Reprogramming towards pluripotency requires AID-dependent DNA demethylation

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Abstract

Reprogramming of somatic cell nuclei to yield induced pluripotent stem (iPS) cells makes possible derivation of patient-specific stem cells for regenerative medicine. However, iPS cell generation is asynchronous and slow (2–3 weeks), the frequency is low (, 0.1%), and DNA demethylation constitutes a bottleneck. To determine regulatory mechanisms involved in reprogramming, we generated interspecies heterokaryons (fused mouse embryonic stem (ES) cells and human fibroblasts) that induce reprogramming synchronously, frequently and fast. Here we show that reprogramming towards pluripotency in single heterokaryons is initiated without cell division or DNA replication, rapidly (1 day) and efficiently (70%). Short interfering RNA (siRNA)-mediated knockdown showed that activation-induced cytidine deaminase (AID, also known as AICDA) is required for promoter demethylation and induction of *OCT4* (also known as *POU5F1*) and *NANOG* gene expression. AID protein bound silent methylated *OCT4* and *NANOG* promoters in fibroblasts, but not active demethylated promoters in ES cells. These data provide the first evidence that mammalian AID is required for active DNA demethylation and initiation of nuclear reprogramming towards pluripotency in human somatic cells.

Reprogramming of somatic cell nuclei towards pluripotency has been achieved by nuclear transfer into enucleated oocytes[1,2] and the introduction of four defined factors to generate iPS cells[3-6]. These remarkable advances enable the generation of patient-specific cells for tissue replacement, modelling human diseases in tissue culture, and drug discovery. However, an understanding of the molecular mechanisms underlying nuclear reprogramming has been elusive, largely due to the technical challenges associated with nuclear transfer and the low efficiency of iPS cell generation.

To identify new early regulators essential for nuclear reprogramming towards pluripotency, we capitalized on our previous experience with heterokaryons–cell fusion products that proved useful in determining the principles inherent to the maintenance of the differentiated state of somatic cells[7-11]. Specifically, these earlier studies, by us and others, showed that the 'terminally differentiated' state of human cells was not fixed, but could be altered, and the expression of previously silent genes typical of other differentiated states could be induced[11-15]. We showed that reprogramming in heterokaryons was influenced by DNA methylation status, tissue of origin, and the relative ratio of nuclei that dictates the balance of regulators[11,15-17], consistent with recent experiments in iPS cells[18-23]. We reasoned that heterokaryons, generated by fusing mouse ES cells and human fibroblasts, could be used to elucidate mechanisms and identify new genes with a role at the onset of reprogramming towards pluripotency because reprogramming takes place in the presence of all ES cell factors, and the onset of reprogramming is synchronously initiated after fusion. The advantages of interspecies heterokaryons over previously used same-species cell fusion hybrids[24,25] are that species differences allow a distinction of transcripts derived from the two fused cell types, and reprogramming is assessed immediately after fusion without the need for cell division or DNA replication. Heterokaryons constitute a third complementary approach to nuclear reprogramming that overcomes some of the limitations of nuclear transfer and iPS cell generation by facilitating mechanistic studies.

The use of heterokaryons in this report allowed us to study epigenetic and transcriptional changes critical to the initiation of reprogramming towards pluripotency. We focused on DNA demethylation–a known block to reprogramming that leads to partially reprogrammed iPS cells[23], and also a key step for reprogramming by nuclear transfer[26]. Despite decades of effort, so far no consensus mammalian DNA demethylase has been identified[27]. Recently, AID has been implicated in DNA demethylation in zebrafish within hours after fertilization, acting in a complex that mediates deamination followed by DNA repair[28]. In mammals, AID is primarily known for its role in the generation of antibody diversity in Blymphocytes[29], but has recently been detected in germ cells[30]. Our studies demonstrate a new role for AID in active DNA demethylation and reprogramming of mammalian somatic cells towards pluripotency.

Efficient reprogramming towards pluripotency in heterokaryons

To produce interspecies heterokaryons, mouse ES cells transduced with a green fluorescent protein (GFP) reporter gene were co-cultured with primary human fibroblasts transduced with a DsRed reporter gene, and fused using polyethylene glycol (PEG) (Fig. 1a and scheme in Supplementary Fig. 1). Fused GFP+ DsRed+ heterokaryons, which were readily sorted by fluorescence-activated cell sorting (FACS) (Fig. 1b) and identified using fluorescence microscopy, contained distinctly stained human and mouse nuclei when visualized with Hoechst dye (Fig. 1c, f). Because the efficiency of PEG fusion is low (0.6–1.0%), GFP+ DsRed+ heterokaryons were sorted twice and enriched to 80% purity (Fig. 1b). Using an antibody for Ki67, a nuclear protein present only in proliferating cells, we determined that cell division did not occur in 98±2% (mean±s.e.m.) of heterokaryons over the 3-day time period assayed after fusion (Fig. 1d, e). Furthermore, BrdU labelling was not detected in 94±4% of heterokaryons over the same time period, indicating the absence of DNA replication (Fig. 1f, g and Supplementary Figs 2 and 3). To favour reprogramming towards a pluripotent state, we skewed the ratio of the input cells so that ES cells outnumbered the fibroblasts as gene dosage and the proportion of proteins contributed by each cell type determines the direction of nuclear reprogramming in somatic cells[16,31].

