Epigenetic Reprogramming of *OCT4* and *NANOG* Regulatory Regions by Embryonal Carcinoma Cell Extract[□]

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Analyses of molecular events associated with reprogramming somatic nuclei to pluripotency are scarce. We previously reported the reprogramming of epithelial cells by extract of undifferentiated embryonal carcinoma (EC) cells. We now demonstrate reprogramming of DNA methylation and histone modifications on regulatory regions of the developmentally regulated *OCT4* and *NANOG* genes by exposure of 293T cells to EC cell extract. *OCT4* and *NANOG* are transcriptionally up-regulated and undergo mosaic cytosine-phosphate-guanosine demethylation. *OCT4* demethylation occurs as early as week 1, is enhanced by week 2, and is most prominent in the proximal promoter and distal enhancer. Targeted *OCT4* and *NANOG* demethylation does not occur in 293T extract-treated cells. Retinoic acid-mediated differentiation of reprogrammed cells elicits *OCT4* promoter remethylation and transcriptional repression. Chromatin immunoprecipitation analyses of lysines K4, K9, and K27 of histone H3 on *OCT4* and *NANOG* indicate that primary chromatin remodeling determinants are acetylation of H3K9 and demethylation of dimethylated H3K9. H3K4 remains di- and trimethylated. Demethylation of trimethylated H3K9 and H3K27 also occurs; however, trimethylation seems more stable than dimethylation. We conclude that a central epigenetic reprogramming event is relaxation of chromatin at loci associated with pluripotency to create a conformation compatible with transcriptional activation.

INTRODUCTION

Reprogramming of a differentiated somatic cell into a pluripotent cell may have applications in regenerative medicine, and as such, several approaches are being examined to produce embryonic stem (ES)-like cells. Nuclear transplantation into oocytes has demonstrated that functional nuclear reprogramming is possible, through the production of nuclear transfer ES cells (Cibelli *et al.*, 1998; Munsie *et al.*, 2000; Wakayama *et al.*, 2001) and cloned animals (Wilmut *et al.*, 2002; Gurdon and Byrne, 2003). Fusion of somatic cells with ES or embryonal carcinoma (EC) cells also elicits a reprogramming of the somatic genome within the hybrids, demonstrated by X chromosome reactivation (Tada *et al.*, 2001), changes in gene expression profile, and acquisition of ES cell properties, including contribution to all germ layers in teratomas and in aggregation chimeras (Tada *et al.*, 1997, 2001; Pells *et al.*, 2002; Terada *et al.*, 2002; Ying *et al.*, 2002; Cowan *et al.*, 2005). Recently, retroviral transduction and constitutive expression of four factors (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*) was also shown to induce an ES cell-like behavior in mouse

fibroblasts, similar to that reported by fusion with ES cells (Takahashi and Yamanaka, 2006). A fourth approach to reprogramming entails treatment of reversibly permeabilized somatic cells with an extract of another differentiated cell type (Håkkelien *et al.*, 2002) or of undifferentiated, pluripotent ES or EC cells (Taranger *et al.*, 2005). Notably, epithelial 293T cells treated with extract of undifferentiated human EC (NCCIT) cells induces expression of genes associated with pluripotency, such as *OCT4* and *NANOG*; causes down-regulation of somatic cell-specific genes, such as lamin A (*LMNA*); and enhances in vitro differentiation capacity (Taranger *et al.*, 2005). From these observations, it is increasingly clear that exposure of a somatic genome to factors derived from pluripotent cells or eggs is sufficient to elicit partial or complete reprogramming of nuclear function.

All reprogramming approaches investigated to date seem to involve modifications of the epigenome. Methylation in the 5-position of a cytosine in a cytosine-phosphate-guanosine (CpG) dinucleotide is a heritable modification that favors genomic integrity, ensures proper regulation of gene expression, and is essential for long-term gene silencing (Antequera, 2003). Partial DNA demethylation in restricted areas in the *Oct4/OCT4* regulatory region has been reported previously (Tada *et al.*, 1997; Simonsson and Gurdon, 2004; Cowan *et al.*, 2005; Takahashi and Yamanaka, 2006), and it is proposed to be required for activation of the gene (Simonsson and Gurdon, 2004). These studies have been extended with the demonstration that the *Nanog* promoter is also demethylated in nuclear transfer ES cells (Blelloch *et al.*, 2006), in fibroblast-ES cell hybrids (Cowan *et al.*, 2005), and in transduced cells (Takahashi and Yamanaka, 2006). Additionally, acetylation and methylation of lysine (K) residues in the amino-terminal tail of histones H3 and H4, which regulate chromatin assembly on promoters and thereby promoter activation (Lachner and Jenuwein, 2002), have been shown in mouse

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Abbreviations used: EC, embryonal carcinoma; ES, embryonic stem; HBSS, Hanks' balanced salt solution; ChIP, chromatin immunoprecipitation; RA, retinoic acid; RT-PCR, reverse transcription-polymerase chain reaction; SLO, Streptolysin O; TSS, transcription start site.

thymocyte-ES cell hybrids (Kimura *et al.*, 2004). A limitation of cell fusion or transduction approaches to nuclear reprogramming, however, is the mixing of genomic sequences, making epigenetic analyses of the reprogrammed cells dependent on single nucleotide polymorphism or species specificity of the sequences examined. In the cell extract system, only a limited nonquantitative assessment of demethylation has been reported on *OCT4* (Taranger *et al.*, 2005), and no quantifiable indications exist to date of epigenetic reprogramming of the human *OCT4* and *NANOG* loci.

