a more potent reprogramming capacity. This enhanced reprogramming (Figure 2B; Table S1) was not a reflection of intrinsic differences in the survival, cloning or fusion efficiency of S/G2, as shown in control experiments (Figures S2A and S2B). Importantly, hybrid clones generated by fusing mouse B cells with either unsynchronized or G2-enriched ESC re-expressed the Oct4-GFP transgene robustly (Figures S2C and S2D), were tetraploid (Figure S2D) and able to differentiate upon LIF withdrawal into mesoderm, endodermal, and ectodermal cell types (as exemplified by clones 1, 6, and 3,10, respectively; Figures S2D–S2F). Consistent with full reprogramming of hybrid cells, a bisulfite analysis of DNA methylation showed a near complete loss of methylated CpG residues across endogenous mouse Oct4 alleles by day 21 (Figure 2C). To estimate when after fusion this reprogramming-associated loss of DNA methylation had occurred, we next performed bisulfite analysis of the promoter region of the somatically derived Oct4-GFP transgene. This region is heavily methylated in the parental mouse B cells (97%, Day 0) but hypomethylated in reprogrammed hybrids (2% methylation, day 21). Time course experiments revealed that DNA demethylation of the reporter was evident as early as 3 days after fusion (Figure 2D), consistent with previous studies showing that Oct4 activation is an early event required for ESC fusion-mediated reprogramming (Han et al., 2008; Pereira et al., 2008).

Rapid and Potent Reprogramming of Human Somatic Cells Fused with S/G2-Enriched Mouse ESCs

To explore the possible mechanisms that underlie the improved reprogramming capacity of S/G2-enriched mouse ESCs we performed heterokaryon analyses using human B cells or human fibroblasts as targets (Figure 3A). This approach allows the earliest steps in reprogramming to be followed by employing a combination of species-specific antibodies, fluorescence in situ hybridization (FISH) probes, and RT-PCR primers to discriminate events that occur within individual mouse and human (somatic) nuclei after cellular fusion (Pereira et al., 2008; Yeom et al., 1996). B cells were predominantly in G1 (≥ 75%), as judged by PI staining. Successfully fused cells were plated in media supplemented with puromycin for 21 days. Immediately following cell fusion, the parental mESC and mB nuclei remained discrete within a single cell body supported by a shared cytoplasm for up to 3 days. Subsequently, the nuclei fuse giving rise to a proportion of stable proliferating hybrid cells resistant to puromycin and positive for alkaline phosphatase activity and Oct4 expression. The reporter was evident as early as 3 days after fusion (Figure 2D), consistent with previous studies showing that Oct4 expression is an early event required for ESC fusion-mediated reprogramming.
Figure 3. Mouse ESCs Enriched for S and G2 Phases of the Cell Cycle Efficiently Reprogram Human B Lymphocytes and Fibroblasts in Heterokaryons

(A) Experimental strategy for generating interspecies heterokaryons. Mouse ESCs (mESC) were fused in a 1:1 ratio with human B (hB) or human fibroblasts (hF) cells using PEG and cultured in ESC media supplemented with puromycin.

(B) mESCs enriched according to cell-cycle stage (G1 = F8/9, G1/S = F11/12, S/G2 = F13/14, and G2/M = F15/16) were fused with hB cells and hES-specific gene expression was assessed using RT-qPCR and species-specific primers. Gene expression was calculated relative to GAPDH, using the human ESC cell line NCL1 as a positive control (Pereira et al., 2008). Two independent experiments are shown as examples.

(C) The potency of mESCs to induce OCT4, NANOG, and CRIPTO expression from hB cells, 2 and 3 days after fusion was assessed in seven independent experiments. Values were normalized to G2-enriched samples, error bars denote SE from the mean and values statistically different from G2 (p < 0.05; single sample t test) are marked with an asterisk.

(D) In similar experiments using human fibroblasts as targets, human pluripotency gene induction was assessed (as above), and expression levels at day 0 (white bars) and day 4 (black bars) are shown. Error bars denote SE from the mean of three independent experiments, and asterisks indicate a significant difference with a p value (t test) of < 0.05. See also Figure S3 and Table S2.

