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Induction of Dedifferentiation, Genomewide Transcriptional Programming, and Epigenetic Reprogramming by Extracts of Carcinoma and Embryonic Stem Cells[D]

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Functional reprogramming of a differentiated cell toward pluripotency may have long-term applications in regenerative medicine. We report the induction of dedifferentiation, associated with genomewide programming of gene expression and epigenetic reprogramming of an embryonic gene, in epithelial 293T cells treated with an extract of undifferentiated human NCCIT carcinoma cells. 293T cells exposed for 1 h to extract of NCCIT cells, but not of 293T or Jurkat T-cells, form defined colonies that are maintained for at least 23 passages in culture. Microarray and quantitative analyses of gene expression reveal that the transition from a 293T to a pluripotent cell phenotype involves a dynamic up-regulation of hundreds of NCCIT genes, concomitant with down-regulation of 293T genes and of indicators of differentiation such as A-type lamins. Up-regulated genes encompass embryonic and stem cell markers, including *OCT4*, *SOX2*, *NANOG*, and Oct4-responsive genes. *OCT4* activation is associated with DNA demethylation in the *OCT4* promoter and nuclear targeting of Oct4 protein. In fibroblasts exposed to extract of mouse embryonic stem cells, Oct4 activation is biphasic and RNA-PolII dependent, with the first transient rise of *Oct4* up-regulation being necessary for the second, long-term activation of *Oct4*. Genes characteristic of multilineage differentiation potential are also up-regulated in NCCIT extract-treated cells, suggesting the establishment of "multilineage priming." Retinoic acid triggers Oct4 down-regulation, de novo activation of A-type lamins, and nestin. Furthermore, the cells can be induced to differentiate toward neurogenic, adipogenic, osteogenic, and endothelial lineages. The data provide a proof-of-concept that an extract of undifferentiated carcinoma cells can elicit differentiation plasticity in an otherwise more developmentally restricted cell type.

INTRODUCTION

Differentiated cells are thought to be stably committed to their fate; however, there is evidence to indicate that dedifferentiation events can take place. Urodele amphibians and teleost fish can replace lost anatomical parts by a process of migration, dedifferentiation, proliferation, and redifferentiation of epithelial cells in the wounded area. Functional reprogramming of differentiated cell nuclei has also been illustrated by the derivation of pluripotent embryonic stem (ES) cells (ESCs) (Cibelli *et al*., 1998; Munsie *et al*., 2000; Wakayama *et al*., 2001; Hwang *et al*., 2004) and by the live birth of cloned animals (Wilmut *et al*., 2002; Gurdon and Byrne, 2003) after nuclear transplantation into unfertilized eggs. Notably, *Xenopus* eggs can reprogram mammalian somatic nuclei to express the POU family member homeodomain transcription factor gene *Oct4* (Byrne *et al*., 2003) by a process requiring DNA demethylation (Simonsson and Gurdon, 2004). DNA demethylation also occurs after fusion of mouse thymocytes with embryonic germ (EG) cells (EGCs) or ESCs (Tada *et al*., 1997, 2001; but interestingly, only EG cells are capable of demethylating imprinted genes (Tada *et al*., 1997). Similarly, fusion of neuronal progenitor cells (Pells *et al*., 2002; Ying *et al*., 2002) or bone marrow-derived cells (Terada *et al*., 2002) with ESCs results in hybrids that express markers of pluripotency (Pells *et al*., 2002), contribute to chimeras (Ying *et al*., 2002), and form teratomas (Terada *et al*., 2002). Similar observations resulted from fusing human fibroblasts with ESCs (Cowan *et al*., 2005). Fusion of embryonal carcinoma (EC) cells (ECCs) with T-lymphoma cells also promotes the formation of colonies expressing pluripotent cell transcripts from the lymphoma genome (Flasza *et al*., 2003). Thus, components of pluripotent EG, ES, or EC cells have the potential of eliciting reprogramming events in a somatic genome.

As an alternative to fusion, somatic nuclear function may also be altered using nuclear and cytoplasmic extracts, with the rationale that extracts provide the necessary regulatory components. Notably, extracts of regenerating newt limbs

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Abbreviations used: CHX, cycloheximide; DRB, 5,6-dichloro-1-D-ribofuranosyl benzimidazole; ECC, embryonal carcinoma cell; EGC, embryonal germ cell; EGFP, enhanced green fluorescent protein; ESC, embryonic stem cell; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; LIF, leukemia inhibitory factor; NTP, nucleotide triphosphate; PBS, phosphate-buffered saline; SLO, streptolysin O.

promote cell cycle reentry and down-regulation of myogenic markers in differentiated myotubes (McGann *et al*., 2001). Furthermore, we have shown that kidney epithelial 293T cells permeabilized with streptolysin O (SLO) and briefly exposed to an extract of Jurkat T-cells take on T-cell properties, including growth in aggregates, chromatin remodeling, expression of T-cell-specific genes and surface receptors, secretion of interleukin-2, and stimulation-dependent assembly of the interleukin-2 receptor (Håkelien *et al*., 2002, 2005; Landsverk *et al*., 2002). Similarly, lysates of cardiomyocytes or insulinoma cells elicit expression of cardiomyocyte or β-cell markers in adipose stem cells (Gaustad *et al*., 2004) and fibroblasts (Håkelien *et al*., 2004), and a pneumocyte extract was recently shown to induce differentiation of ESCs into a pneumocyte phenotype (Qin *et al*., 2005). 293T cells were also shown to express pluripotency markers such *OCT4* and *GCAP* and down-regulate a kidney marker after coculture with extract of *Xenopus* eggs (Hansis *et al*., 2004). Despite these observations, evidence for induction of epigenetic reprogramming events in large numbers of cells by extracts is lacking.

Teratocarcinomas are a particular type of germ cell tumors that contain undifferentiated stem cells and differentiated derivatives that can include endoderm, mesoderm, and ectoderm germ layers (Chambers and Smith, 2004). Undifferentiated carcinoma cells can be cultured to give rise to lines of ECCs. ECCs form malignant teratocarcinomas when transplanted into ectopic sites; however, some ECC lines can also contribute to tissues of the developing fetus when introduced into a blastocyst (Blelloch *et al*., 2004). Undifferentiated human teratocarcinoma NCCIT cells were established from a mediastinal mixed germ cell tumor (Teshima *et al*., 1988). NCCIT is at a stage intermediate between a seminoma (a precursor of germ cell tumors) and an embryonal carcinoma (Damjanov *et al*., 1993). NCCIT is a developmentally pluripotent cell line that can differentiate into derivatives of all three embryonic germ layers and extraembryonic cell lineages (Damjanov *et al*., 1993).

This study tests the hypothesis that an extract of undifferentiated somatic cells can elicit dedifferentiation in a somatic cell line. Based on morphological and immunolabeling observations, gene expression profiling, DNA methylation assays, and functional assessments, we show that 293T and NIH3T3 cells can be programmed by extracts of undifferentiated NCCIT cells or mouse ES cells to acquire characteristics of pluripotency.

MATERIALS AND METHODS

Cells

NCCIT, Jurkat (clone E6-1), and 293T cells (American Type Culture Collection, Vanassas, MD) were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, and nonessential amino acids (complete RPMI 1640 medium). NIH3T3 Swiss-Albino fibroblasts (American Type Culture Collection) were cultured in DMEM (Sigma-Aldrich) with 10% FCS, L-glutamine, and 0.1 mM β-mercaptoethanol. Mouse ESCs were isolated from inner cell masses of strain sv129 blastocysts and plated on mouse fibroblast γ-irradiated feeder layers in ESC medium (DMEM, 15% FCS, 0.1 mM β-mercaptoethanol, nonessential amino acids, and 1% penicillin/streptomycin) supplemented with 1000 U/ml (10 ng/ml) recombinant leukemia inhibitory factor (LIF; Sigma-Aldrich) on gelatin-coated plates. Before harvesting for preparing extracts, ESCs were passaged and cultured under feeder-free conditions in RPMI 1640 medium containing 10 ng/ml LIF. Cells treated with NCCIT or 293T extract were seeded at 100,000 cells per well in a 48-well plate and cultured in 250 μl of complete RMPI 1640 medium with antibiotics. Cells exposed to ESC extract were cultured as ESCs with 10 ng/ml LIF under feeder-free conditions.

Cell Extracts

To prepare NCCIT extracts, cells were washed in phosphate-buffered saline (PBS) and in cell lysis buffer (100 mM HEPES, pH 8.2, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and protease inhibitors), sedimented at 400 × *g*, resuspended in 1 volume of cold cell lysis buffer, and incubated for 30–45 min on ice. Cells were sonicated on ice in 200-μl aliquots using a Labsonic-M pulse sonicator fitted with a 3-mm-diameter probe (B. Braun Biotech, Melsungen, Germany) until all cells and nuclei were lysed, as judged by microscopy (our unpublished data). The lysate was sedimented at 15,000 × *g* for 15 min at 4°C to pellet the coarse material. The supernatant was aliquoted, frozen in liquid nitrogen, and stored for up to 9 mo at −80°C. Lysate of 95,583 ± 10,966 NCCIT cells was used to generate 1 μl of extract. Protein concentration of the NCCIT extract was 29.5 ± 4.6 mg/ml (Bradford) and pH was 7.0 ± 0.0 (4 batches). ESC extracts (25–30 mg/ml protein) were similarly prepared from LIF-adapted ESC cultures. 293T, Jurkat, and NIH3T3 extracts were also prepared as described above. If necessary, extracts were diluted with H_2O before use to adjust osmolarity to ~300 mOsM.