Here, we provide evidence of reprogramming of DNA methylation and histone modifications on the *NANOG* promoter and throughout the *OCT4* regulatory region in human epithelial cells as a result of transient exposure to EC cell extract. Bisulfite sequencing analysis of *OCT4* and *NANOG* regulatory regions reveals mosaic DNA demethylation over time. Assessment of six modifications of histone H3 by using a novel quick and quantitative chromatin immunoprecipitation (Q²ChIP) assay indicates that chromatin remodeling also takes place on *OCT4* and *NANOG* to establish a conformation compatible with transcriptional activation. Subsequent stimulation of extract-treated cells with retinoic acid (RA) promotes a remethylation of *OCT4*, arguing for specificity of the methylation changes elicited by the extract. Because only the somatic cell genome is present in the extract, the approach constitutes a useful tool for investigating the molecular processes behind nuclear reprogramming.

MATERIALS AND METHODS

Antibodies and Reagents

Antibodies against H3K9ac (catalog no. 06-942), H3K9m2 (07-441), H3K9m3 (07-442), and H3K27m3 (05-851) were from Upstate Biotechnology (Lake Placid, NY). Antibodies against H3K4m2 (Ab7766) and H3K4m3 (Ab8580) were from Abcam (Cambridge, United Kingdom). Other reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Cells

293T cells and undifferentiated human EC cells (NCCIT) were cultured in RPMI 1640 medium containing 10% fetal calf serum (complete RPMI medium) (Taranger *et al.*, 2005). Cells treated with extracts were seeded at 100,000 cells/well in a 48-well plate and cultured in 250 μ l of complete RPMI medium after membrane resealing. In some experiments, cells were also induced to differentiate with 10 μ M RA in bacterial culture plates for up to 3 wk as described previously (Taranger *et al.*, 2005).

Reprogramming Extracts

Extracts of NCCIT cells or control 293T cells were prepared as described (Taranger *et al.*, 2005). In short, cells were washed in phosphate-buffered saline (PBS) and in cell lysis buffer, and then they were sedimented and resuspended in cell lysis buffer. Cells were sonicated on ice; the lysate was sedimented at 15,000 $\times$ g for 15 min; and the supernatant was aliquoted, frozen in liquid nitrogen, and stored at -80°C . Extracts were diluted with H₂O before use to adjust osmolarity to $\sim$ 300 mOsm.

Plasma Membrane Permeabilization and Extract Treatment

The procedure was as reported previously (Taranger *et al.*, 2005) with minor modifications. In short, 500,000 293T cells were washed in 500 μ l of cold Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution (HBSS) and resuspended in 490 μ l of ice-cold HBSS. Tubes were placed in a H₂O bath at 37°C for 2 min, and 10 μ l of Streptolysin O (SLO; 100 μ g/ml stock diluted 1:10 in cold HBSS; Sigma-Aldrich) was added (final concentration, 200 ng/ml). Samples were incubated horizontally in a H₂O bath for 30 min at 37°C with occasional agitation and placed on ice. Note that optimal SLO concentration and time of incubation need to be adjusted for each SLO batch. Samples were diluted with 1 ml of cold HBSS, and cells were sedimented at 120 $\times$ g for 5 min at 4°C. Permeabilization was assessed by uptake of a fluorescent dextran in separate samples 24 h after resealing and replating the cells (Taranger *et al.*, 2005).

Permeabilized cells (500,000) were suspended in 500 μ l of NCCIT or 293T extract (control) containing an ATP-regenerating system and 1 mM of each nucleotide triphosphate. Tubes were incubated horizontally for 1 h at 37°C in a H₂O bath with occasional agitation. To reseal membranes, the extract was

diluted with complete RPMI medium containing 2 mM CaCl₂, and cells were seeded at 100,000 cells/well in a 48-well plate. After $\sim$ 4 h, floating cells were removed, and plated cells were cultured in complete RPMI medium.

Bisulfite Sequencing

DNA was purified by two phenol chloroform isoamylalcohol extractions, followed by one extraction with chloroform isoamylalcohol, and then the DNA was ethanol-precipitated. DNA was dissolved indifferently in H₂O or TE buffer (10 mM Tris-HCl, pH 8.0, and 10 mM EDTA). Bisulfite conversion (Warnecke *et al.*, 2002) was performed using the MethylEasy DNA bisulfite modification kit as described by the manufacturer (Human Genetic Signatures, Sydney, Australia). Converted DNA was used fresh or stored at -20°C . Converted DNA was amplified by polymerase chain reaction (PCR) by using primers published previously (Deb-Rinker *et al.*, 2005) or designed with MethPrimer (www.urogene.org/methprimer/index1.html) (Supplemental Table 1). PCR conditions were 95°C for 10 min and 40 cycles of 95°C for 1 min, 50/55/58°C for 1 min (temperature was primer-dependent; see Supplemental Table 1), and 72°C for 1 min, followed by 10 min at 72°C. PCR products were purified with the GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich) and then cloned into bacteria by TOPO TA cloning (Invitrogen, Carlsbad, CA) and reverse-sequenced using M13 primers (MWG Biotech, High Point, NC). Sequences of 10 bacterial clones per genomic region examined are represented as rows of circles, with each circle symbolizing the methylation state of one CpG. Chi-square tests were done to compare percentages of methylation between cell types or treatments. Unpaired *t* tests were performed to compare 1) the extent of methylation of a specific CpG deduced from 10 sequences, and 2) numbers of methylated CpGs in a given sequence, between cell populations. The *t* test results are provided in Tables 1 and 2 and throughout the text.