To determine whether ES-cell-specific genes were induced in the human fibroblast nuclei, the induction of human *OCT4* and *NANOG* was assayed relative to ubiquitous *GAPDH* using species-specific primers (Supplementary Fig. 4). Messenger RNA isolated from sorted

heterokaryons 1, 2 and 3 days after fusion was assessed by reverse transcription followed by semiquantitative PCR (RT–PCR) and real-time PCR (Fig. 2a,b). The day 0 controls tested initially were (1) human fibroblasts alone, (2) pre-PEG, unfused co-cultures of mouse ES cells and human fibroblasts, or (3) human fibroblasts treated with PEG to control for the effects of PEG and fusion. All of these day 0 controls gave similar results (data not shown) and the unfused co-culture control was used for all subsequent experiments. Notably, the induction of both human *OCT4* and *NANOG* transcripts was evident as early as day 1 after fusion in heterokaryons but not in controls (Fig. 2a, b and Supplementary Fig. 5), indicating that the onset of expression of two key human pluripotency genes is rapid in heterokaryons. By day 1, expression of human *OCT4* and *NANOG* (normalized to *GAPDH* in the same samples), had increased 5-fold relative to the control (day 0) and persisted at 10-fold higher levels on days 2 and 3 (Fig. 2b). Human-specific primers were used to determine whether other key pluripotency genes in addition to *OCT4* and *NANOG* were induced using real-time PCR, including *KLF4*, *ESRRB*, *TDGF1* (also known as *CRIPTO*), *SOX2* and *c-Myc* (also known as *MYC*) (Supplementary Fig. 6). *ESRRB*[32] and *TDGF1* (ref. [33]), which have been shown to be essential for maintaining ES cell self-renewal[32] and are targets of OCT4 and NANOG[34], were found to be upregulated 3-fold and 2.5-fold, respectively, in heterokaryons on day 2 after fusion (Supplementary Fig. 6). *SOX2* is already expressed in human fibroblasts and its promoter is extensively demethylated pre-fusion, in agreement with findings in mouse fibroblasts[23]; its expression did not increase after fusion. Expression of *KLF4* (ref. [35]), which is functionally interchangeable with ESRRB, did not change in heterokaryons at day 2 after fusion (Supplementary Fig. 6).

To assess the efficiency of nuclear reprogramming in human fibroblasts after fusion, single FACS-sorted heterokaryons were analysed by nested RT–PCR for the three human transcripts *OCT4*, *NANOG* and *GAPDH*(control), using two sets of human-specific primers in each case (Fig. 2c). As expected, no human gene products were detected in mouse ES cells (control), and only human *GAPDH* was detected in human fibroblasts (control) (Supplementary Fig. 4). Notably, 70% of single FACS-sorted heterokaryons from three independent fusion experiments isolated on day 3 after fusion expressed both human *OCT4* and *NANOG* (Fig. 2c, d and Supplementary Fig. 7), showing that a high proportion of heterokaryons initiated reprogramming towards pluripotency. This is in marked contrast to the slow and inefficient induction of *OCT4* and *NANOG* expression in iPS cells (, 0.1% of the total population) in 2–3 weeks[3-5,36].

Active DNA demethylation in heterokaryons

Because DNA demethylation has been shown to be a major limiting step in reprogramming fibroblasts towards iPS cells[23], we analysed the time course and extent of demethylation of the human *OCT4* and *NANOG* promoters in heterokaryons. DNA was isolated from heterokaryons on days 1, 2 and 3 after fusion and subjected to bisulphite conversion. Human *OCT4* and *NANOG* promoter regions were amplified by PCR using human- and bisulphite-specific primers (Supplementary Fig. 4) and the products were cloned and sequenced. DNA demethylation was evident at the human *OCT4* and *NANOG* promoters and progressively increased through day 3 (Fig. 3a). In contrast, the β-globin (HBB) HS2 locus remained methylated throughout, indicating that the DNA demethylation was specific (data not shown). The time course and progressive accumulation of demethylated CpG sites in the human *OCT4* and *NANOG* promoters (Fig. 3b) parallel the progressive increase in transcript accumulation observed over the same 3-day time period using real-time PCR (Fig. 2b). Notably, promoter demethylation and activation of pluripotency genes in human somatic cells take place in the absence of Ki67 or BrdU labelling (Fig. 1e, g); thus, demethylation is active and independent of cell division and DNA replication.

AID requirement for DNA demethylation and pluripotency

We focused on AID as a candidate factor with a role in mammalian DNA demethylation as it has been detected in mammalian pluripotent germ cells[30] and implicated in DNA demethylation in zebrafish post fertilization[28]. We assayed mouse ES cells and human fibroblasts for AID expression using real-time PCR. Although AID expression in somatic cells is generally thought to be restricted to B lymphocytes, we detected *AID* mRNA in human fibroblasts as well as mouse ES cells, albeit at greatly reduced levels (5% and 15%, respectively) compared to Ramos, a B-lymphocyte cell line (Supplementary Fig. 8). To investigate the role of AID in these cells, we performed a transient knockdown of mouse and human *AID* mRNAs by transfection of three distinct, non-overlapping siRNAs to different sequences within the *AID* coding region, and a fourth siRNA specific to the non-coding 3′ untranslated region (UTR) of *AID*, to rule out off-target effects and ensure that the results were specific to *AID* (Supplementary Fig. 9). A fifth siRNA with 50% identity to the *AID* coding region was used as a control. The extent and timing of knockdown was first confirmed in control mouse ES cells in which siRNA-1, -2, -3 and -4 reduced *AID* transcripts at day3 post-transfection as compared to the control siRNA (Supplementary Fig. 10, top). AID protein was detected in ES cell lysates. Knockdown of AID protein by 88% correlated well with the mRNA reduction by 81% (Supplementary Fig. 11). In human fibroblasts, *AID* transcripts were reduced by all of the siRNAs (Supplementary Fig. 10, bottom). These data show that AID is present and can be efficiently reduced by four distinct siRNAs in both ES cells and fibroblasts.