Low levels of human OCT4 (POU5F1), NANOG, CRIPTO (Figure 3B), DNMT3b, REX1, FGFR1, FGF2, and TLE1 (Figure S3A) transcripts were detected 2 days after fusion with mouse ESCs (Pereira et al., 2010, 2008). The expression of human pluripotency genes was consistently higher in fusions performed with S/G2 ESCs compared with either G1 or asynchronous cells (Figures 3B and S3B). Transcripts were detectable in these heterokaryons was compared using qPCR (Pereira and Fisher, 2009).

The expression of human pluripotency genes was consistently higher in fusions performed with S/G2 ESCs as compared with either G1 or asynchronous cells (Figures 3B and S3B, top). This enhanced reprogramming capacity of late S/G2 and G2-enriched mouse ESC fractions was reproducible and statistically significant (Figure 3C).

To determine whether somatic targets other than lymphocytes were also susceptible, we also fused elutriated mouse ESC fractions with human fibroblasts. As shown in Figures 3D and S3B (lower), reprogramming of fibroblasts was also significantly enhanced following fusion with S/G2- and G2-enriched mESCs, as indicated by increased induction of human OCT4, NANOG, and CRIPTO transcripts.

Piccolo et al., 2011). Human B cells were fused in a 1:1 ratio with unsynchronized or G1-, S-, G2-enriched mouse ESC fractions and expression of human pluripotency genes induced within significantly enhanced following fusion with S/G2- and G2-enriched mESCs, as indicated by increased induction of human OCT4, NANOG, and CRIPTO transcripts.
To understand the basis of this improved reprogramming capacity we initially examined the possibility that factors that are known to potentiate or inhibit iPS-based reprogramming (Yamanaka and Blau, 2010) might fluctuate during ESC cell cycle. We were, however, unable to detect any significant changes in the levels of mouse Oct4, Klf4, Sox2, c-Myc, Nanog, p53, or p21 transcripts in cell-cycle-enriched mouse ESCs (Figure S3C) and protein levels remained unchanged for most candidates (Figure S3D). Although western blotting and immunofluorescence analysis showed slight increases in Oct4 and Sox2 levels upon cell-cycle progression (Figures S3D and S3E; Tables S2A and S2B), careful comparison of unsynchronized and G2-enriched samples indicated broadly comparable levels in both (Figure S3D, red box). We did not see any experimental evidence that these factors were either prematurely dissociated from the chromatin or exported to the cytoplasm ahead of mitosis (Figure S3F). Likewise, Nanog expression, which displays a characteristic periodicity in ESC cultures (Chambers et al., 2007) and has been shown to enhance experimental reprogramming (Silva et al., 2006; Theunissen et al., 2011), was independent on ESC cell-cycle stage (Table S2C; Figures S3C and S3D).

S/G2 ESCs Induce Precocious Nucleotide Incorporation in Somatic Nuclei Shortly after Fusion
An alternative explanation for the superior efficiency of S/G2-enriched ESCs to reprogram somatic cells is that S- or G2-phase cells were capable of inducing premature DNA synthesis and chromosome condensation in G1-phase targets (Johnson and Rao, 1970; Rao and Johnson, 1970) and that this may facilitate chromatin remodeling, DNA demethylation, and activation of critical genes, as has been previously suggested (De Carvalho et al., 2010; Mikkelsen et al., 2008). To examine this possibility, we fused human B and mouse ESCs in a 1:1 ratio and after 24 hr applied a pulse of BrdU (45 min, 100 μM) to enumerate nuclei in these cultures that were undergoing DNA synthesis. Heterokaryons containing BrdU-labeled nuclei were then visualized by DAPI (blue), phalloidin (red), and anti-BrdU (green) containing as shown in Figure 4A. Most heterokaryons contained...
Within 1 day Heterokaryons Formed with Mouse ESCs

| Experiment | BrdU+ hB | Sample Size | % |
|------------|---------|-------------|---|
| 1 | hB × unsynchronized mESC | 8 | 24 | 33 |
|  | hB × S/G2-enriched mESC | 30 | 35 | 86 |
| 2 | hB × unsynchronized mESC | 11 | 23 | 48 |
|  | hB × S/G2-enriched mESC | 14 | 19 | 74 |
| 3 | hB × unsynchronized mESC | 10 | 30 | 33 |
|  | hB × S/G2-enriched mESC | 27 | 35 | 77 |
|  | hB × G1-enriched mESC | 0 | 80 | <1.3 |

**DNA Replication Is Critical for Initiating Successful Reprogramming of Somatic Nuclei toward Pluripotency in ESC-Derived Heterokaryons**