Chromatin Immunoprecipitation (Q²ChIP)

To minimize sample loss during the ChIP procedure and maximize ChIP specificity, we recently developed and validated a quick and quantitative Q²ChIP assay (Dahl and Collas, 2007) also used in this study. To prepare antibody-bead complexes, paramagnetic beads (Dynabeads protein A; Dynal Biotech, Oslo, Norway) were washed twice in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 M EGTA, 1% Triton X-100, 0.1% SDS, 0.1% Na-deoxycholate, and 140 mM NaCl), and then they were resuspended in 1 volume of RIPA buffer. Beads (10 μ l) were added to 90 μ l of RIPA buffer containing 2.4 μ g of primary antibody in a 0.2-ml PCR tube, and then they were incubated on a rotator for 2 h at 4°C. For DNA-protein cross-linking, 20 mM of the histone deacetylase inhibitor sodium butyrate was added to cells immediately before harvesting (and to all solutions thereafter). Cells were fixed in suspension with 1% formaldehyde for 8 min in PBS at 1–2 $\times$ 10⁶ cells/ml, and fixation was stopped with 125 mM glycine for 5 min. All subsequent steps were performed on ice or at 4°C. Cross-linked cells were washed twice in PBS/20 mM butyrate and lysed by a sixfold dilution in lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, and protease inhibitors) containing 20 mM butyrate. Aliquots of 200 μ l were sonicated each for 10 $\times$ 30 s on ice to generate chromatin fragments of $\sim$ 500 base pairs. The lysate was sedimented at 10,000 $\times$ g for 10 min, the supernatant was collected, and chromatin concentration was determined by A₂₆₀ from an aliquot diluted 100-fold.

Chromatin diluted (2 A₂₆₀ units) in RIPA buffer/20 mM butyrate was transferred to a 0.2-ml tube containing antibody-bead complexes (see above), and the sample was rotated at 40 rpm for 2 h at 4°C. Immune complexes were washed three times in RIPA buffer and once in TE buffer, each for 4 min at 4°C on a rotator set at 40 rpm. The ChIP material was transferred to a new tube, and TE was replaced with 150 μ l of elution buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 20 mM butyrate, and 50 mM NaCl) containing 1% SDS and 50 μ g/ml proteinase K. Samples were incubated for 2 h at 68°C on a Thermomixer at 1300 rpm (Eppendorf, Hamburg, Germany). Elution buffer was recovered, the ChIP material was reextracted for 5 min, and both supernatants were pooled. Another 200- μ l elution buffer was added to the eluted material, and DNA was extracted once with phenol-chloroform isoamyl alcohol, once with chloroform isoamyl alcohol, and then ethanol-precipitated.

Immunoprecipitated DNA was analyzed in triplicates by real-time PCR starting from 5 μ l of DNA (from a total of 150 μ l). PCR conditions were 95°C for 3 min and 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. ChIP PCR primers are listed in Supplemental Table 1. Data are presented as -fold enrichment of precipitated DNA associated with a given histone modification, relative to a 1/100 dilution of input chromatin (Feldman *et al.*, 2006; Dahl and Collas, 2007). ChIPs were performed in two separate experiments as well as from 293T extract-treated cells, 293T cells, and NCCIT cells.

Real-Time Reverse Transcription (RT)-PCR

RT-PCR was carried from 1 μ g of total RNA by using the Iscript cDNA synthesis kit (Bio-Rad, Hercules, CA). cDNA (20 μ l) was diluted 1:10, and 5 μ l was used in each of triplicate quantitative PCRs on a MyiQ real-time PCR detection system with IQ SYBR Green (Bio-Rad). Primers used are listed in Supplemental Table 2. PCR conditions were 95°C for 3 min and 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Data were analyzed using

Figure 1. Morphological changes of 293T cells elicited by EC extract treatment are associated with transcriptional up-regulation of pluripotency genes. (A) Morphology of 293T cells, NCCIT cells, and 293T cells 4 wk after treatment with two different EC extracts (experiments 1 and 2) or with a 293T cell extract. Bar, 200 μ m. (B) Quantitative RT-PCR analysis of expression of *OCT4*, *NANOG*, *LMNA*, and *LMNB1* in each cell group relative to mRNA level in 293T cells (mean $\pm$ SD from 2 separate analyses in each experiment, each with triplicate RT-PCRs). mRNA levels in NCCIT cells are also shown.

formulas of (Pfaffl, 2001) with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as normalization control.

RESULTS

Up-Regulation of *OCT4* and *NANOG* Expression in EC Extract-treated Cells

Permeabilized epithelial 293T cells exposed for 1 h to a whole-cell postchromosomal supernatant of undifferentiated NCCIT cells, resealed and cultured, formed colonies of tightly packed cells over time with a morphology characteristic of NCCIT cells (Figure 1A). Colony formation was independent of cell density, and it did not occur among cells treated with a control 293T cell extract. Quantitative RT-PCR analysis of gene expression in these cultures 4 wk after extract treatment, in two separate experiments, indicates that *OCT4* and *NANOG* were strongly up-regulated from levels barely detectable by real-time RT-PCR (Figure 1B). Moderate down-regulation of *LMNA* expression also occurred, supporting the absence of detection of lamins A and C in the nuclear envelope of EC extract-treated cells (Taranger *et al.*, 2005). None of these changes were detected in 293T extract-treated cells (Figure 1B). As expected, expression of the constitutively expressed lamin B1 (*LMNB1*) gene was not altered by extract treatment (Figure 1B).