To assess the initiation of reprogramming in heterokaryons subjected to AID knockdown, the expression of human *OCT4* and *NANOG* relative to *GAPDH* was assessed by real-time PCR. For heterokaryon experiments, siRNAs were transfected into both the mouse ES cells and the human fibroblasts 24 h before fusion (see Supplementary Fig. 1 for scheme). A persistent knockdown of *AID* was detected by real-time PCR in heterokaryons using each of the four siRNAs on days 2 and 3 after fusion relative to heterokaryons transfected with the control siRNA (Fig. 4a). Notably, both *OCT4* and *NANOG* gene induction was greatly inhibited using siRNA-1 and siRNA-2 on day 2 after fusion, and siRNA-3 and siRNA-4 on day 3 after fusion, as compared to using the control siRNA (Fig. 4a). All siRNAs resulted in a similar block of *OCT4* and *NANOG* activation. These observations indicate that the effect of AID is extremely dosage-sensitive, as a 35% knockdown led to a similar inhibition of pluripotency gene induction as a 96% knockdown. Together, these data show that all four siRNAs to *AID* used here efficiently inhibited *OCT4* and *NANOG* activation by at least 80%.

To assess the effect of AID knockdown on promoter demethylation, we assayed the CpG methylation status of the human *OCT4* and *NANOG* promoters in heterokaryons. Both day 2 and 3 heterokaryons subjected to AID knockdown using each of the four siRNAs showed a profound inhibition of CpG demethylation in the human *OCT4* and *NANOG* promoters as compared to the control (Fig. 4b, c). A summary of the bisulphite sequencing data for all the siRNA knockdown experiments is shown in Supplementary Fig. 12. In parallel with the reduction in demethylation of the *OCT4* and *NANOG* promoters after AID knockdown, the induction of *OCT4* and *NANOG* transcripts was reduced by at least 80% on days 2 and 3, relative to the control (Fig. 4a). These data show that promoter demethylation is critical to the expression of these two pluripotency genes and that AID is required for mammalian DNA demethylation in somatic cell reprogramming.

To investigate further the requirement and specificity of AID for initiating reprogramming, we tested its ability to accelerate reprogramming, and to rescue the DNA demethylation block caused by the siRNA knockdown in heterokaryons. Therefore, human AID (hAID) was transiently overexpressed in mouse ES cells before siRNA transfection (see scheme in Supplementary Fig. 1). In two separate experiments, when AID was overexpressed twofold or

fourfold relative to the control in heterokaryons at day 1 after fusion, there was no acceleration in promoter demethylation or reprogramming (Supplementary Fig. 13). However, after overexpression of hAID in heterokaryons undergoing transient knockdown by siRNA-3, there was a complete rescue of *NANOG* promoter demethylation and gene expression and a partial rescue of *OCT4* promoter demethylation and gene expression (Supplementary Fig. 14). The lack of reprogramming acceleration and partial rescue observed for *OCT4* could possibly be due to the kinetics of human *OCT4* and *NANOG* promoter demethylation, which in heterokaryons may require at least 1 day to occur, or by the lack of other factors that work together with AID. These data show that the added hAID is functional and rule out any non-specific effects of the siRNA, further confirming the specific and essential role of AID in DNA demethylation at the onset of reprogramming towards pluripotency.

Binding of AID to methylated pluripotency gene promoters

To validate the role of AID in DNA demethylation of human *OCT4* and *NANOG* promoters further, we tested whether AID specifically binds to these promoter regions by performing chromatin immunoprecipitation (ChIP) experiments using an anti-AID antibody, previously validated for ChIP[37]. The promoter regions assessed in ChIP experiments were designed to be within the *OCT4* and *NANOG* promoter regions that were analysed for CpG demethylation by bisulphite sequencing (Fig. 5 and Supplementary Fig. 15). In human fibroblasts, the ChIP analyses showed significant binding of AID to both human *OCT4* (sixfold) and human *NANOG* (eightfold) promoters (Fig. 5). Thus, AID binds to the heavily methylated promoter regions of human *OCT4* and *NANOG* in fibroblasts that undergo demethylation during reprogramming. As controls, AID binding to the promoter of the IgM constant region (Cμ) was significant, as expected[38], whereas no binding was observed for THY1.1 (also known as CD90.1), which is expressed in fibroblasts.

In contrast to fibroblasts, no AID binding was observed at the promoter regions of *Oct4* and *Nanog* in mouse ES cells despite the higher levels of AID protein in ES cells, presumably because these promoters are active and demethylated (Fig. 5). As controls, AID binding was detected at the promoter of *Cdx2*, a gene not expressed in undifferentiated ES cells[39], but was absent from the *p53* (also known as *Trp53*) promoter, as previously reported[37]. Together, these findings provide strong support for a direct involvement of AID in DNA demethylation and the sustained expression of human *NANOG* and *OCT4* leading to the onset of reprogramming towards pluripotency.

Discussion

DNA demethylation is essential to overcome gene silencing and induce temporally and spatially controlled expression of mammalian genes, yet no consensus mammalian DNA demethylase has been identified despite years of effort[27]. Evidence of DNA demethylation by 5-methyl-cytosine DNA glycosylases has been shown in plants[40,41], but mammalian homologues such as thymine DNA glycosylase (TDG) or the methyl-CpG-binding domain protein 4 (MBD4) have not exhibited comparable functions[42,43]. As an alternative to direct demethylation, it has been postulated that the deamination of cytosine followed by DNA repair could lead to DNA demethylation[44]. AID belongs to a family of cytosine deaminases (AID, APOBEC1, 2 and 3 subgroups) that have established roles in generating antibody diversity in B cells, RNA editing and antiviral response[45]. Both AID and APOBEC1 are expressed in progenitor germ cells, oocytes and early embryos and have a robust 5-methyl-cytosine deaminase activity *in vitro*[30], resulting in a T-G mismatch that is repaired through the base excision DNA repair (BER) pathway, and could theoretically lead to DNA demethylation without replication. Recently in zebrafish embryos, AID was implicated as a member of a tripartite protein complex along with MBD4 and GADD45A, causing cytosine deamination

and leading to base excision by MBD4 (ref. [28]). The third component GADD45A lacks enzymatic activity and its role in repair-mediated DNA demethylation and gene activation in *Xenopus* oocytes remains a matter of debate[46,47].