SLO-mediated Permeabilization and Cell Extract Treatment

293T and 3T3 cells were washed in cold PBS and in cold Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA). Cells were resuspended in aliquots of 100,000 cells/100 μl of HBSS, or multiples thereof; placed in 1.5-ml tubes; and centrifuged at 120 × *g* for 5 min at 4°C in a swing-out rotor. Sedimented cells were suspended in 97.7 μl of cold HBSS, tubes were placed in a H_2O bath at 37°C for 2 min, and 2.3 μl of SLO (Sigma-Aldrich) (100 μg/ml stock diluted 1:10 in cold HBSS) was added to a final SLO concentration of 230 ng/ml. Samples were incubated horizontally in a H_2O bath for 50 min at 37°C with occasional agitation and set on ice. Samples were diluted with 200 μl of cold HBSS, and cells were sedimented at 120 × *g* for 5 min at 4°C. Permeabilization was assessed by monitoring uptake of a 70,000-M_r Texas Red-conjugated dextran (50 μg/ml; Invitrogen) in a separate sample 24 h after resealing and replating the cells (our unpublished data). Permeabilization efficiency under these conditions was ~80%.

After permeabilization, cells were suspended at 1000 cells/μl in 100 μl of NCCIT, ESC or indicated control extract (or multiples thereof) containing an ATP-regenerating system (1 mM ATP, 10 mM creatine phosphate, and 25 μg/ml creatine kinase; Sigma-Aldrich), 100 μM GTP (Sigma-Aldrich), and 1 mM each nucleotide triphosphate (NTP; Roche Diagnostics, Mannheim, Germany). The tube containing cells was incubated horizontally for 1 h at 37°C in a H_2O bath with occasional agitation. To reseal plasma membranes, the extract was diluted with complete RPMI 1640 medium containing 2 mM $CaCl_2$ and antibiotics, and cells were seeded at 100,000 cells per well on a 48-well plate. After 2 h, floating cells were removed, and plated cells were cultured in complete RPMI 1640 medium.

Microarray Analysis of Gene Expression

Microarrays. Gene expression analysis was performed using Human Genome Affymetrix U133A GeneChips as described previously (Ji *et al*., 2004). Total RNA was isolated using a Stratagene RNA Nanoprep isolation kit, treated with DNAse I, and purified with RNeasy Mini columns (QIAGEN, Valencia, CA).

PCRcDNA. First-strand cDNA was prepared as described previously (Ji *et al*., 2004) using a SMART PCRcDNA synthesis kit (BE Biosciences Clontech, Palo Alto, CA). Briefly, total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen). cDNA was amplified by PCR as follows: 95°C for 1 min, 20–30 cycles of 95°C for 15 s, 65°C for 30 s, and 68°C for 3 min. PCRcDNA was purified with QIAquick columns (QIAGEN) and ethanol precipitation and dissolved in H_2O.

Biotin-labeling of cRNA. Biotin-labeled cRNA was prepared from PCRcDNA using a T7 RNA polymerase MEGAscript T7 kit (Ambion, Austin, TX), purified with an RNeasy mini kit and fragmented as described (Ji *et al*., 2004). Fragmented cRNA was used for hybridization or stored at −80°C.

Hybridization to GeneChips, Labeling, and Scanning. Hybridization was performed using 50 μg of fragmented cRNA at 45°C for 16 h as described previously (Ji *et al*., 2004). Chips were washed, stained at 35°C for 15 min with a phycoerythrin-strepavidin conjugate (Invitrogen), washed, and scanned on an HP GeneArray scanner Hewlett Packard (Palo Alto, CA).

GeneChip Image Quantification and Data Processing. GeneChip images were quantified and gene expression values calculated using the Affymetrix Microarray suite version 5.0 (MAS 5.0; Affymetrix, Santa Clara, CA). Expression ratios were calculated relative to mean hybridization level of three glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotide spots on the arrays, and plots were drawn using Microsoft Excel 2002 (Microsoft, Redmond, WA).

Polymerase Chain Reaction

PCR amplification of the simian virus SV40 large T antigen was performed using primers 5′-GTGGCTATGGGAACTGGAG-3′ and 5′-CTCTACAGAT-

GTGATATGGCTG-3′, which cover nucleotides 39–265 of GenBank locus AF168998. PCR conditions were 95°C for 3 min and 30 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s followed by 10 min at 72°C. PCR products were visualized by ethidium bromide staining in a 2% agarose gel.

Reverse transcription (RT)-PCR reactions were carried from 200 to 1000 ng of total RNA using the Iscript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative RT-PCR reactions were performed in triplicates on a MyiQ real-time PCR detection system using either IQ SYBR Green (Bio-Rad) or ProbeLibrary probes (Exiqon, Vedbæk, Denmark) as indicated in Table S1. SYBR Green PCR conditions were 95°C for 4.5 min and 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, using *GAPDH* as normalization control. ProbeLibrary PCR conditions were 95°C for 7 min and 40 cycles of 94°C for 20 s and 60°C for 1 min using *ACTB* as standard.

Bisulfite Sequencing

DNA was purified by phenol-chloroform-isoamylalcohol extraction or by using the GenElute mammalian genomic DNA miniprep kit (Sigma-Aldrich). Bisulfite conversion was performed using the MethylEasy DNA bisulphite modification kit (Human Genetic Signatures, Sydney, Australia) as described by the manufacturer. Converted DNA was amplified by seminested PCR using primers (Human Genetic Signatures) specific for the human *OCT4*, *LMNA*, and *LMNB1* genes (see *Results*), and PCR products were sequenced. PCR conditions were, for each of the nested PCRs, 95°C for 3 min and 30 cycles of 95°C for 1 min, 50°C for 2 min, and 72°C for 2 min, followed by 10 min at 72°C.

Induction of Neuronal Differentiation

To generate neuronal derivatives (Stewart *et al*., 2003), cells were seeded in complete RPMI 1640 medium at 5×10^5 cells per 90-mm sterile bacterial culture dish. Suspension cultures were maintained for 24 h before adding 10 μM all-*trans*-retinoic acid (Sigma-Aldrich). Cells were cultured for 3–5 wk in retinoic acid, replacing the medium every 2–3 d. Subsequently, cell aggregates were washed in complete RPMI 1640 medium and plated onto poly-L-lysine (10 μg/ml; Sigma-Aldrich)-coated plates in complete RPMI 1640 medium containing the mitotic inhibitors fluorodeoxyuridine (10 μM; Sigma-Aldrich), cytosine arabinosine (1 μM; Sigma-Aldrich), and uridine (10 μM; Sigma-Aldrich).

Mesodermal Lineage Differentiation

Cells were cultured for 21 d in complete RPMI 1640 medium containing 10 μM all-*trans*-retinoic acid and washed in complete RPMI 1640 medium. For adipogenic differentiation, cells were cultured for a further 21 d in DMEM/Ham's F-12 supplemented with 10% FCS, dexamethasone, insulin, and indomethacin (Boquest *et al*., 2005). Cells were fixed with 4% formalin, washed in 5% isopropanol, and stained for 15 min with Oil-Red-O (Sigma-Aldrich). For osteogenic differentiation, cells were cultured for 21 d in DMEM containing 10% FCS, dexamethasone, β-glycerophosphate, and L-ascorbate-2-phosphate (Boquest *et al*., 2005). Extracellular matrix mineralization nodules were visualized by Alzarin red staining. Endothelial differentiation was performed as described previously (Planat-Benard *et al*., 2004). Briefly, NCCIT extract-treated cells and controls were harvested by flask shaking and plated at 2×10^5 cells per milliliter in 3 ml of methylcellulose (Methocult GF H4434; Stem Cell Technologies, Vancouver, British Columbia, Canada) and cultured for 7 d.

Immunological Procedures

For immunofluorescence, cells were seeded onto coverslips, fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with bovine serum albumin, and hybridized with relevant antibodies. Antibodies used were a rabbit polyclonal anti-Oct4 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), an anti-lamin A/C monoclonal antibody (mAb) (1:50 dilution; mAb XB10; BAbCO, Covance Research Products, Grand Rapids, MI), and a rabbit polyclonal antibody against a peptide of human B-type lamins (1:1000) (Chaudhary and Courvalin, 1993). Rabbit polyclonal antibodies (1:200) against neurofilament NF200 were from Sigma-Aldrich, and anti-NeuN (mAb377) and anti-nestin antibodies (mAb5362; 1:200) were from Chemicon International (Temecula, CA). Secondary antibodies were Cy2- and Cy3-conjugated anti-mouse and anti-rabbit antibodies and Cy3-conjugated anti-rabbit antibodies (1:1000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). For direct immunolabeling, cells (~300,000) were incubated with fluorescein isothiocyanate-conjugated mouse anti-human CD31 or rabbit anti-human CD144 antibodies (1:10 dilution; Serotec, Oxford, United Kingdom) in 100 μl of PBS. After extensive washing in PBS, cells were fixed with 3% paraformaldehyde before viewing. For Western blotting, antibodies used were anti-Oct4 (1:250), anti-lamin A/C (1:500), anti-lamin B (1:5000), and anti-tubulin (1:250; Santa Cruz Biotechnology). Immunodepletion of Oct4 from mouse ESC extract was performed using anti-Oct4 antibodies (1:50 dilution) bound to protein A/G-Sepharose beads. After 1-h incubation in extract at 4°C, bead complexes were removed by sedimentation at $4000 \times g$ for 5 min, and a second round of immunoprecipitation was carried out for 30 min at 4°C. After sedimentation of the beads, an aliquot of the supernatant was removed for Western blotting, whereas the extract was used for cell treatment.