OCT4 and *NANOG* Promoters Undergo Partial DNA Demethylation in EC Extract-treated Cells

To determine whether the EC extract was capable of eliciting epigenetic modifications on exogenous chromatin templates, we first examined DNA methylation changes in the *OCT4*, *NANOG*, and *LMNA* promoter regions. Bisulfite sequencing analysis was carried out to establish 5'-3' CpG methylation profiles across the *OCT4* proximal promoter (PP, which included the transcription start site, or TSS), the proximal enhancer (PE), and the distal enhancer (DE). Nine amplicons (referred to as *OCT4* regions 1–9) were examined, collectively covering 47 potentially methylated CpG dinucleotides within nucleotides –2995 to +66 relative to the TSS (Figure 2, bisulfite sequencing [BiS] primers; see Supplemental Figure 1 for sequence information). Three regions were also

examined in the *NANOG* promoter, encompassing a total of 14 CpGs within nucleotides –1503 to –163 relative to the TSS (Figure 2 and Supplemental Figure 1). The proximal *LMNA* promoter region examined encompassed nine CpGs within nucleotides –277 to +92 relative to the TSS (Figure 2 and Supplemental Figure 1).

The *OCT4* region examined was methylated in 293T cells and largely unmethylated in NCCIT cells ($p < 10^{-4}$; Figure 3A; see Table 1 for statistical analysis). This methylation pattern was consistent with the pattern of expression of

Figure 2. *OCT4*, *NANOG*, *LMNA*, and *GAPDH* regulatory regions examined by bisulfite sequencing and by ChIP in this study. Localization of amplicons generated with BiS and ChIP primers are shown. Numbers are nucleotide numbers in relation to the transcription start site (TSS; +1). Sequence coverage in the *OCT4* locus is shown in Supplemental Table 1. CR1–CR4 on *OCT4* refer to conserved regions 1–4; DE, distal enhancer; PE, proximal enhancer; and PP, proximal promoter.

Figure 3. Transient exposure of 293T cells to EC extract elicits DNA demethylation of *OCT4* and *NANOG*. (A) Bisulfite sequencing analysis of *OCT4*, *NANOG*, and *LMNA* methylation in 293T and NCCIT cells. Top numbers indicate CpG number relative to the TSS. Global percentages of methylated cytosines (%Me) are shown. Each row of circles for a given amplicon represents the methylation status of each CpG in one bacterial clone for that region. Series of 10 clones are shown. Bottom numbers (under B) refer to amplicon number, i.e., the *OCT4* and *NANOG* regions examined (see Figure 2). (B) 293T cells treated with EC extract (2 experiments, experiments 1 and 2) or with 293T extract were analyzed as described in A. (C and D) Percentages of methylated cytosines in each position in *OCT4*, *NANOG*, and *LMNA* determined from data shown in A and B. On the x-axes, CpG no. 1 is the 5'-most cytosine examined in each region. Positions of genomic regions examined are shown. For *t* test analysis, see Table 1 and text.

OCT4 in NCCIT and 293T cells. Nevertheless, in NCCIT cells, the 5' end of the DE (region 1) was overall methylated, whereas regions 6 and 7 in the PE were more, and mosaically, methylated than the rest of the region (Figure 3A). This observation was consistent with *OCT4* methylation profiles reported in the undifferentiated EC cell line, NT2 (DeB-Rinker *et al.*, 2005). Likewise, the *NANOG* promoter was 67%

methylated in 293T cells but unmethylated in NCCIT cells ($p < 10^{-4}$; Figure 3A; Table 1). Thus, the *OCT4* and *NANOG* regulatory regions examined display sufficiently distinct methylation patterns to be analyzed in extract-treated cells. Last, we found that the *LMNA* promoter was hypomethylated both in 293T and NCCIT cells, with, however, CpG no. 1 being more methylated in 293T cells ($p < 0.001$; Figure 3A).

Table 1. *t* test analysis of the numbers of methylated CpGs between treatments or cell types at wk 4 after EC or 293T extract treatment

Gene	Comparison of nos. of methylated CpGs between cell types (p values) ^a				
	Exp. 1	Exp. 2	293T extract	293T	NCCIT
<i>OCT4</i> ^b					
p values	p = 0.365 p < 0.001	X p < 0.001	X X p = 0.629 p < 0.0001	X X X p < 0.0001	X X X X
<i>NANOG</i> ^c					
p values	p = 0.198 p = 0.01 p < 0.001	X p = 0.01 p < 0.001	X X p = 0.827 p < 0.0001	X X X p < 0.0001	X X X X

^a Unpaired *t* tests. Data expressed as two-tailed p values with X referring to treatment or cell type with which comparison is made.

^b Regions 1–9 were analyzed (see Figure 3).

^c Regions 1–3 were analyzed (see Figure 3).

Absence of methylation in the *LMNA* promoter in NCCIT cells, despite the lack of *LMNA* expression, is reminiscent of the unmethylated state of silent gene promoters poised for transcription in undifferentiated ES cells (Azuara *et al.*, 2006). Profiles of 5'-3' CpG methylation in the *OCT4*, *NANOG*, and *LMNA* regulatory regions in 293T and NCCIT cells are represented graphically in Figure 3C as the average methylation state of a given CpG on the basis of 10 sequences per amplicon.

CpG methylation in the *OCT4* and *NANOG* promoters were next examined in two independent reprogramming experiments, 4 wk after exposure to EC extract (experiments 1 and 2) or to 293T extract (Figure 3, B and D). *OCT4* was partially demethylated in both experiments to reach methylation levels of 73 and 66%, compared with 91% in 293T extract-treated controls (p < 0.001; chi-square test) and 89% in untreated 293T cells (p < 0.001; chi-square test; see Table 1 for *t* test analyses). Nevertheless, demethylation did not occur consistently throughout the *OCT4* regulatory locus. The most susceptible areas were *OCT4* regions 2 and 3 in the DE, region 5 in the PE, and region 9 surrounding the TSS in the PP. *OCT4* regions 6 and 7, which are relatively methylated in NCCIT cells, remained unaffected by extract treatment. Furthermore, although *OCT4* methylation was slightly mosaic between 293T cells (presumably as an artifact of extended culture; Figure 3A), mosaicism was enhanced after extract treatment (Figure 3B), most likely due to a variable response of the cells to extract. We concluded that EC extract promotes partial demethylation of *OCT4*, in agreement with transcriptional activation of the gene.