Our data provide new evidence implicating AID in active DNA demethylation in mammalian cells, and demonstrate that AID-dependent DNA demethylation is an early epigenetic change necessary for the induction of pluripotency genes in human fibroblasts. Knockdown of AID in heterokaryons prevented DNA demethylation of the human *OCT4* and *NANOG* promoters and the expression of these pluripotency factors by fibroblast nuclei. Indeed, initiation of nuclear reprogramming towards pluripotency was inhibited in human somatic fibroblasts when AID-dependent DNA demethylation was reduced, providing strong evidence that AID is a crucial regulator for the onset of reprogramming. The inhibitory effects of AID reduction were rescued by hAID overexpression with a complete rescue observed for *NANOG* and a partial rescue observed for *OCT4*. Moreover, AID binding was observed at silent methylated *OCT4* and *NANOG* promoters in fibroblasts but not in active unmethylated *OCT4* and *NANOG* promoters in ES cells, demonstrating its specific role in DNA demethylation. Previous studies that used cell fusion to analyse reprogramming to pluripotency could not distinguish the products of the two nuclei because the cells were derived from the same species[24,25]. A recent study used heterokaryons to reprogram lymphocytes towards pluripotency, however, only modest demethylation changes at the *OCT4* promoter were observed that did not increase with time[48], possibly owing to differences in the cell types and methods used for isolating heterokaryons. The high efficiency of reprogramming in heterokaryons achieved here allowed the discovery of a regulator critical to the induction of five pluripotency genes including *OCT4* and *NANOG*, the first known markers of stable reprogramming leading to the generation of iPS cells. The heterokaryon platform can now be exploited (1) to determine the other components of the mammalian DNA demethylation complex (glycosylase and other DNA repair enzymes) that are likely to work together with AID to mediate active DNA demethylation (Fig. 6), and (2) to perform an unbiased search for other regulators of nuclear reprogramming by screening for human genes that are expressed immediately after cell fusion. Future studies will determine whether expression of AID alone or in conjunction with these other molecules will enhance the generation of iPS cells.

METHODS SUMMARY

Heterokaryon generation and isolation

GFP+ mouse ES cells and DsRed+ human fetal lung primary fibroblasts were generated by transduction with retroviral constructs and fused to form non-dividing, multinucleated heterokaryons as previously described[31]. GFP+ DsRed+ heterokaryons were sorted twice by flow-cytometry (FACSVantage SE, BD Biosciences) and analysed for gene expression and methylation.

Immunofluorescence

For antibody staining, GFP+ DsRed+ heterokaryons were cytospun and stained. Images were acquired using an epifluorescent microscope (Axioplan2, Carl Zeiss MicroImaging), Fluar ×20/0.75 or ×40/0.90 objective lens, and a digital camera (ORCA-ER C4742-95, Hamamatsu Photonics). The software used for acquisition was OpenLab 4.0.2 (Improvision).

BrdU was added to mouse ES cell and human fibroblast co-cultures 3 h after PEG-induced fusion. Labelling and antibody staining was performed using the BrdU Labelling and Detection Kit I (Roche).

Single-cell RT–PCR

Single cells were directly sorted by FACS. Collection of cells and the subsequent two-step multiplex nested RT–PCR were performed directly from cell lysates using all three human and gene-specific primers (*OCT4*, *NANOG* and *GAPDH*) as described in Methods.

DNA methylation analyses

Bisulphite treatment was performed on DNA isolated from FACS sorted heterokaryons (2,000–10,000 cells) using the Epitect Bisulfite Kit (Qiagen). Nested PCR for regions of the human *OCT4* (ref. [18]) and *NANOG* promoters was performed using human- and bisulphite-specific primers. PCR products from second-round bisulphite-specific PCR amplification were cloned and sequenced as described previously[17].

siRNA transfection

For siRNA transfection, ES cells and primary fibroblasts were plated at 50–60% confluence the day before transfection. siRNAs (Dharmacon) were transfected using siImporter (Millipore).

ChIP

ChIP was performed as previously described[49]. ChIP data was presented as normalized to input DNA and as the mean± s.e.m.

Statistical analysis

Data are presented as the mean±s.e.m. Comparisons between groups used the Student's *t*-test assuming two-tailed distributions.

METHODS

Heterokaryon generation and isolation by flow cytometry

GFP+ mouse ES cells and DsRed+ human fetal lung primary fibroblasts were generated by transduction with retroviral constructs as previously described[31], and fused to form non-dividing, multinucleated heterokaryons. Cells were first co-cultured for 12 h in ES cell media and then treated with PEG 1500 (Roche) for 2 min at 37 °C, followed by four successive washes with DMEM. ES cell media was replaced after washing and every 12 h thereafter. GFP+ DsRed+ heterokaryons were sorted twice by flow-cytometry (FACSVantage SE, BD Biosciences) and analysed for gene expression and methylation.

Immunofluorescence

Heterokaryons were sorted twice in PBS with 2.5% (v/v) goat serum and 1 mM EDTA, and cytospun for 5 min. The cytospun GFP+DsRed+ heterokaryons were stained with Hoechst 33342, and imaged. For antibody staining, cytospun cells were fixed, permeabilized and blocked using 20% FBS in PBS. Cells were incubated with the mouse anti-Ki67 antibody (Dako Denmark A/S) at 1:100 dilution in blocking buffer for 1 h, rinsed three times in PBS, and then incubated with a goat anti-mouse Cascade blue secondary antibody (Millipore) at 1:500 dilution for 30 min, rinsed three times, mounted with Fluoromount-G and imaged. Images were acquired using an epifluorescent microscope (Axioplan2, Carl Zeiss MicroImaging), Fluar ×20/0.75 or ×40/0.90 objective lens, and a digital camera (ORCA-ER C4742-95, Hamamatsu Photonics). The software used for acquisition was OpenLab 4.0.2 (Improvision).