Alkaline Phosphatase Assay

Relative intracellular ALP levels were determined using a dot-blot assay. Two microliters of soluble lysate ($15{,}000 \times g$ supernatant at 20 μg/μl protein) from indicated cell types were spotted on a dry 45-μm nitrocellulose membrane (Bio-Rad). The membrane was wetted in 50 mM NaCl, 10 mM Tris-HCl, pH 7.0, and ALP was revealed by applying an Alk-Phos Direct detection solution (GE Healthcare, Piscataway, NJ). Light emission on film was quantified by densitometry within a linear signal range.

RESULTS

Treatment of 293T Cells with NCCIT Extract Promotes Colony Formation

293T cells were permeabilized with SLO and exposed for 1 h to an extract of undifferentiated NCCIT cells. In vitro culture of extract-treated cells was accompanied by morphological, immunological, gene expression, and functional analyses over 8–12 wk to evaluate induction of dedifferentiation in two to six independent experiments. As controls, permeabilized 293T cells were treated with extract of 293T or Jurkat T-cells.

A first result of NCCIT extract exposure was a change in morphology of 293T cells. Within 2 wk, colonies with defined edges developed and resembled NCCIT colonies (Figure 1A and B, a–e). This phenotype was maintained for at least 12 wk in culture, corresponding to >50 population doublings and 23 passages (Figure 1B, a–e). The phenotype was not a mere consequence of treatment with any extract as 293T cells incubated in their own extract did not form colonies (Figure 1B, f–j), and 293T cells treated with an extract of Jurkat cells formed clearly morphologically distinct aggregates (Figure 1C). The latter were reminiscent of Jurkat T-cell clusters (Håkelien *et al*., 2005).

The NCCIT Extract Elicits Expression of Oct4 and Oct4-responsive Genes, Expression of ESC Markers, and Repression of A-Type Lamins

Expression of the homeodomain protein Oct4, a POU family transcription factor, is restricted to germ cells, preimplantation embryos, the epiblast of early postimplantation embryos and ESCs (Chambers and Smith, 2004). As expected, Oct4 was detected in the nucleus of NCCIT but not 293T cells (Figure 2A), and identity of the protein was confirmed by immunoblotting (our unpublished data). One week after treatment with NCCIT extract, >60% of 293T cells displayed intranuclear Oct4, whereas Oct4 remained undetectable in 293T extract-treated cells (Figure 2, B and C).

We next monitored the loss A-type nuclear lamins, a marker of differentiated cells (Hutchison and Worman, 2004), from 293T cells. mAbs against lamins A and C (lamin A/C) decorated the nuclear periphery of 293T but not NCCIT cells (Figure 2A). In contrast, 1 wk after NCCIT extract treatment, lamin A/C was undetectable in >60% of the cells, whereas controls displayed lamin A/C labeling (Figure 2, B and C). Notably, Oct4 expression paralleled the loss of lamin A/C expression in the same cells (Figure 2C), suggesting that these cells were undergoing dedifferentiation. Expression of the ubiquitously expressed B-type lamins (Hutchison and Worman, 2004) remained unaltered (Figure 2, A–C).

Induction of *OCT4* (*POU5F1*) transcription and loss of *LMNA* (lamin A) gene expression over time were demonstrated by quantitative RT-PCR analysis 4 wk after extract treatment (Table 1). Several target genes of Oct4 were also up-regulated, including *UTF1*, *OXT2*, *REX1*, and *NANOG*. Interestingly, Oct4 is known to act in cooperation with Sox2

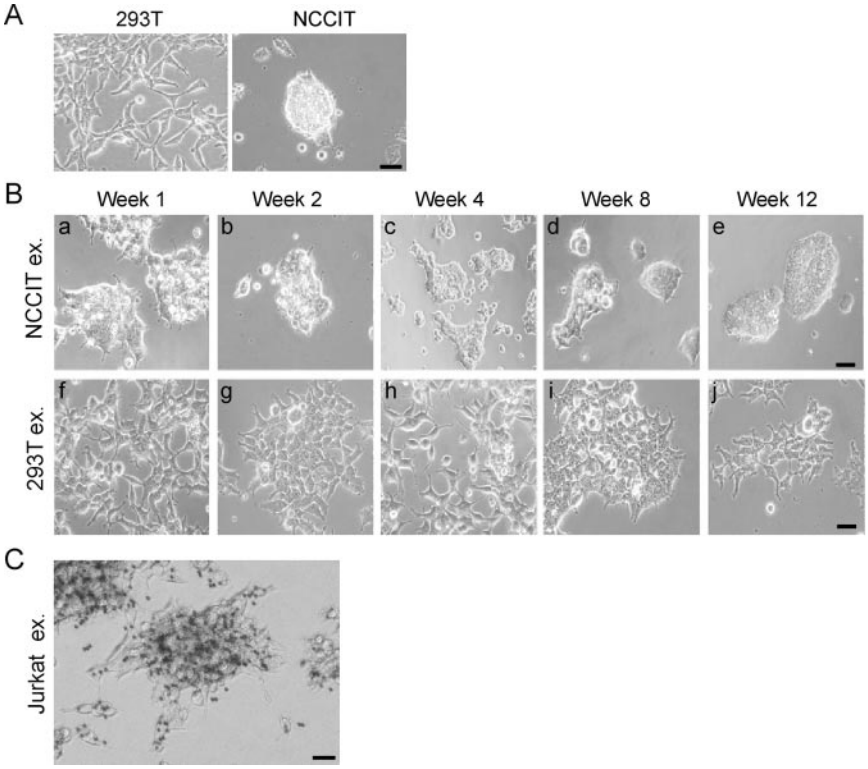

Figure 1. Morphology of 293T cells treated with NCCIT extract. (A) Untreated 293T and NCCIT cells. (B) 293T cells at indicated time points after exposure to NCCIT (a–e) or 293T (f–j) extract. (C) 293T cells 10 d after exposure to Jurkat extract and cultured under T-cell growth conditions. Dark spots are Dynal Biotech (Montebello, Norway) magnetic beads bearing antibodies against CD3 and CD28 surface antigens and used to promote T-cell expansion. Bars, 30 μm.

(Avilion *et al*., 2003), which was also found to be induced in extract-treated cells. Additional markers of pluripotentiality (Hoffman and Carpenter, 2005) up-regulated in NCCIT extract-treated cells and verified by quantitative RT-PCR included ALP 1 (*APL*), *STELLA*, *AC133*, *CD9*, *DMNT3B*, and *DNMT3L* (Table 1). Expression of these genes was examined and confirmed at 2, 4, and 6 wk after extract treatment but not examined thereafter (our unpublished data). However, genes such as *PDGFαR*, *FGF2*, *LEFTY1*, *LEFTY2*, *CD135*, or *CD117* were not expressed in any cell type or were not altered by extract treatment (our unpublished data). As expected, transcripts for the ubiquitously expressed lamin B1 (*LMNB1*) were not altered by exposure to either extract (Table 1), supporting our immunofluorescence observations. None of the pluripotency marker transcripts examined by real time RT-PCR were elicited in 293T cells treated with their own extract (our unpublished data).

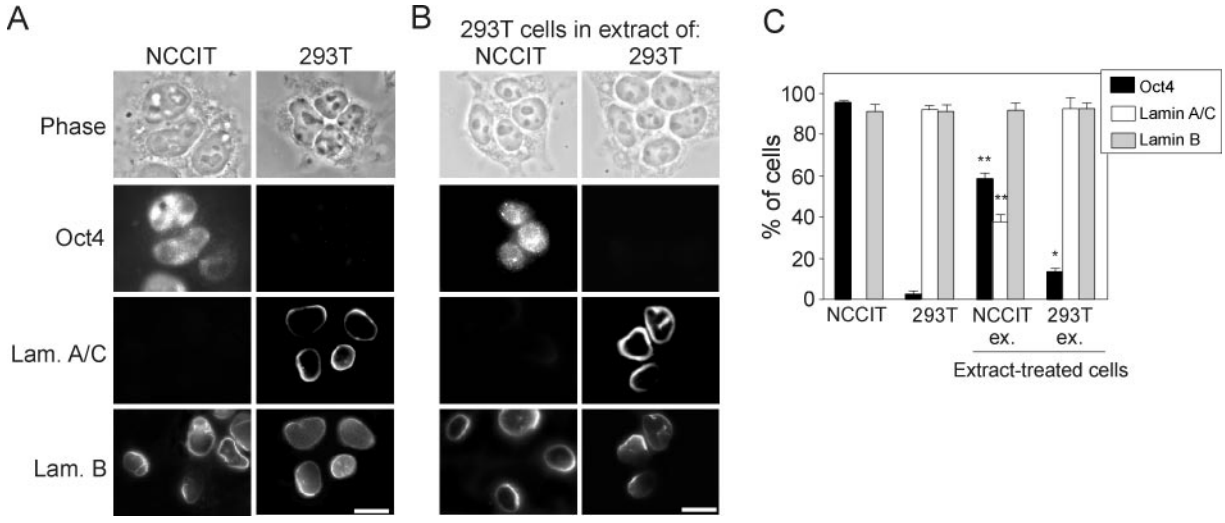

Figure 2. Immunofluorescence analysis of Oct4, lamin A/C and B-type lamin expression in 293T cells exposed to NCCIT extract. Untreated NCCIT and 293T cells (A) and 293T cells (B) treated with NCCIT or 293T extract were immunolabeled with antibodies against Oct4, lamin A/C, and B-type lamins (B, 1 wk after extract treatment). Bars, 20 μm. (C) Proportions (mean ± SD) of untreated NCCIT and 293T cells and of extract-treated cells expressing Oct4, lamin A/C, and B-type lamins. Three sets of 200 cells were examined for each marker. *$p < 0.05$ compared with 293T cells (*t* test); **$p < 0.001$ compared with 293T cells and 293T cells treated with 293T extract (*t* test).