The *NANOG* promoter was also demethylated within 4 wk of extract exposure in both reprogramming attempts (Figure 3, B and D; p < 0.001, chi-square tests; see *t* test analyses in Table 1). As little as 39% methylation was detected in the regions examined (Figure 3B, experiment 2). Again, demethylation did not occur in all cells or alleles, and it resulted in a mosaic methylation pattern (Figure 3B). Nonetheless, all regions examined were affected. In particular, CpGs no. 3, 4, and 11–14 were significantly demethylated in both experiments relative to 293T extract-treated

cells (p < 0.001). We did not notice any changes in methylation of the *LMNA* promoter in treated or control cells relative to 293T cells (Figure 3, B and D).

We next determined how early *OCT4* demethylation took place after extract treatment, focusing on regions that showed the most pronounced demethylation in the previous experiment, namely, regions 2, 3, and 9. Demethylation occurred as early as 1 wk after treatment with EC extract in two additional experiments (experiments A and B) but not in 293T extract-treated cells (57 and 56% methylation in experiment A and B versus 89% in 293T extract-treated cells; p < 10⁻³ [chi-square test] and p < 10⁻⁴ [*t* tests]; Figure 4, A and B). Demethylation was most pronounced in DE region 2 and PP region 9, near the TSS. Demethylation was enhanced by week 2 (region 9; p < 0.001 [*t* test] relative to week 1; Figure 4, A and B) and correlated with transcriptional activation of *OCT4* (Figure 4D). To our surprise, however, demethylation of *NANOG* (region 1) was not detected by week 1 or 2 (Figure 4C), despite activation of the gene (Figure 4D). This suggests that demethylation may have occurred elsewhere in the *NANOG* promoter. Note that *NANOG* expression at week 1 in experiment A and B (Figure 4D) was apparently higher than that at week 4 in experiment 1 and 2 (Figure 1B), despite the relatively higher methylation level of region 1 (compare Figures 3B and 4C). However, *NANOG* expression in untreated 293T cells is barely detectable by real-time RT-PCR, and small variations in PCR efficiency and/or in background *NANOG* mRNA levels in 293T cells between experiments may translate into dramatic differences in the relative mRNA level calculated in extract-treated cells. Thus, expression levels in experiment 1 and 2 (Figure 1B) may not be compared with those of Figure 4D.

Collectively, our results indicate that EC extract is capable of inducing demethylation of *OCT4* and *NANOG* regulatory regions in exogenous genomes. Different regions across the *OCT4* promoter and enhancer are differentially demethylated, and CpGs around the TSS of *OCT4* seem to be particularly susceptible to demethylation. Demethylation in the *NANOG* promoter was more uniform than that of *OCT4*, but this might have been due to fewer numbers of CpGs examined.

Figure 4. DNA demethylation of *OCT4* correlates with transcriptional activation in EC extract-treated cells. (A) Bisulfite sequencing analysis of *OCT4* regions 2, 3, and 9 at weeks 1 and 2 after EC extract (experiments A and B) and 293T extract treatments. (B) Percentages of methylation of each CpG in *OCT4* region 9 at weeks 1 and 2. (C) Bisulfite sequencing analysis of *NANOG* region 1 at weeks 1 and 2 in cells as described in A. (D) Real-time RT-PCR analysis of *OCT4* and *NANOG* expression in two reprogramming experiments 1 wk after EC extract treatment. Means of triplicate RT-PCRs (SE bars are negligible).

EC Extract Modifies Histone Lysine Methylation and Acetylation on *OCT4* and *NANOG*

In addition to DNA methylation, posttranslational modifications of the amino-terminal tails of core histones, notably histone H3, contribute to the regulation of gene expression. We determined whether treatment of 293T cells with EC extract modified lysines (K) 4, 9, and 27 on histone H3. To accommodate relatively small cell numbers available for ChIP analysis in this study, and to optimize detection of small differences in lysine methylation and acetylation, we recently modified a conventional ChIP protocol (Spencer *et al.*, 2003). Q²ChIP minimizes sample loss with a cross-linking step in suspension, it preserves acetylated epitopes by inhibiting histone deacetylase activity early in the process, and it enhances ChIP specificity by eliminating background through a tube-shift step after washes of the ChIP material (Dahl and Collas, 2007). Four weeks after EC extract (experiments 1 and 2; see above) or 293T extract treatment, chromatin was prepared for ChIP analysis of changes in three marks of transcriptionally active chromatin (H3K9ac, H3K4m2, and H3K4m3) and in three repressive marks (H3K9m2, H3K9m3, and H3K27m3) on *OCT4*, *NANOG* and on the constitutively active *GAPDH* promoter. Regions examined are shown in Figure 2, and the data are illustrated in Figure 5.