To evaluate whether DNA synthesis was required to initiate pluripotent gene expression from somatic nuclei, we fused human B cells and mouse ESCs and then monitored human pluripotent gene induction following treatment with agents that block DNA synthesis. Treatment of heterokaryons with aphidicolin (a drug that inhibits DNA polymerase activity), mimosine (that arrests cells in late G1) or with hydroxyurea (to prevent late origin firing) for 48 hr severely compromised the induction of a panel of human pluripotency genes as compared with untreated controls (Figure 4C, top). DNA demethylation at endogenous human OCT4 alleles was evident in untreated cultures, as indicated by increased sensitivity to HpaII digestion (Figure 4C, bottom, black). However, in parallel cultures in which DNA polymerase activity was inhibited by aphidicolin treatment, we detected no corresponding change in sensitivity of the OCT4 locus (Figure 4C, bottom, red). Taken together, these data suggested that precocious DNA synthesis within somatic nuclei was critical for remodeling and demethylating the human OCT4 locus prior to its reactivation in heterokaryons.

**Oct4 Re-Expression and Precocious DNA Synthesis**

The incidence of precocious DNA synthesis among successfully reprogrammed ESC heterokaryons was evaluated in BrdU tracing experiments, using Oct4-GFP as a reporter. Briefly, mouse B cells carrying Oct4-GFP were fused with nonsynchronized ESCs and pulse labeled with BrdU (100 μM, 45 min) at different times early after fusion (at 6, 18, or 24 hr, or double-pulsed at 6 and 18 hr), washed and returned to culture. Two to 3 days after fusion heterokaryons that contained “reprogrammed lymphocytes” were identified on the basis of GFP re-expression and then examined to ask whether these cells retained BrdU that had been acquired during the time window of the pulse. A schematic representation of this experiment is depicted in Figure 5A. As shown in Figure 5B and illustrated in Figure 5C, most Oct4-GFP positive heterokaryons (green) identified 2-3 days after fusion had incorporated BrdU (red) within the first few hours of fusion. Specifically, we demonstrated that about a third of all successfully reprogrammed cells were marked by BrdU applied 6 hr postfusion and about two thirds of all successfully reprogrammed cells were marked by BrdU...
applied 18 hr postfusion. By applying a double pulse of BrdU at 6 and 18 hr after fusion, virtually all (90%) successfully reprogrammed B cells identified on the basis of Oct4 re-expression, were shown to have undergone DNA synthesis within a day of fusion with ESCs.

**DISCUSSION**

Here we provide quantitative evidence that dominant reprogramming of somatic cells by ESCs is enhanced using S/G2-enriched samples. This extends claims made from hybrid studies (Sullivan et al., 2006) showing that the potency of S/G2 ESCs occurs in heterokaryons when pluripotent gene expression is initiated. We found no evidence that the improved reprogramming efficiency of S/G2 ESCs was due to reprogramming factors present in cells at later stages of the cell cycle or their “release” into the cytoplasm prior to mitotic chromosome condensation. Rather, our data indicate that ESCs in S/G2 induce B cells or fibroblasts to undergo DNA synthesis within a day of fusion and heterokaryon formation. This is important for reprogramming as inhibitors of DNA synthesis such as hydroxyurea, mimosine and aphidicolin, blocked pluripotent gene induction in heterokaryons. We also found that virtually all heterokaryons that re-expressed a somatically derived Oct4-GFP transgene showed evidence of premature DNA synthesis occurring within the first day after fusion. Collectively, this indicates that early DNA synthesis is a critical feature of successful stem cell fusion-mediated reprogramming that has not been fully appreciated until now.