Table 1. Quantitative RT-PCR analysis of expression of indicated stem cell genes in 293T cells treated with NCCIT extract

Gene	Fold up- or down-regulation		
	293T extract	NCCIT extract	NCCIT cells
AC133	2▲	260 ▲	271▲
APL	2▲	35 ▲	28▲
CD9	1	3 ▲	4▲
DNMT3B	1	16 ▲	11▲
DNMT3L	3▲	36 ▲	23▲
NANOG	1	2513 ▲	1541▲
OXT2	0	20 ▲	23▲
POU5F1	1	600 ▲	410▲
REX1	1	1994 ▲	1985▲
SOX2	1	18 ▲	27▲
STELLA	1	88 ▲	48▲
UTF1	2▲	121 ▲	133▲
LMNA	2▼	370 ▼	15▼
LMNB1	1	1.5▲	1

Table indicates fold up-regulation (▲) or down-regulation (▼) of indicated genes in 293T cells exposed to 293T or NCCIT extract and cultured for 4 wk. Reference level (1) was that of untreated 293T cells. Transcript levels in NCCIT cells relative to 293T cells are also shown.

To verify that expression of Oct4 and reduction of A-type lamin expression did take place in 293T cells, 293T cells stably expressing an enhanced green fluorescent protein (EGFP) and a geneticin-resistance gene (293T-EGFP-Gen^R cells) were treated with NCCIT extract. After 2 wk of culture with 700 μg/ml geneticin, which kills NCCIT cells (our unpublished data), the majority of 293T-EGFP-Gen^R cells stained positive for Oct4 (Figure 3A). Strong up-regulation of *OCT4*, SOX2, and *APL* and moderate up-regulation of *STELLA* (~2-fold) gene expression were also detected in these cells, whereas *LMNA* was repressed (Figure 3B). We also took advantage of the large T antigen marker carried by 293T cells. PCR analysis indicated that 293T-EGFP-Gen^R cells, but not NCCIT cells, contained the SV40 large T antigen transgene (Figure 3C), confirming that cells expressing Oct4 were of 293T origin. Of note, karyotyping analysis of 293T cells before and after extract treatment was inconclusive due to the severe aneuploidy and genomic instability of untreated 293T cells (our unpublished data). 293T cell aneuploidy was expected to occur as a result of large T antigen transformation, which is known to cause endoreplication (Wu *et al.*, 2004).

Last, treatment of NCCIT extract with 500 μg/ml DNAse I, which eliminates most detectable DNA in the extract (our unpublished data), before incubating 293T-EGFP-Gen^R cells did not affect induction of *OCT4*, *SOX2*, *APL*, and *STELLA* expression or *LMNA* repression (Figure 3B; +DNAse). This argues that Oct4 expression in 293T cells did not emanate from intact DNA derived from the NCCIT extract.

The NCCIT Extract Promotes OCT4 DNA Demethylation

Transfer of mammalian cells into the germinal vesicle of *Xenopus* oocytes elicits DNA demethylation in the *OCT4* promoter, a prerequisite for *OCT4* expression in this system (Simonsson and Gurdon, 2004). The *OCT4* region analyzed in our study was from nucleotide 1433–1671 (GenBank sequence AJ297527), encompassing eight potentially methylated cytosines in CpG dinucleotides between conserved regions CR2 and CR3 in the *OCT4* promoter (Nordhoff *et al.*, 2001) (Figure 4A). Bisulfite sequencing showed that this region was unmethylated in NCCIT but methylated in 293T cells (Figure 4A). In 293T cells exposed to their own extract, *OCT4* remained methylated. However, in NCCIT extract-treated cells, *OCT4* demethylation was evident after 4 and 9 wk of culture (Figure 4A) and provided molecular support for long-term transcriptional activation of *OCT4*. Two CpGs showed apparent partial demethylation (Figure 4A), which was interpreted as the expected presence of a mixed cell population in which cytosines −1686 and −1676 did not undergo demethylation. We concluded therefore that the NCCIT extract was capable of eliciting *OCT4* demethylation in 293T cells. *OCT4* demethylation was specific for the NCCIT extract and it did not occur in 293T extract (Figure 4A).

We also examined the DNA methylation status of *LMNA*, whose expression is virtually repressed in NCCIT extract-treated cells. The *LMNA* region analyzed spanned nucleo-

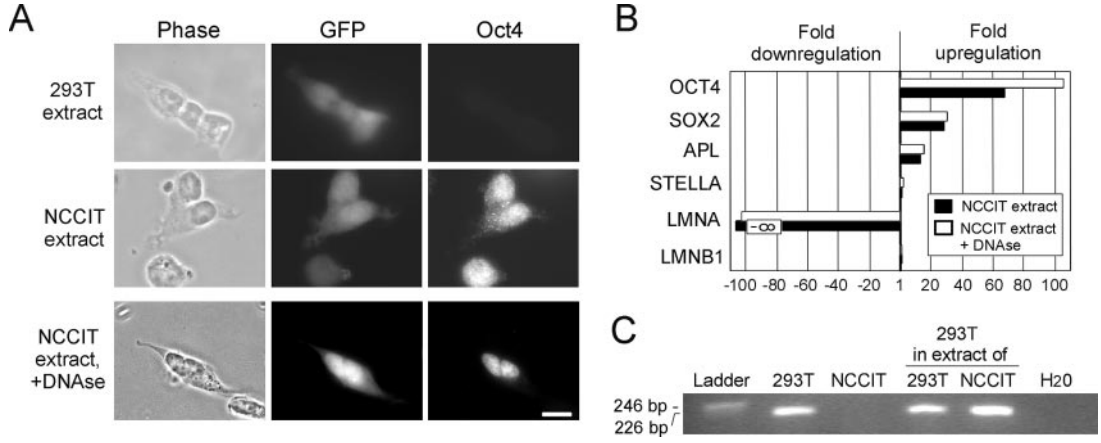

Figure 3. Oct4 expression in EGFP-labeled 293T cells. (A) 293T cells stably expressing EGFP and a geneticin resistance (Gen^R) gene were treated with 293T or NCCIT extract and cultured for 2 wk with 700 ng/ml geneticin before immunolabeling with anti-Oct4 antibodies. NCCIT extract was also treated with 500 μg/ml DNAse I before incubating cells (bottom row). Bar, 20 μm. (B) Quantitative RT-PCR analysis of expression of indicated genes in 293T-EGFP-Gen^R cells 2 wk after incubation in intact or DNAse I-treated NCCIT extract (relative to 293T extract-treated controls). (C) PCR analysis of the presence of SV40 large T antigen in 293T, NCCIT, and extract-treated cells. Ladder is a 123-base pair DNA ladder.

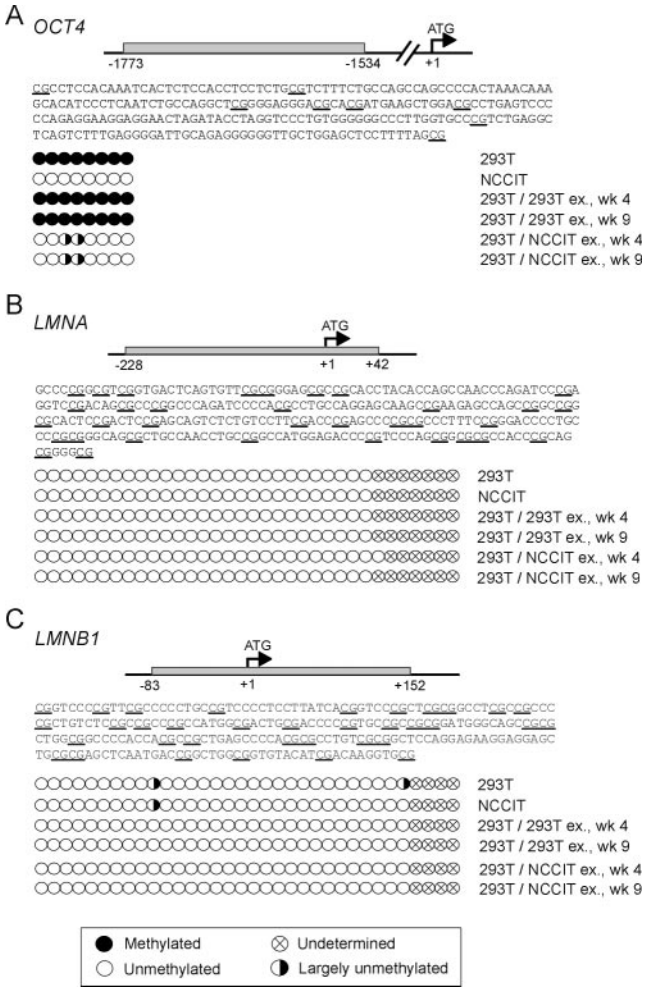

Figure 4. Bisulfite sequencing analysis of DNA methylation changes in extract-treated cells. 293T cells, NCCIT cells, and cells treated with 293T or NCCIT extract were examined for cytosine methylation in underlined CpG dinucleotides within shown genomic regions of the human *OCT4* (A), *LMNA* (B), and *LMNB1* (C) genes. Diagrams show localization (nucleotide numbers) of regions examined relative to the ATG translation start (+1).