BrdU was added to mouse ES cell and human fibroblast co-cultures 3 h after PEG-induced fusion. Labelling and antibody staining was performed using the BrdU Labelling and Detection Kit I (Roche).

Analysis of gene expression

RNA was prepared from ES cells, fibroblasts and twice-sorted heterokaryons at different times post fusion or after siRNA treatment using the RNeasy micro kit (Qiagen). Total RNA for each sample was reverse transcribed using the Superscript First-Strand Synthesis System for RT–PCR (Invitrogen). The reverse-transcribed material was subjected to PCR using Go GreenTaq DNA polymerase (Promega). Human-specific primers were designed for analysing the expression of *OCT4*, *NANOG* and *GAPDH*. Primers used for *AID* and *GAPDH* in the siRNA treatment experiments amplify both human and mouse transcripts to assess the total levels of AID and *GAPDH* in heterokaryons. Human-specific primers used for RT–PCR and quantitative PCR are: *OCT4* F 5′-TCGAGAACCGAGTGAGAGGC-3′, R 5′-CACACTCGGACCACATCCTTC-3′; *NANOG* F 5′-CCAACATCCTGAACCTCAGCTAC-3′, R 5′-GCCTTC TGCGTCACACATT-3′; *GAPDH* F 5′-TGTCCCCACTGCCAACGTGTCA-3′, R 5′-AGCGTCAAAGGTGGAGGAGTGGGT-3′. Non-species-specific primer sequences for assessing knockdown after siRNA treatment are as follows: *GAPDH* F 5′-ACCACAGTCCATGCCATCAC-3′, R 5′-TCCACCACCCTGTTGCTGTA-3′; *AID* F 5′-AAAATGTCCGCTGGGCTAAG-3′, R 5′-AGGTCCCAGTCCGAGATGTAG-3′.

Real-time PCR

Real-time PCR was performed using an ABI 7900HT Real time PCR system using the Sybr Green PCR mix (Applied Biosystems). Samples were cycled at 94 °C for 2 min, 40 cycles of: 94 °C for 20s, 58 °C for 45 s.

DNA methylation analyses

FACS-sorted heterokaryons (2,000–10,000 cells) were collected in 20 μl PBS. DNA was extracted using the DNeasy Tissue Kit (Qiagen). Bisulphite treatment was performed using the Epitect Bisulfite Kit (Qiagen). Nested PCR for regions of the human *OCT4* and *NANOG* promoters was performed using human- and bisulphite-specific primers. Samples were cycled for the first and second nested PCR at 94 °C for 2 min, 30 cycles of 94 °C for 20 s, 61 °C for 30 s, 68 °C for 30 s. PCR products from second-round bisulphite-specific PCR amplification were cloned and sequenced as described before[17].

Single heterokaryon nested RT–PCR

Single-cell collection. Single heterokaryons were directly sorted by FACS (FACSVantage SE, BD Biosciences) into PCR tubes containing 9-μl aliquots of RT–PCR lysis buffer. The buffer components included commercial RT–PCR buffer (SuperScript One-Step RT–PCR Kit Reaction Buffer, Invitrogen), RNase inhibitor (Protector RNase Inhibitor, Roche) and 0.15% IGEPAL detergent (Sigma). After a short pulse-spin, the PCR-tubes were immediately shock-frozen and stored at −80 °C for subsequent analysis.

Two-step multiplex nested single-cell RT–PCR. Cell lysates were first reverse-transcribed using the human-specific primer pairs for *OCT4*, *NANOG* and *GAPDH* (Supplementary Table 2, external primers; Supplementary Fig. 1b) using SuperScript One-Step RT–PCR Kit (Invitrogen). In brief, the RT–PCR was performed in the same PCR cell-lysis tubes by addition of an RT–PCR-reaction mix containing the gene-specific primer pairs and RNase inhibitor. Genomic products were excluded by designing and using intron-spanning primer sets for the first and second round PCR and nested RT–PCR to ensure greater specificity. In the first step,

the reverse transcription reactions were carried out at 55 °C for 30 min, followed by a 2-min step at 94 °C. Subsequently, 30 cycles of PCR amplification were performed as follows: 94 °C for 30 s; 58 °C for 30 s; 68 °C for 30s. In the final PCR step, the reactions were incubated for 3 min at 68 °C. The completed reactions were stored at 4 °C.

In the second step of the PCR protocol, the completed RT–PCR reaction from the first step was diluted 1:1 with water. One per cent of these reactions were replica transferred into new reaction tubes for the second round of PCR, which was performed for each of the genes separately using nested gene-specific internal primers, for greater specificity, in a total reaction volume of 20 μl (Platinum Taq Super-Mix HF, Invitrogen). Thirty cycles of PCR amplification were performed as follows: 94 °C for 30 s; 58 °C for 30 s; 68 °C for 30 s. In the final PCR step, the reactions were incubated for 3 min at 68 °C. The completed reactions were stored at 4 °C. The second-round PCR products were then subjected to gel electrophoresis using one-fifth of the reaction volumes and 1.4% agarose gels.

THY1.1 enrichment of heterokaryons

GFP$^-$ (non-GFP) mES and DsRed$^+$ hFb co-cultures treated with PEG were trypsinized and resuspended in 3 ml FACS buffer. Cells were incubated for 30 min at room temperature with biotin mouse anti-human CD90.1 (BD Pharmingen) at a dilution of 1:5,000. The cells were washed once, resuspended in 3 ml FACS buffer, and incubated for 30 min at room temperature with 10 μl of Dynabeads Biotin Binder (Invitrogen). Beads were collected by magnetic isolation, washed twice and the enriched heterokaryons were cytospun.