We first examined histone modifications in 293T and NCCIT cells. As expected from expression in NCCIT cells, the *OCT4* promoter and enhancer contained acetylated H3K9 and barely detectable di- and trimethylated H3K9 or trimethylated H3K27 (Figure 5A). In contrast, 293T cells harbored H3K9m2, H3K9m3, and H3K27m3 but low levels of acetylated H3K9. Di- and trimethylated H3K4 was detected in both cell types, in agreement with expression, or potential for expression, of the gene (Figure 5A). All histone modifications occurred similarly throughout the *OCT4* proximal promoter (*OCT4*-E:PP amplicon), the proximal enhancer (*OCT4*-D:PE and *OCT4*-C:PE amplicons), and the distal enhancer (*OCT4*-B:DE and *OCT4*-A:DE amplicons). The *NANOG* promoter displayed high levels of H3K9ac, H3K4m2, and H3K4m3 together with low levels of H3K9 and H3K27 methylation in NCCIT cells (Figure 5B), again consistent with expression of the gene. In contrast, heterochromatin marks (H3K9m2, H3K9m3, and H3K27m3) were abundant in 293T cells (Figure 5B). Last, no significant differences were detected for any histone H3 modification on the *GAPDH* promoter (Figure 5C). In agreement with its constitutive expression, *GAPDH* exhibited acetylated H3K9, di- and trimethylated H3K4, and background levels of methylation on H3K9 or H3K27.

Figure 5. EC extract treatment elicits changes in histone H3 methylation and acetylation on *OCT4* and *NANOG*. 293T cells were treated with EC extract in two separate experiments (experiments 1 and 2) or exposed to 293T extract. Cells were cultured for 4 wk and analyzed by Q²ChIP for indicated histone modifications. Untreated 293T and NCCIT cells were also examined. Data are presented as -fold enrichment of precipitated DNA associated with a given histone modification relative to a 100-fold dilution of input chromatin. (A) Five genomic regions on *OCT4* (*OCT4-A* to *OCT4-E*) were analyzed (see Figure 2, ChIP primers). (B) *NANOG* promoter. (C) *GAPDH* promoter. Each data point is from a triplicate real-time PCR (error bars are negligible and are not shown).

Treatment with EC extract promoted acetylation and demethylation of H3K9 (m2 and m3) together with a reduction of H3K27m3 on *OCT4* (Figure 5A, blue and green columns). Demethylation of H3K9m2 was consistently more pronounced than that of H3K9m3 or H3K27m3, suggesting that a trimethylated state is more stable than a dimethylated state. As expected from our observations in 293T and NCCIT cells, no changes in H3K4 methylation were detected. Furthermore, 293T extract treatment maintained low H3K9ac and elevated H3K4m2 and H3K4m3 levels (Figure 5A). Demethylation of H3K9m2 and H3K9m3 occurred, however, albeit to a lesser extent than in EC extract-treated cells, particularly in the *OCT4*-E:PP, *OCT4*-D:PE, and *OCT4*-B:DE regions. *OCT4*-C:PE and *OCT4*-A:DE regions were demethylated to the same extent. In addition, 293T extract-treated cells maintained elevated H3K27m3 particularly in the DE (*OCT4*-A:DE, *OCT4*-B:DE) but showed similar H3K27m3 patterns as in EC extract-treated cells in the PE and PP (Figure 5A).

The *NANOG* promoter also underwent H3K9 acetylation in EC extract-treated cells, together with moderate increases in di- and trimethylated H3K4, demethylation of H3K9m2, moderate demethylation of H3K9m3, and weak demethylation of H3K27m3 (Figure 5B). Moreover, histone modification profiles in 293T extract-treated cells were similar to those of 293T cells, except for some demethylation of H3K9m2 (Figure 5B). Last, no changes took place on the *GAPDH* promoter (Figure 5C), indicating that changes on *OCT4* and *NANOG* were specific.

In summary, histone modification changes detected on *OCT4* and *NANOG* regulatory regions after EC extract treatment are indicative of a remodeling of chromatin on these promoters to acquire an epigenetic state characteristic of pluripotent cells. Acetylation and demethylation of H3K9 occur in an EC extract-specific manner and are indicative of transcriptional activation of these genes.

Retinoic Acid Causes Remethylation of the *OCT4* Promoter in Reprogrammed Cells

To ascertain the specificity of *OCT4* demethylation elicited by EC extract, we determined whether the promoter was responsive to induction of differentiation with RA. First, stimulation of NCCIT cells with 10 μ M RA for 3 wk strongly down-regulated *OCT4* and *NANOG* expression and activated nestin (*NES*) transcription, an early marker of neuronal differentiation (Figure 6A). *OCT4* repression correlated with heavy DNA methylation in the PP (region 9) and DE (regions 2 and 3), establishing the responsiveness of these regions to RA in NCCIT cells (Figure 6B; compare with Figure 3A, NCCIT).

Second, stimulation of reprogrammed cells with RA starting 3 wk after extract treatment (experiments A and B) down-regulated *OCT4* expression (Figure 6C). Remarkably, remethylation of *OCT4* occurred in both batches of reprogrammed cells (Figure 6, D and E; see Table 2 for *t* test analyses). Note that only region 9 was examined here, because it was previously shown to be very responsive to EC extract treatment. *OCT4* remethylation in reprogrammed cells occurred to the same extent as in RA-treated NCCIT cells (Figure 6E and Table 2). In contrast, reprogrammed cells kept in culture for 6 wk without RA maintained a relatively hypomethylated profile, in agreement with elevated *OCT4* mRNA levels (Figure 6, C–E). Finally, as anticipated, *OCT4* remained highly methylated in 293T extract-treated cells exposed to RA (Figure 6E). We concluded that demethylation of *OCT4* elicited by EC extract treatment is a functionally significant epigenetic response, because it can be reverted by induction of differentiation.