tides 2379–2648 (GenBank L12399), encompassing 34 potentially methylated cytosines found in CpG dinucleotides flanking the ATG translation start (Figure 4B). This region is unmethylated in 293T cells and clearly remained unmethylated in NCCIT extract-treated cells (Figure 4B). However, we also found an absence of *LMNA* DNA methylation in this region in NCCIT cells despite the repression of *LMNA* in these cells (Figure 4B). Thus, we concluded that NCCIT extract-treated cells did not undergo aberrant DNA methylation in this region. We cannot ascertain, however, that other regions in the LMNA locus were modified by extract treatment. Last, *LMNB1* remained unmethylated regardless of extract treatment (Figure 4C), in agreement with its ubiquitous expression. The *LMNB1* region examined spanned nucleotides 1594–1828 (GenBank L37737), encompassing 35 potentially methylated cytosine nucleotides in a CpG island around the ATG start. *GAPDH*, also ubiquitously expressed and used as reference in our quantitative RT-PCR analyses, also remained largely unmethylated in any cell type examined (our unpublished data).

Transcriptional Profiling of NCCIT Cells Relative to 293T Cells

To evaluate the extent of transcriptional alterations elicited by the NCCIT extract, an Affymetrix U133A GeneChip microarray analysis of 293T cell gene expression was carried out for 8 wk after extract treatment. We took advantage of a SMART PCRcDNA approach that combines PCR amplification and T7 RNA polymerase to amplify submicrogram RNA samples (Ji *et al*., 2004). Although some distortion of within-sample stoichiometry occurs with this method, one can assume the same distortion between samples, thus maintenance of between-sample stoichiometry allows comparative analyses.

We first assessed genes significantly ($p < 0.001$) up- or down-regulated at a more than threefold difference level in NCCIT compared with 293T cells. A total of 2950 genes were up-regulated, whereas 2528 genes were down-regulated in NCCIT cells, in two independent analyses performed in duplicate. Distributions of up- and down-regulated genes into functional classes were similar, with most genes encoding elements involved in transcription regulation (22 and 19%, respectively), cytoskeletal organization (6 and 3%), metabolism (4 and 10%), protein synthesis and processing (6 and 4%), cell signaling (10 and 8%), and chromatin organization (6 and 4%) (Figure S1).

NCCIT Extract Induces Expression of NCCIT-specific Genes and Down-Regulation of 293T Genes

The NCCIT extract elicited up- and down-regulation of ~1700–2000 and ~1650–1800 genes, respectively, on any given week relative to 293T cells (Figure 5B, green and red bars). Of these, ~70 and ~34%, respectively, were shared with NCCIT cells and qualified as "NCCIT genes" (Figure 5, A and B, yellow bars; E). Furthermore, the likelihood that expression of these genes was altered by chance rather than as a result of extract treatment was extremely low ($p < 10^{-5}$ and $p < 10^{-4}$, respectively; *t* tests), indicating that changes were elicited by the NCCIT extract.

We then addressed the specificity of gene expression changes elicited by NCCIT extract. First, exposure of 293T cells to their own extract induced up- or down-regulation of ~1600 and ~600 genes, respectively (Figure 5C, green and red bars), of which only ~6% were identified as NCCIT genes (Figure 5C, yellow bars; E; listed in Table S2). Similarly, treatment of 293T cells with Jurkat T-cell extract altered expression of a negligible proportion of NCCIT genes (Figure 5, D and E). Furthermore, nearly all NCCIT genes affected by 293T or Jurkat extract were the same (Table S2; annotations[a,b]), and probabilities that these genes were altered by chance rather than by extract treatment were relatively high ($p > 0.07$ and $p > 0.08$, respectively; *t* tests). Resulting numbers of NCCIT genes specifically up- or down-regulated by NCCIT extract and reproducibly in both experiments are shown in Figure 5F (green and red bars, respectively). Thus, the NCCIT extract elicits specific alterations in the 293T cell expression profile. Genes not differentially expressed in NCCIT compared with 293T also seem to be affected.

The consistency of NCCIT gene expression changes in NCCIT extract-treated cells over time was subsequently assessed. Figure 5G (gray bars) shows that 686 genes were consistently up-regulated from weeks 1–8, whereas 161 genes were consistently down-regulated (these genes were shared between both experiments). These genes are listed in Table S3. The remaining affected genes included those with an onset of up- or down-regulation later than week 1, or