Western blots

To visualize AID protein knockdown in mouse ES cells, cell lysates were collected 3 days after transfection with control siRNA or siRNA-3. Detection of AID in these samples was performed from 170 μg of whole cell lysate using anti mouse-AID (L7E7, Cell Signaling, dilution 1:500). The membrane was stripped and probed with anti-mouse α-tubulin (Sigma, dilution 1:20,000) for the loading control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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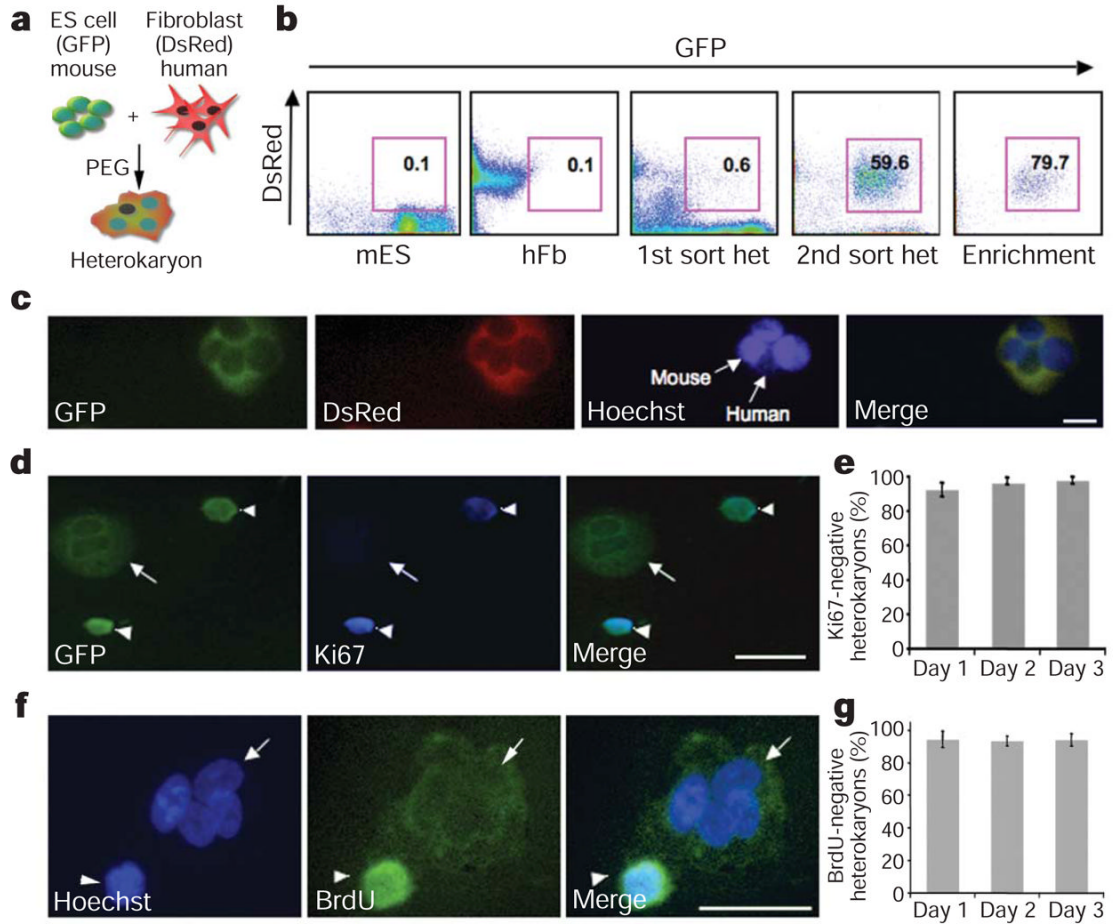
Figure 1. Absence of cell division and DNA replication in heterokaryons

a, Heterokaryon fusion scheme. GFP[+] mouse ES (mES) cells were co-cultured with DsRed[+] primary human fibroblasts (hFb) and then fused using PEG. b, FACS profiles of GFP[+] mES, DsRed[+] hFb, and GFP[+] DsRed[+] day 2 heterokaryons (het). c, Representative image of GFP[+] DsRed[+] day 2 heterokaryons. Hoechst 33342 (blue) denotes the nuclei. The heterokaryon shown has three distinct, unfused bright mouse nuclei, and one uniformly stained human nucleus. Scale bar, 50 μm. d, GFP[+] DsRed[+] day 2 heterokaryons were cytospun and stained for Ki67 (blue) to assess cell division. The GFP[+] heterokaryon has two distinct nuclei (arrow) that are negative for Ki67 (blue) in contrast to the mononuclear cells (arrowheads) that stain positive for Ki67. Scale bar, 50 μm. e, Heterokaryons on days 1, 2 and 3 after fusion were scored on the basis of Ki67 staining, and 98±2% heterokaryons were non-dividing (mean ±s.e.m., P, 0.05). **f,** Heterokaryons, generated using GFP[2] ES cells, were enriched using a human fibroblast marker THY1.1 (see Methods) on day 1 post fusion, and stained for BrdU (green) and nuclei (blue) using Hoechst 33258. The heterokaryon (arrow) has three uniformly stained human nuclei and one bright, punctate mouse nucleus, and is negative for BrdU staining. In contrast, the indicated human mononuclear cell (arrowhead) stains positive for BrdU. Scale bar, 50 μm. g, Day 1, 2 and 3 heterokaryons were scored on the basis of nuclear and BrdU staining. DNA replication did not occur in 94±3% heterokaryons (mean±s.e.m., P, 0.05).
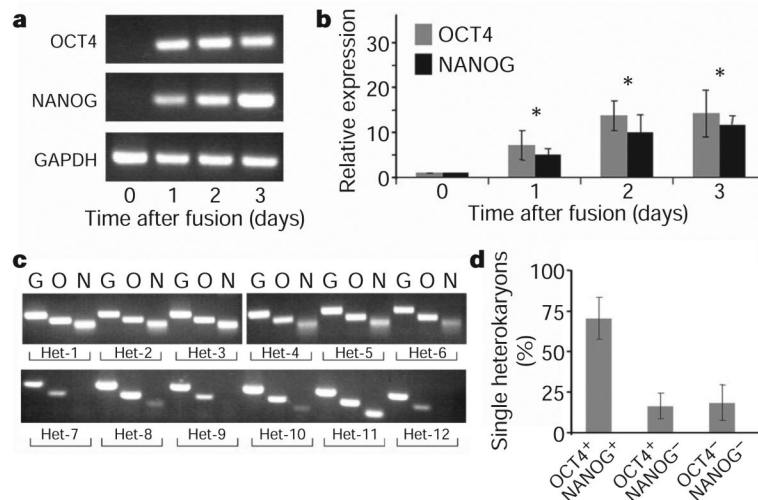
Figure 2. Time course of human fibroblast pluripotency gene expression in heterokaryons at the single-cell level