DISCUSSION

This report demonstrates the epigenetic reprogramming of *OCT4* and *NANOG* as a result of transient treatment of 293T epithelial cells with extract of EC cells. We previously reported a nonquantitative assessment of demethylation of eight CpGs in EC extract-treated cells within *OCT4* region 5 in the PE (Taranger *et al.*, 2005). We now show mosaic CpG demethylation throughout the *OCT4* regulatory region and in the *NANOG* promoter. Targeted *OCT4* and *NANOG* demethylation is specific for EC extract, and it does not occur in 293T extract-treated cells. *OCT4* demethylation is physiologically relevant, because it is associated with activation of the gene, whereas RA-mediated differentiation elicits its remethylation along with transcriptional repression. DNA demethylation is accompanied by methylation and acetylation changes on lysines 4, 9, and 27 of histone H3 on the *OCT4* PP, PE, and DE as well as on the *NANOG* promoter, to create a chromatin configuration compatible with transcriptional activation.

Reprogramming of DNA Methylation on *OCT4* and *NANOG* Regulatory Regions

EC extract-induced demethylation produces mosaic methylation profiles on *OCT4* and *NANOG* upstream regulatory sequences. On the basis of previous immunological observations of Oct4 protein expression (Taranger *et al.*, 2005), not all cells are expected to be reprogrammed to the same extent. Our results are reminiscent of partial demethylation of *OCT4* and *Nanog* in mouse fibroblasts constitutively overexpressing Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006). Apparent partial reprogramming in our system may be due the examination of a heterogeneous cell population, or assuming that enzyme(s) causing demethylation originate from the EC extract, to a restricted enzyme access to target sequences. This may be alleviated by incubation of purified nuclei or deproteinized chromatin, which in *Xenopus* eggs accelerates demethylation (Simonsson and Gurdon, 2004), rather than cells. Interestingly, reprogramming of *OCT4* methylation is as efficient in somatic–ES cell hybrids (Cowan *et al.*, 2005) as by nuclear transplantation (Simonsson and Gurdon, 2004), two situations where nuclei are directly exposed to the putative reprogramming factors.

Reprogramming of *OCT4* DNA methylation by extract treatment is targeted to specific, nonrandom areas, which may be more sensitive to demethylation. The most significant DNA demethylation detected occurs in regions 2 and 3 in the DE and in region 9 in the PP. Regions 2 and 3 encompass putative elements for transcription factors, including COUP-T, MZF1, GATA-2, HNF4, and three Sp1 elements. Region 9, surrounding the TSS, covers several MZF1, ADR1 (whose promoter binding is promoted by loss of histone deacetylation; Verdone *et al.*, 2002), HSF, GATA-1, GATA-2, and Sp1 elements (www.cbrc.jp/htbin/nph-tfsearch). The *NANOG* promoter region demethylated by extract treatment is rich in putative HSF; ADR1, CdxA, AP-1/4, IRF-1, Cap, and c-Rel elements. Whether all these elements are involved in transcription activation and whether methylation modulates their binding to DNA remain uncertain. Nonetheless, DNA demethylation, together with hyperacetylation and hypomethylation of H3K9 (see below), contribute to loosening chromatin structure and thereby to the binding of transcription factors.

Demethylation in the *OCT4* PP (region 9) took place within 1 wk of extract treatment and was slightly enhanced by week 2. By week 1, extract-treated cells have undergone three rounds of replication (cells are quiescent for the first

Figure 6. Retinoic acid induces remethylation of the *OCT4* promoter in EC extract-treated cells. (A) Real-time RT-PCR analysis of *OCT4*, *NANOG*, and *NES* expression in RA-stimulated NCCIT cells (mean of triplicate PCRs; error bars negligible). (B) DNA methylation of *OCT4* regions 2, 3, and 9 in NCCIT cells after 3 wk of RA stimulation. (C) Real-time RT-PCR analysis of *OCT4* expression in EC extract-treated cells (experiments A and B) 3 wk after exposure to extract (Start) and after one additional week of culture with 0 (–RA) or 10 μ M RA (+RA). (D) Methylation of *OCT4* region 9 in EC extract-treated cells exposed for 3 wk to RA, starting 3 wk after extract exposure as described in C. (E) Percentage of methylation of individual CpGs in indicated cell types. For *t* test analyses, see Table 2.

48 h after extract treatment), so whether reprogramming-associated demethylation is a replication-dependent process or an active replication-independent process remains unknown. This also holds true for cell fusion or transduction reprogramming strategies, because clonal selection of the reprogrammed cells occurs before analysis (Cowan *et al.*, 2005; Takahashi and Yamanaka, 2006). However, reprogramming of *Oct4* methylation in *Xenopus* oocytes occurs in the absence of replication, transcription, or protein synthesis (Simonsson and Gurdon, 2004), and as such, it may involve active demethylating activity. Interestingly, the Aid/Apobec 1 members of the family of 5-methylcytosine deaminases are expressed in a cluster containing *Nanog* and other pluripotency genes in oocytes, embryonic germ cells, and ES cells (Morgan *et al.*, 2004), three cell types known to be able to reprogram somatic genomes (Tada *et al.*, 1997, 2001; Wilmut *et al.*, 2002; Gurdon *et al.*, 2003). Activity of Aid/Apobec deaminases results in C→T transitions in methylated DNA,

or to demethylation in connection with repair of the T:G mismatch (Morgan *et al.*, 2004). As such, Aid/Apobec deaminases may play a role in epigenetic reprogramming.