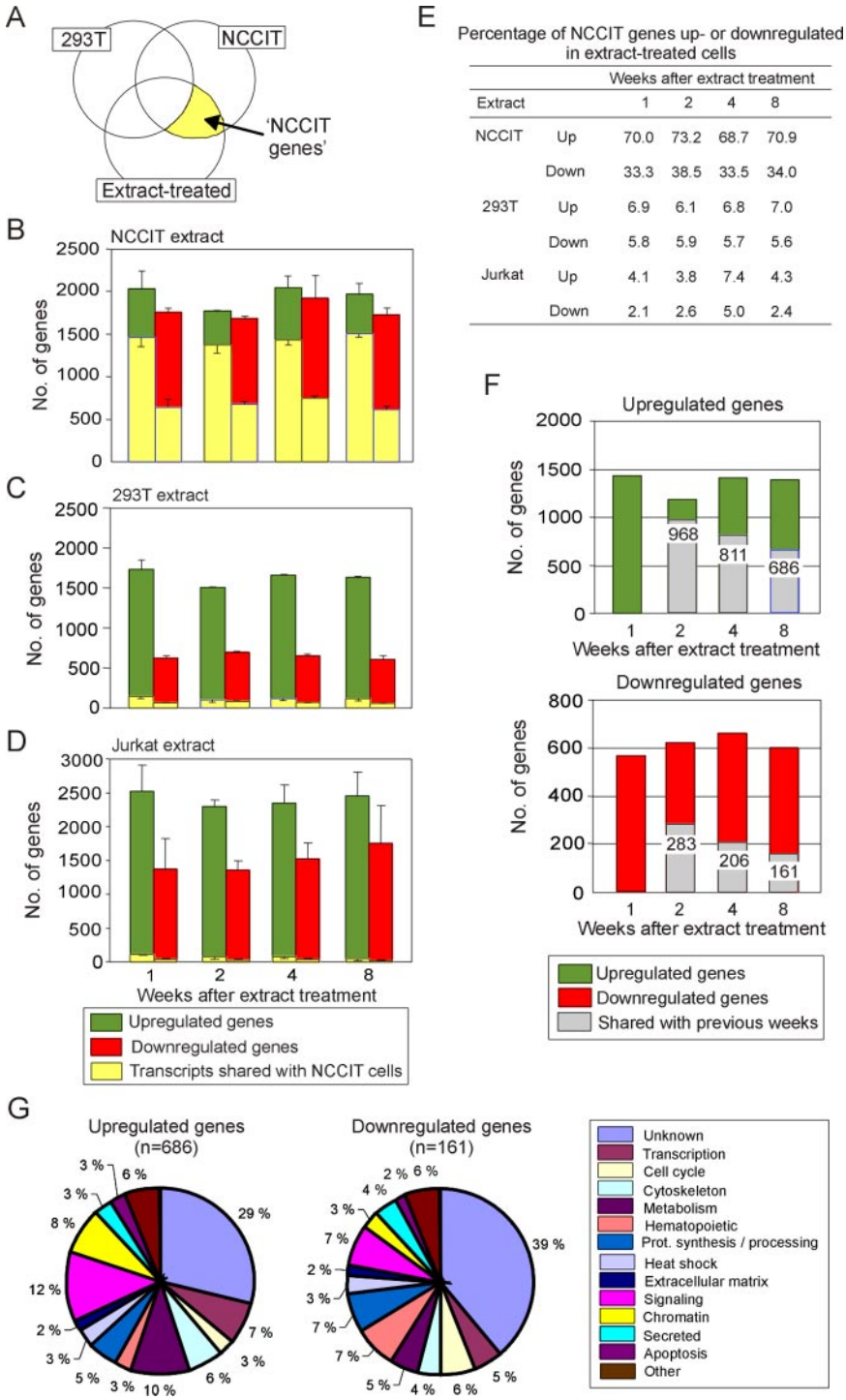

Figure 5. Microarray analysis of gene expression in extract-treated 293T cells. (A) Venn diagram identifying "NCCIT-specific" genes (yellow area). Numbers of genes up- or down-regulated more than threefold (relative to input 293T cells) in cells incubated in extract of NCCIT (B), 293T (C), and Jurkat (D) cells (mean ± SD of two [B and C] and four [D] experiments). Yellow bars indicate genes up- or down-regulated in extract-treated cells and shared with NCCIT cells. In B, the likelihood that NCCIT genes are up- or down-regulated by chance rather than by extract treatment is extremely low ($p < 10^{-5}$ and $p < 10^{-4}$, respectively; *t* tests). By contrast, in C and D these probabilities are relatively high ($p > 0.07$ and $p > 0.08$, respectively; *t* tests). (E) Percentage of NCCIT genes up- or down-regulated in extract-treated cells (percentage of total up- or down-regulated genes). (F) Number of NCCIT genes specifically up- or down-regulated by treatment with NCCIT extract, over time. (G) Consistency of gene up- or down-regulation over time after treatment with NCCIT extract. Numbers of up- and down-regulated NCCIT genes in cells exposed to NCCIT extract are shown in green and red. Gray bars represent genes consistently up- or down-regulated at weeks 1 and 2 (gray bars at week 2), weeks 1, 2, and 4 (gray bars at week 4), etc., and shared between the two experiments. (H) Functional class distribution of genes consistently up- or down-regulated over 8 wk in two experiments (gray bars in G). These genes are listed in Table S3.

those with a more fluctuating expression level. For example, a large number of genes were up-regulated from week 2 onward, and thus they were not taken into account in the above-mentioned analysis. Functional class distribution of the consistently up- or down-regulated genes (Figure 5H) shows that most annotated up-regulated genes encoded elements involved in transcription, cytoskeletal organization, metabolism, signaling, and chromatin remodeling, whereas down-regulated genes were more evenly distributed across functional classes.

Treatment with NCCIT Extract Up-Regulates Markers of Pluripotency and Genes Indicative of Multilineage Priming

Table 2 lists markers of pluripotency represented in the array and that were up-regulated in extract-treated cells. In agreement with our immunolabeling and DNA methylation data, *OCT4* (*POU5F1*) was up-regulated from week 2 onward. Notably, the Oct4-responsive genes *UTF1* and *REX1/DRN3* (Hosler *et al.*, 1989; Okuda *et al.*, 1998) were also up-regulated together with *SOX2*, suggesting the induction

Table 2. Changes in expression level of selected markers of dedifferentiation and multi-lineage differentiation potential in NCCIT extract-treated cells

Name	GenBank accession no.	Description	NCCIT cells (fold up-regulation)[a]	NCCIT extract-treated cells (fold up-regulation)[a]			
				Wk 1	Wk 2	Wk 4	Wk 8
Somatic cell markers							
LMNA	M13452.1	Nuclear lamin A	0.0015	0.9	0.0011	0.0014	0.0011
LMNB1	NM_005573.1	Nuclear lamin B1	2.5	1.7	1.0	1.5	1.4
LMNB2	M94363	Nuclear lamin B2	0.8	1.1	1.1	0.9	1.1
NPR3	NM_000908.1	Atrionatriuretic peptide receptor C	0.001	0.001	0.001	0.001	0.001
Embryonic, germ cell and stem cell markers							
OCT4 (*POU5F1*) and Oct4-responsive genes							
POU5F1	NM_002701.1	POU domain, class 5, TF1 (Oct4)	36	2	17	35	36
UTF1	N_003577.1	Undifferentiated embryonic cell transcription factor 1	317	2	213	293	318
REX1	AJ243797	Deoxyribonuclease III (Drn3)	5	1	2.5	6	9
FOXD3	N_012183.1	Forkhead box D3 (FoxD3)	847	38	283	295	254
Telomerase and telomerase-associated factors							
TERT	N_003219.1	Telomerase reverse transcriptase	102	2.5	22	76	89
TERF1	N_017489.1	Telomerase-associated factor 1	3.5	3	3.2	4.5	3.5
TERF2	N_005652.1	Telomerase-associated factor 2	0.7	0.5	0.9	0.6	0.6
SOX2	AI356682	Sex determining region Y-box 2 (Sox2)	119	1	12	118	121
POU3F1	L26494.1	POU domain, class 3, TF1 (Oct-6)	164	1.5	3	106	84
ALP1	M13077	Placental alkaline phosphatase 1	1311	38	292	928	927
ALP1	J04948.1	Placental alkaline phosphatase 1	2124	56	344	651	1337
CD44	N_000610.1	CD44 antigen (homing function)	1028	102	205	622	663
LIF	N_002309.2	Leukemia inhibitory factor	221	7	27	127	271
FZD9	N_003508.1	Frizzled (*Drosophila*) homologue 9	331	10	101	102	107
TEF	U06935.1	Thyrotroph embryonic factor	423	69	126	222	273
SCGF	D86586.1	Stem cell growth factor β	934	234	917	939	906
GCNF	AF004291.1	Germ cell nuclear factor	1105	27	71	424	696
SPINK2	N_021114.1	Serine protease inhibitor, Kazal type, 2	662	2.5	65	257	555
DKK2	N_014421.1	Dickkopf (*Xenopus laevis*) homolog 2[b]	1122	345	457	473	476
INTA6	AV733308	Integrin α6	3.1	1.5	4.5	10	15
Markers of potential lineage- specific differentiation							
Osteogenic lineage							
BMP1	N_006129.2	Bone morphogenic protein 1	870	67	169	367	470
BMP2	NM_001200.1	Bone morphogenic protein 2	655	166	333	653	663
OGN	N_014057.1	Osteoglycin	17	2	4	14	17
CTSK	N_000396.1	Cathepsin K	8	4	5	5	6
TNFRSF11B	N_002546.1	Osteoprotegerin	82	1	14	94	65
Endothelial lineage							
VWF	N_000552.2	Von Willebrand Factor	629	604	598	734	647
NOS3	N_000603.1	Nitric oxide synthase 3 (endothelial cell)	421	127	167	129	306
Myogenic lineage							
MYF5	N_005593.1	Myogenic factor 5	13	10	13	30	23
TMP1	N_000366.1	Tropomyosin 1 alpha	46	14	12	11	16
MYH11	N_022844.1	Myosin, heavy polypeptide 11	98	4	22	28	69
Neurogenic lineage							
NTS	N_006183.2	Neurotensin	73	7	20	27	48
NRG1	N_004495.1	Neuregulin 1 isoform gamma	857	60	56	66	86
MBP	M13577.1	Myelin basic protein	21	2	7	11	14
MOBP	D28114.1	Myelin-associated oligodend. basic protein	43	22	24	24	22
NCAM1	BF348061	Neural cell adhesion molecule 1	18	1	7	17	20
CD56	U63041.1	Neural cell adhesion molecule CD56	16	1.5	2	4	11
Adipogenic lineage							
APOA2	N_001643.1	Apolipoprotein A-II	13	4	5	9	9
APOD	N_001647.1	Apolipoprotein D	11	3	11	10	12
APOE	N33009	Apolipoprotein E	21	2	11	22	2
APOC1	N_001645.2	Apolipoprotein C1	4	0.5	1.5	3	4
PPARG2	N_015869.1	Peroxisome prolif. activated receptor γ2	80	1	1.5	10	22
FAD1	BG165833	Fatty acid desaturase 1	10	3	4	7	5
Chondrogenic lineage							
COL4A3	U02520	Collagen type IV alpha 3	36	3	9	27	31
COL5A2	N_000393.1	Collagen, type V, alpha 2	44	2	4	12	16
COL8A1	BE877796	Collagen, type VIII, alpha 1	19	1	5	9	15
COL11A1	J04177	Collagen, type X1, alpha 1	251	1	10	42	230
CSPG2	N_004385.1	Chondroitin sulfate proteoglycan 2	85	1	10	92	87
AGC1	N_013227.1	Aggrecan 1	15	5.5	4.5	50	46
DSPG3	N_004950.2	Dermatan sulphate proteoglycan 3	310	1.5	3	52	157
CSPG1	X17406.1	Aggrecan 1	9	1	2	13	16
FNP	X02761.1	Fibronectin precursor	9	4	9	4	2
FN1	AK026737.1	Fibronectin 1	12	4	10	7	6

[a] Relative to 293Tcells.

[b] Also up-regulated in cells exposed to 293T or Jurkat extract.