a, Human-specific primers against *OCT4*, *NANOG* and *GAPDH* were used for transcript analysis by RT–PCR of unfused co-cultures on day 0 and heterokaryons (mES 3 hFb) isolated on days 1, 2 and 3 after fusion. b, Real-time PCR to assess the upregulation of *OCT4* (grey) and *NANOG* (black) in day 1, 2 and 3 heterokaryons using human-specific primers (mean ± s.e.m.; *P, 0.03). Unfused co-cultures served as day 0 controls and the expression of *OCT4* and *NANOG* was normalized to *GAPDH* expression. Data shown are from three independent fusion experiments. c, Single heterokaryon nested PCR was used to assess the efficiency of reprogramming in the heterokaryon population. Direct reverse transcription and nested PCR were performed simultaneously on day 3 single heterokaryons, using human-specific primers for *GAPDH* (G), *OCT4* (O) and *NANOG* (N) as indicated. Twelve heterokaryons analysed from a single fusion experiment are shown. Supplementary Fig. 3 shows 41 heterokaryons analysed from two additional fusion experiments. d, The frequency of heterokaryons expressing both *OCT4* and *NANOG* is 70 ± 13%, showing that a high proportion of heterokaryons initiate reprogramming towards pluripotency. Data shown are a summary of three independent fusion experiments (mean ± s.e.m.).
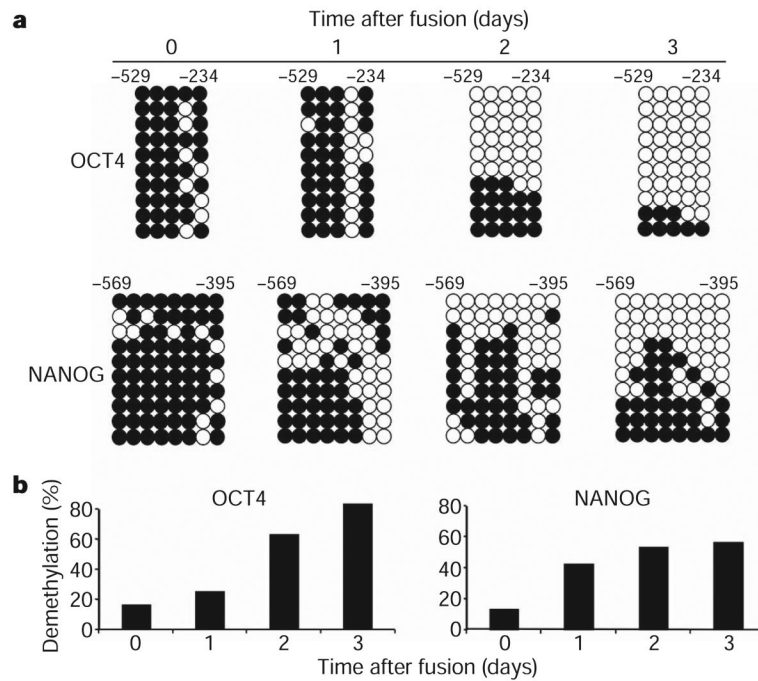
Figure 3. Time course of DNA demethylation at pluripotency gene promoters in heterokaryons
a, Bisulphite sequencing analysis of methylation status of the human *OCT4* and *NANOG*
promoter in heterokaryons. Both human *OCT4* and *NANOG* promoters in heterokaryons show
rapid and progressive DNA demethylation on days 1, 2 and 3 after fusion compared to the co-
culture control. White circles indicate unmethylated, black circles indicate methylated CpG
dinucleotides. **b,** The percentage of demethylation at the human *OCT4* and *NANOG* promoters
in heterokaryons after fusion shows a progressive increase in demethylation. Thirty clones
were analysed at each time point in two to three independent experiments; ten representative
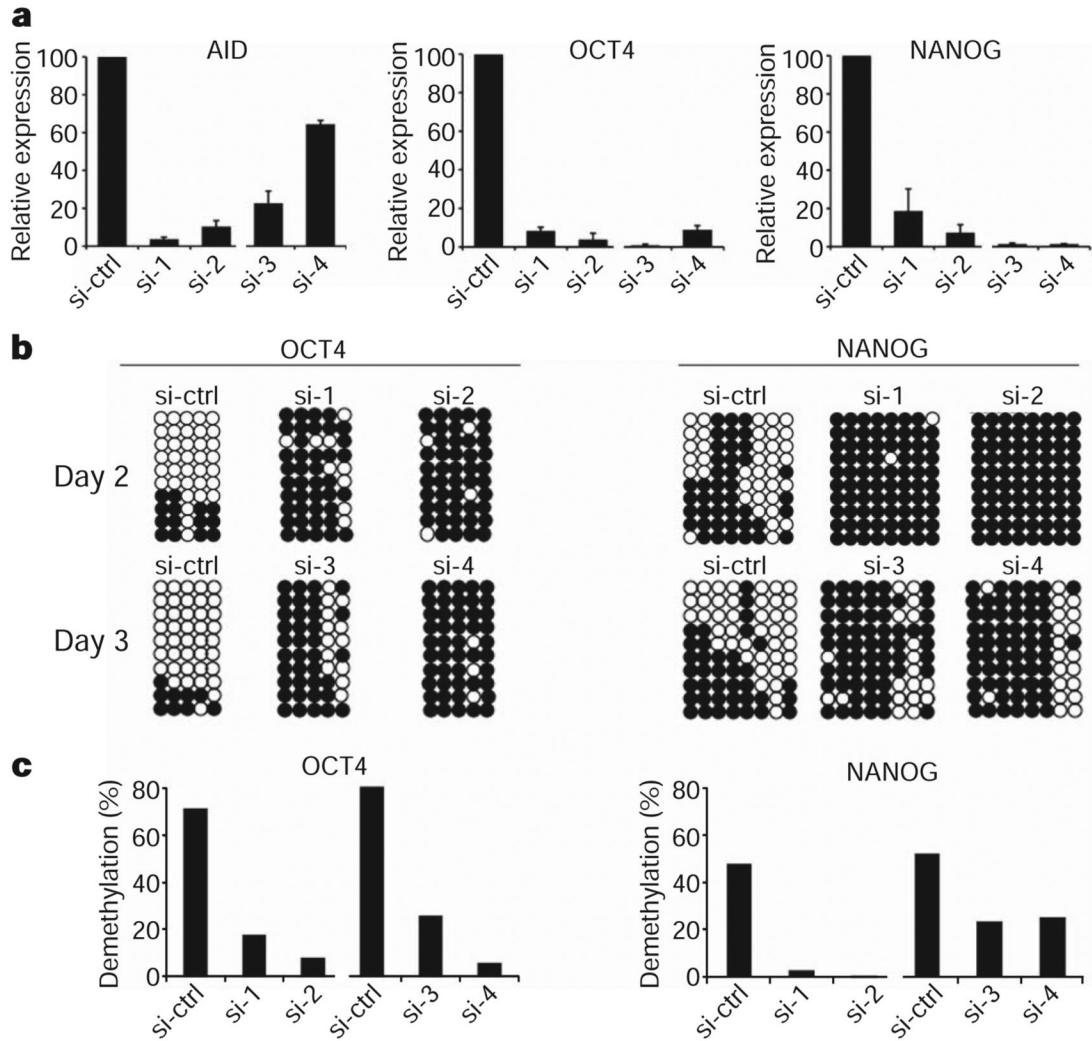clones are shown.