Retinoic acid stimulation of the reprogrammed cells promotes *OCT4* remethylation and transcriptional down-regulation. Down-regulation of *OCT4* is expected to correlate with the establishment of a repressive chromatin structure as demonstrated previously in NCCIT cells (Dahl and Collas, 2007), and with the dissociation of transcription regulators from the PP (Minucci *et al.*, 1996) to ensure long-term silencing. Our data indicate that in reprogrammed cells, the *OCT4* locus behaves epigenetically as in NCCIT cells, in which *OCT4* is fully methylated in regions 2, 3 (DE), and 9 (PP) after RA stimulation (Figure 6; also see Deb-Rinker *et al.*, 2005). So, the (partially) demethylated *OCT4* promoter in reprogrammed cells retains the ability to undergo further ad hoc epigenetic modifications upon differentiation.

Table 2. *t* test analysis of the numbers of methylated CpGs in the *OCT4* proximal promoter (region 9) between extract-treated cells, 293T cells, and NCCIT cells exposed to 0 or 10 μ M retinoic acidComparison of nos. of methylated CpGs between cell types (p values)^a

Exp. A		Exp. B		293T extract		293T	NCCIT
+RA	–RA	+RA	–RA	+RA	–RA	+RA	+RA
p < 0.0001	X						
p > 0.1		p = 0.04	X	X		X	X
		p < 0.05		X		X	
		p = 0.147		p < 0.01	X		X
				p = 0.621		X	X
	p < 0.0001		p = 0.004		X		
					X		
						p = 0.15	X

^a Unpaired *t* tests. Data expressed as two-tailed p values with X referring to treatment or cell type with which comparison is made. Cells not exposed to RA (–RA) were analyzed 6 wk after EC extract (exp. A and exp. B) or 293T extract treatment. Cells treated with RA were exposed to 10 μ M RA for 3 wk starting 3 wk after extract treatment. 293T and NCCIT cells were also exposed to 10 μ M RA.

Remodeling Chromatin through Posttranslational Modifications of Histone H3 on *OCT4* and *NANOG*

The most prominent histone modification specifically elicited by EC extract on the *OCT4* PP, PE, and DE and on the *NANOG* promoter is acetylation of H3K9. This takes place in the context of minimal hypermethylation of already di- and trimethylated H3K4, which mark genes either transcriptionally active (H3K4m3) or competent for transcription (H3K4m2) (Santos-Rosa *et al.*, 2002). These changes are consistent with chromatin remodeling on the *Oct4* promoter in mouse thymocyte–ES cell hybrids, except for the marked trimethylation of (initially unmethylated) H3K4 detected in the thymocyte nuclei (Kimura *et al.*, 2004). Transcriptional activation of *Oct4* in mouse fibroblasts treated with ES cell extract requires ATP hydrolysis, most likely for nuclear import of transcription factors (Håkelién *et al.*, 2002; Landsverk *et al.*, 2002) and for the activity of chromatin remodeling complexes (Kingston and Narlikar, 1999; Aalfs *et al.*, 2001). *Oct4* activation by ES cell extract or *Xenopus* egg extract also requires the Brg1 subunit of the SWI/SNF complex (Hansis *et al.*, 2004; Taranger *et al.*, 2005). Promoter-specific targeting of SWI/SNF may involve H3K4 (hyper)methylation on *OCT4* and *NANOG* and prime the loci for further transcription-permissive remodeling (H3K4 methylation per se is not sufficient to allow transcription as H3K4 is methylated on both *OCT4* and *NANOG* in 293T cells). This additional remodeling presumably occurs by recruitment of histone acetyl transferases, whose activity results in the marked acetylation of H3K9 on *OCT4* and *NANOG*. Therefore, a key reprogramming event is relaxation of chromatin to create a conformation compatible with transcriptional activation.

Trimethylation of H3K27 is a facultative heterochromatin mark that promotes the recruitment of Polycomb group proteins for gene silencing (Cao *et al.*, 2002; Czermin *et al.*, 2002; Orlando, 2003). Interestingly, in ES cells, H3K27m3 can also mark transcriptionally silent, albeit acetylated, promoters for activation upon differentiation (Azuara *et al.*, 2006). We detected some H3K27m3 demethylation on *OCT4* in EC extract-treated cells but essentially none on *NANOG*. Because both promoters are acetylated on H3K9, this again illustrates the heterogeneity of the cell populations examined and suggests that the loci have been partially reprogrammed. To support this view, these modifications were

not as prominent as in NCCIT cells, in which both genes are transcribed at a higher level than in reprogrammed cells. Notably, H3K27m3 seems to be fully demethylated in thymocyte–ES cell hybrids, suggesting a more extensive chromatin remodeling in this system (Kimura *et al.*, 2004). Globally, however, the changes reported as a result of EC extract treatment reflect a remodeling of chromatin on *OCT4* and *NANOG* indicative of a transition from a potentially active to an active promoter.

Our results indicate that the primary epigenetic determinants of *OCT4* and *NANOG* reprogramming by EC cell extract are DNA demethylation, and acetylation and demethylation of H3K9. Demethylation of H3K9m2 clearly occurs; however, trimethylated histone marks tend to remain more stable: demethylation of H3K9m3 or H3K27m3 was less pronounced than that of H3K9m2. Interestingly, modulation of repressive histone modifications such as H3K9 trimethylation is a feature of fertilized embryos, which is also not faithfully reproduced by somatic cell nuclear transfer (Santos *et al.*, 2003). Furthermore, nuclear transplantation into *Xenopus* oocytes has shown that, indeed, repressive complexes do not readily disassemble (Kikyo and Wolffe, 2000). It is clear, therefore, that demethylation of trimethylated repressive histone marks remains a limiting factor in nuclear reprogramming, irrespective of the approach. Identification of the molecular mechanism driving histone demethylation (Shi *et al.*, 2004; Armstrong *et al.*, 2006; Schneider and Shilatifard, 2006) is likely to constitute a significant step toward improving nuclear reprogramming efficiency.

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