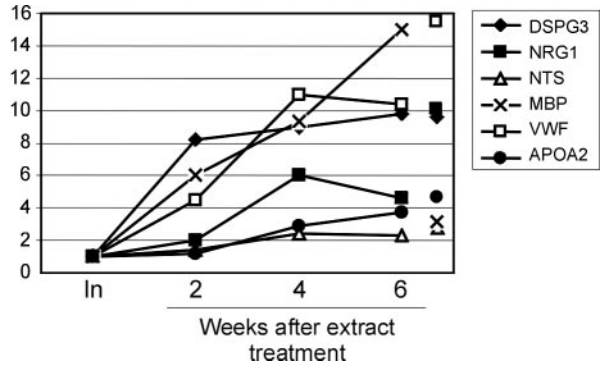

Figure 6. Quantitative RT-PCR analysis of expression of indicated multilineage priming genes in 293T cells treated with NCCIT extract relative to transcript levels in 293T cells exposed to 293T extract. Expression levels were adjusted to those of *GAPDH* in triplicate samples. Single data points show mean expression level in NCCIT cells.

of Oct4-dependent functions in reprogrammed cells. Telomerase (*TERT*) and telomerase-associated factor 1 (*TERF1*) were also increasingly up-regulated. Other pluripotency markers up-regulated were the CD44 stem cell antigen, placental ALP (*APL1*), *LIF*, stem cell growth factor β (*SCGF*), germ cell nuclear factor (*GCNF*), and integrin α6 (*INTA6*), a putative marker of "stemness" shared between three gene expression profile analyses of mouse ESCs (Fortunel *et al*., 2003). Remarkably, except for Dikkopf2 (*DKK2*), none of the stem cell marker genes listed in Table 2 was affected by treatment with 293T or Jurkat extract, illustrating the extract specificity of changes elicited (Table S4). In parallel, *LMNA* was essentially repressed, whereas expression of B-type lamins (*LMNB1* and *LMNB2*) persisted, consistent with our RT-PCR and immunolabeling data. The kidney-derived 293T cell marker natriuretic peptide receptor C (*NPR3*) was also strongly down-regulated (Hansis *et al*., 2004).

We also noted the up-regulation of markers of lineage-specific differentiation to levels comparable with NCCIT expression levels. These included markers of osteogenic, endothelial, myogenic, neurogenic, adipogenic, and chondrogenic lineages (Boquest *et al*., 2005). Expression of markers of chondrogenic (*DSPG3*), neuronal (*NRG1*, *NTS*, and *MBP*), endothelial (*VWF*), and adipocyte (*APOA2*) lineages was confirmed by real-time RT-PCR (Figure 6). Furthermore, expression of several housekeeping genes, including *18S*, *28S*, *GAPDH*, *HPRT1*, and *ACTB* and 35 genes for ribosomal proteins, was unaffected in extract-treated cells (Table S5). Collectively, these results indicate that the NCCIT extract promotes the up-regulation of markers of pluripotency typically expressed in ECCs or ESCs and suggest, in addition, the establishment of a "multilineage priming" in 293T cells treated with NCCIT extract.

Oncogenes and Tumor Suppressor Genes Are Not Affected by NCCIT Extract Treatment

NCCIT is a tumor cell line that bears genetic mutations required for its expansion and phenotypic characteristics. We determined whether mRNA levels for oncogenes and tumor suppressor genes were altered in 293T cells exposed to NCCIT extract, relative to untreated cells. We did not observe any up-regulation or induction of oncogene expression in NCCIT extract-treated cells. Genes such *c-MYC*, *c-MYC*-responsive genes, genes encoding Myc-interacting or Myc-regulated proteins, and genes encoding RAB and RAF isoforms were not significantly expressed in any of the cell types examined nor were they altered by NCCIT extract. Among tumor suppressor genes, *P53* was strongly up-regulated in NCCIT compared with 293T ($p < 10^{-4}$) but remained unaltered in NCCIT extract-treated cells ($p > 0.05$). Other tumor suppressor genes, however, were either not significantly expressed in 293T or NCCIT [*RB1*, *TSC1*, *TSC22*, *BRCA1*, *BRCA2*, *CDKN2A* (p16), *CDKN1A* (p21, Cip1), *CDKN1C* (P57, Kip2), *MSH2*, *STK11*, *MEN1*, and *MEN2*] or were expressed at similar levels in both cell types (*PTCH*, *PTEN*, and *WT1D*). These genes remained unaltered by treatment with NCCIT extract ($p > 0.05$). Similarly, genes encoding enzymes involved in DNA repair (*XPA*, *ERCC5*, *FANCA*, *-C*, *-E*, *-F*, and *-G*) were not significantly expressed in either cell types nor altered by extract treatment. *ATM* was highly expressed in 293T and NCCIT cells and remained unchanged in extract-treated cells. Because it is unlikely that NCCIT genetic lesions are passed onto the 293T cell genome through extract treatment, acquisition of an NCCIT phenotype by 293T cells implies that either the phenotype obtained is independent of NCCIT lesions or that genetic mutations that gave rise to the NCCIT tumor phenotype are dispensable for the maintenance of this state.

Retinoic Acid Stimulation of NCCIT Extract-treated Cells Induces Neuronal Differentiation

To determine whether NCCIT extract-treated 293T cells acquired a potential for pluripotency, we attempted to induce neuronal differentiation in vitro with retinoic acid (Stewart *et al*., 2003). 293T, NCCIT (our unpublished data), and extract-treated cells were exposed to 10 μM all-*trans*-retinoic acid and were maintained as suspended aggregation cultures (Figure 7A). Suspensions of all cell types formed disorganized aggregates in bacteriological dishes but after 2 wk in retinoic acid, the cells formed spheres that sometimes fused with one another (Figure 7A). This was particularly evident for NCCIT extract-treated 293T cells (Figure 7A, top). After washing and replating, cells adhered to poly-L-lysine-coated coverslips. However, only NCCIT and NCCIT extract-treated cells showed evidence of neurite outgrowth already after culture for 2 d in the absence of retinoic acid but in the presence of mitotic inhibitors (Figure 7A, bottom, and S2A). This suggested that neuronal progenitor cells emanated from NCCIT extract-treated 293T cells.

Immmunolabeling and real-time RT-PCR analyses confirmed the induction of a neuronal phenotype. Spheres of NCCIT cells and of NCCIT extract-treated cells showed a reduction of Oct4 protein to a level nearly undetectable by immunofluorescence in >90% of the cells (Figure 7B). This was confirmed by a threefold reduction of *OCT4* transcript levels in these cells compared with cells not stimulated with retinoic acid (Figure 7C). Moreover, expression of the intermediate filament nestin (*NES*), a marker of neuronal precursor cells (Cattaneo and McKay, 1990), was induced at the transcriptional and translational levels by retinoic acid in NCCIT extract-treated but not 293T extract-treated, cells (Figure 7, B and C). Furthermore, *LMNA*, repressed after exposure to NCCIT extract (Figure 2 and Table 1) was strongly up-regulated de novo by retinoic acid but only in NCCIT extract-treated cells (Figure 7C). This indicated that the cells redifferentiated upon retinoic acid exposure. Last, the majority of NCCIT extract-treated cells, as NCCIT cells (Figure S2B and S2C) were positive for the neuronal markers NeuN and NF200 (Figure 7D). We concluded that NCCIT extract-treated 293T cells can be induced to differentiate into a neuronal phenotype in vitro.