Interestingly, reprogramming studies using nuclear transfer have indicated that cell-cycle synchronization between the donor nucleus and recipient cytoplasm appears to be important (Campbell and Alberio, 2003; Campbell et al., 1996) and the ability of mammalian embryonic cytoplasm to support reprogramming has been shown to fluctuate with the cell cycle (Egli et al., 2007). Although synchronization of the cell cycle is not sufficient to determine successful reprogramming in heterokaryons per se (i.e., G1-phase ESCs do not efficiently reprogram G1-phase B cells), our data may highlight the importance of cell-cycle “compatibility” between nuclei inducing and nuclei undergoing reprogramming. Consistent with classical studies of cell-cycle progression (Blow and Laskey, 1988) and the cell fusion experiments reported by Johnson and Rao (1970) and Rao and Johnson (1970), we showed that fusing S/G2-phase ESCs with somatic cells induced precocious DNA synthesis in somatic nuclei. As 70%–75% of cultivated human B cells are in G1, most of these targets would be expected to be already licensed for DNA replication (reviewed in Blow and Dutta, 2005) and therefore susceptible to S-phase promotion by S/G2 ESCs. In this regard, recent experiments using Xenopus egg extracts at the metaphase (M phase) stage have also shown that M phase can both drive DNA synthesis and improve reprogramming efficiency in nuclear transfer and in IPS assays (Ganier et al., 2011). IPS-based studies have, in addition, indicated that reprogramming can be accelerated by DNA synthesis and cell division (Hanna et al., 2009) as well as by agents that inhibit histone deacetylation (Huangfu et al., 2008) or interfere with the maintenance of DNA methylation (De Carvalho et al., 2010; Feng et al., 2009; Mikkelsen et al., 2008).

The demonstration that nucleotide incorporation is widespread among somatic nuclei in heterokaryons raises the possibility that DNA demethylation of genes that are critical
for reprogramming, such as Oct4 (Simonsson and Gurdon, 2004) could be achieved by replication-dependent (passive) means. Previous studies have implicated AID-mediated deamination in active DNA demethylation in heterokaryons (Bhutani et al., 2010), and Gadd45 (growth arrest and DNA damage 45 protein) in active DNA demethylation during differentiation and stress response (Niehrs and Schäfer, 2012). In preimplantation embryos, both active and passive mechanisms have been implicated in genome-wide DNA demethylation in which Tet3-mediated conversion of 5-methylcytosine to 5-hydroxy-methylcytosine appears to be central (Gu et al., 2011; Inoue and Zhang, 2011). In ESCs, Tet1 and Tet2 family members are actively expressed (Ficz et al., 2011; Wu and Zhang, 2011; Xu et al., 2011) and recent studies have implicated Tet2 and Parp1 in iPS-based reprogramming (Doege et al., 2012). In an accompanying manuscript (Piccolo et al., 2013) we show that Tet1 and Tet2 participate in ESC- and EG-mediated heterokaryon reprogramming and are required to reset DNA methylation within the somatic genome. Interestingly, as conversion of 5-methylcytosine to 5-hydroxy-methylcytosine can occur in the presence of drugs that inhibit DNA replication (Piccolo et al., 2013), it seems likely that both active (DNA replication-independent) and passive (DNA replication-dependent) mechanisms may contribute to pluripotent reprogramming in ESC heterokaryons. This duality may be helpful in reconciling some of the conflicting data arising from reprogramming studies in vivo, as well as providing an explanation for the recent proposal that heterokaryon-mediated reprogramming is mechanistically bifasic (Foshay et al., 2012).

The observation that early DNA synthesis is prevalent in somatic nuclei fused with ESCs is however at odds with a previous report in which BrdU incorporation was not seen in heterokaryons formed between human fibroblasts and mouse ESCs (Bhutani et al., 2010). Although we do not at present have an explanation for this, it is unlikely that this discrepancy is due to the use of different somatic cells or to interspecies incompatibilities as we observed extensive BrdU incorporation using both human and mouse fibroblasts. In our hands AID expression (a putative mediator of 5-methylcytosine demethylation) was not detectable in fused or unfused cells, as has been reported by others (Foshay et al., 2012). Regardless of the explanation for these differences, our study offers a fresh perspective on how reprogramming works as well as providing a simple and reliable method for enriching ESCs at different cell-cycle stages. This will be important for future reprogramming studies and to better understand the importance of the unusual cell-cycle structure in pluripotent stem cell self-renewal.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

E14Tg2a Hprt−/−/E14 mouse ESCs, EBV-transformed human B cells, and Abelson-transformed mouse B cells were cultured as described previously (Pereira et al., 2008).

**Quantitative RT-PCR Analysis**

RNA extraction and RT-qPCR was carried out as described previously (Pereira et al., 2008; primer sequences shown in Table S1).
proteinase K treatment (30 min at 40°C). HpaII-resistance was quantified using qPCR and primers that flanked an HpaII/MspI-sensitive site in the promoter region of OCT4. Ct values were normalized to a region lacking an HpaII/MspI site and to untreated controls. HpaII resistance was calculated as the percentage difference between HpaII (test) and MspI (total) digested samples. Primers are available in Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.01.012.

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