Figure 4. Requirement of AID-dependent DNA demethylation for initiation of reprogramming towards pluripotency in heterokaryons

a, *AID* and human pluripotency gene expression in heterokaryons subjected to siRNA treatment, as assessed by real time PCR. Day 2 heterokaryons were treated with siRNA-1 and -2 (si-1 and si-2), and day 3 heterokaryons were treated with si-3 and si-4. Total levels of mouse and human *AID* transcripts were assessed using a set of degenerate primers, whereas human-specific primers were used for human *OCT4* and *NANOG*. Gene expression was normalized internally to *GAPDH* (degenerate primers) for *AID* expression, and to human *GAPDH* for human *OCT4* and *NANOG* expression. The samples were then normalized to the corresponding day 2 or 3 sample treated with the control siRNA (si-ctrl), represented as 100%. **b,** Human *OCT4* and *NANOG* promoters on days 2 and 3 post fusion after AID knockdown remain methylated. **c,** The percentage of demethylation at the human *OCT4* and *NANOG* promoters after AID knockdown shows a block in demethylation compared to their respective day 2 and day 3 control samples treated with control siRNA.
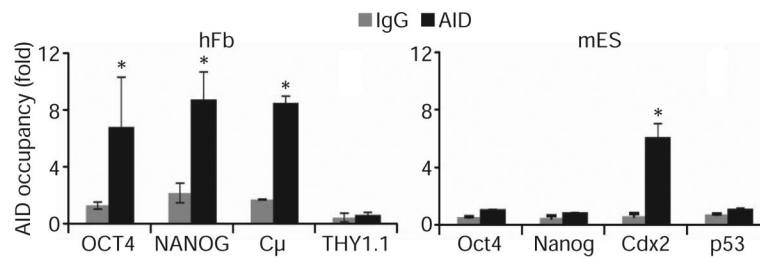
Figure 5. AID binding to pluripotency gene promoters by chromatin immunoprecipitation
ChIP with anti-AID antibody was performed in human fibroblasts (left) and mouse ES cells (right). AID occupancy is shown relative to background IgG signal (mean±s.e.m.). Significant AID binding was detected in human fibroblasts for the methylated *NANOG* and *OCT4* promoters as well as for the positive control Cμ ($P<0.002$). In mouse ES cells, AID binding was detected only in the positive control, *Cdx2* promoter region ($P<0.007$), whereas no significant binding was observed for the unmethylated *Oct4* and *Nanog* promoters.
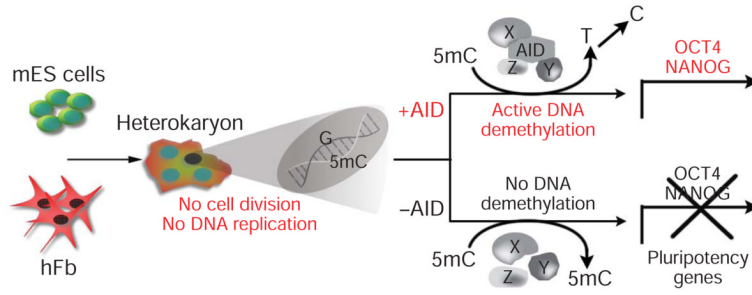
Figure 6. Model for AID-dependent active DNA demethylation in reprogramming
AID-mediated reprogramming and DNA demethylation in heterokaryons takes place in the absence of cell division or DNA replication, showing that AID is a component of an active DNA demethylation complex in mammals. The other putative components of this mammalian DNA demethylase complex (X, Y and Z) that may act together with the deaminase, AID, remain to be identified. 5mC, 5-methyl-cytosine.