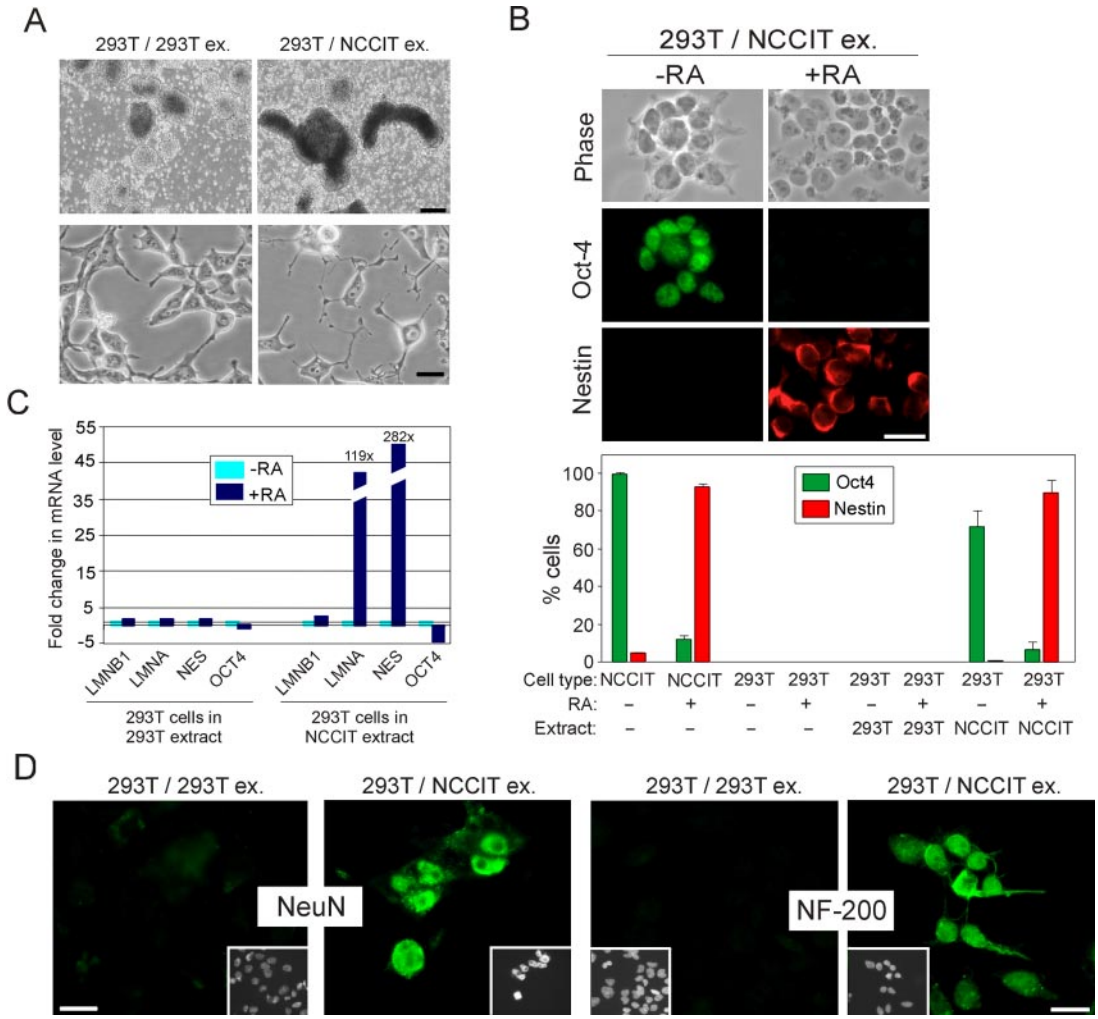

Figure 7. Neuronal differentiation of 293T cells treated with 293T or NCCIT extract. (A) Top, induction of differentiation. Suspended aggregates after 2 wk of culture with 10 μM all-*trans*-retinoic acid. Bottom, differentiation. Cells were plated onto poly-L-lysine-coated coverslips after 2 d of culture in the absence of retinoic acid but with mitotic inhibitors. Note neurite extensions in NCCIT extract-treated cells. (B) NCCIT extract-treated cells either treated with retinoic acid (+RA) or not (−RA) for 3 wk were immunolabeled using antibodies against Oct4 and nestin. Graph shows proportions (mean ± SD) of cells immunolabeled with anti-Oct4 and anti-nestin antibodies (n = 200 cells in each of a triplicate analysis for each treatment). (C) Quantitative RT-PCR analysis of expression of *OCT4*, *NES* (nestin), *LMNA* and *LMNB1* in 293T cells treated with 293T or NCCIT extract, in absence (−RA) or presence (+RA) or retinoic acid for 3 wk. (D) NeuN and NF200 immunofluorescence analysis of indicated cell types induced to differentiate as described in A. Insets, DNA labeled with Hoechst 33342. Bars, 400 μm (A, top); 40 μm (A, bottom); 40 μm (B and D).

NCCIT Extract Enhances Differentiation Potential toward Adipogenic, Osteogenic, and Endothelial Lineages

To provide additional evidence for induction of differentiation potential in 293T cells treated with NCCIT extract, we determined whether the cells would acquire phenotypic characteristics of adipocytes and osteoblasts. After 3 wk of retinoic acid treatment and 3 wk of stimulation in appropriate differentiation medium (see *Materials and Methods*), a proportion of 293T cells, NCCIT cells, and of cells treated with 293T or NCCIT extract were induced to differentiate toward adipogenic and osteogenic pathways (Figures 8, A and B, and S2D and S2E). Intracellular lipid staining with Oil-Red-O showed enhanced differentiation of NCCIT extract-treated cells relative to any other cell type toward the adipogenic pathway (Figure 8A). Moreover, significantly more Alzarin red-stained mineralized nodules were detected in NCCIT extract-treated 293T cells compared with any other cell type (Figure 8B, arrows; $p < 10^{-6}$; *t* tests). Additionally, induction of endothelial differentiation of NCCIT extract-treated cells in methylcellulose triggered the appearance of an endothelial cell phenotype after 7 d. With cells forming elongated "tracks" in methylcellulose (Figure 8C; see controls in Figure S2F). Morphological changes were supported by immunoreactivity to CD31 and CD144, two endothelial cell surface markers (Boquest *et al*., 2005) (Figure 8D), and by induction of expression of *CD31* (2 ± 0.15-fold up-regulation compared with undifferentiated, extract-treated cells) and *CD144* (347 ± 97.6-fold up-regulation). Collectively, these results indicate that treatment with NCCIT extract enhances the ability of 293T cells to differentiate into ectoderm and several mesoderm lineages.

A Mouse ES Cell Extract Promotes A-Type Lamin Down-Regulation and Oct4 Transcription in NIH3T3 Fibroblasts

A nuclear and cytoplasmic extract of mouse ESCs was prepared to determine whether it was capable of eliciting mark-

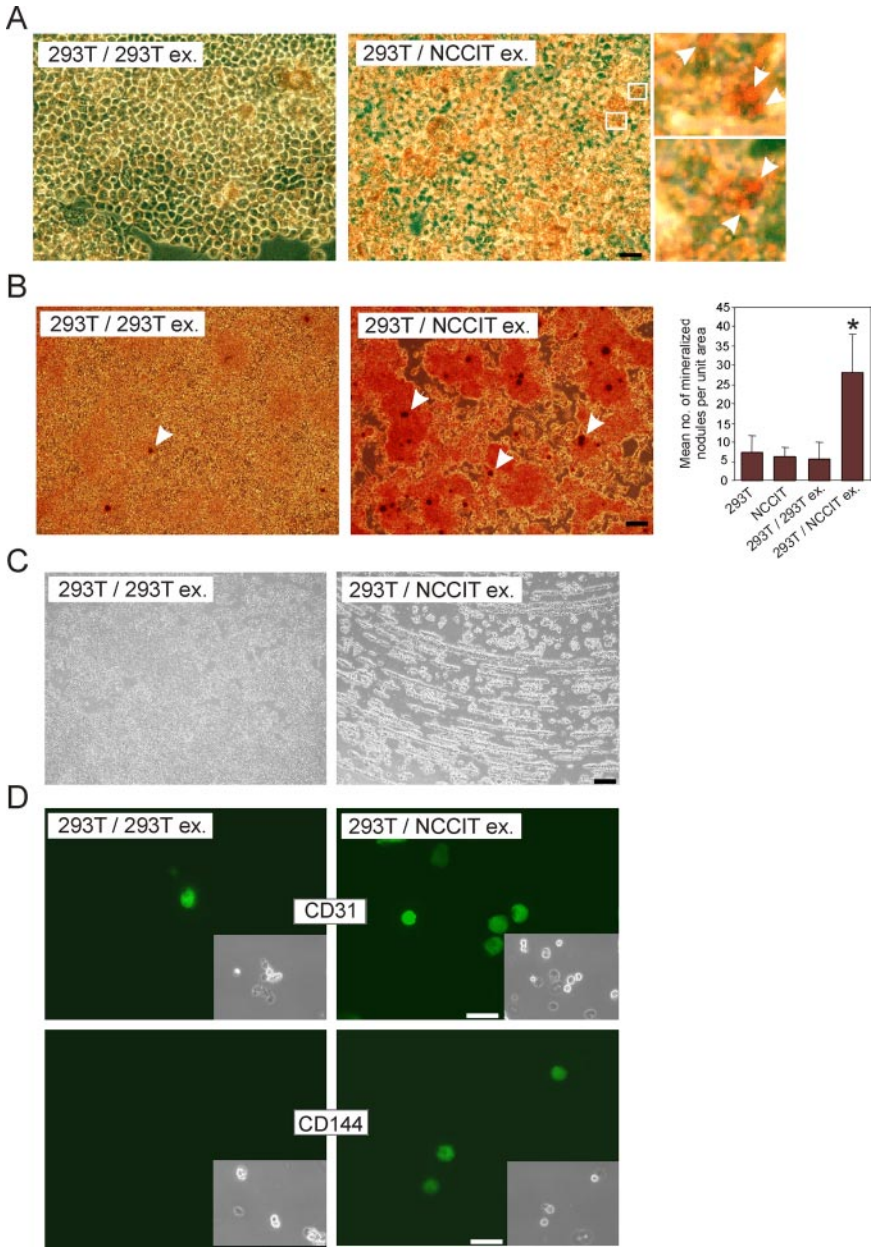

Figure 8. Induction of adipogenic, osteogenic, and endothelial differentiation of 293T cells treated with 293T or NCCIT extract. (A and B) Cells were exposed to 10 μM retinoic acid for 21 d, washed, and cultured in the absence of retinoic acid in adipocyte (A) or osteoblast (B) differentiation medium for 21 d. (A) Cells were stained with Oil-Red-O to reveal lipid droplets. Images on the right are enlargements of two areas framed in white in the adjacent panel (top right-hand quadrant). Arrows point to strongly stained lipid droplets. (B) Cells were stained with Alzarin red to visualize mineralized nodules (arrows). Graph shows mean ± SD number of distinct strongly mineralized nodules (arrows) per unit area (area shown in B). Twenty-four to 27 areas were analyzed within wells of six-well culture plates. *$p < 10^{-6}$ relative to other treatments (ANOVA). (C) 293T and NCCIT extract-treated cells were passaged onto methylcellulose for 7 d to elicit endothelial differentiation. Note the formation of a track phenotype characteristic of cultured endothelial cells (right). (D) Direct immunofluorescence labeling of CD31 and CD144 surface antigens in extract-treated cells induced to differentiate as described in C. After differentiation, cells were loosened from the methylcellulose semisolid substrate by dilution with PBS and thus lost their elongated phenotype. Bars, 40 μm (A), 200 μm (B and C), and 40 μm (D).

ers of pluripotency, as with the NCCIT extract. Within 4–9 d after ESC extract treatment, a proportion 3T3 cells formed distinct colonies of small round cells that lifted from the surface to form embryoid-like bodies (Figure 9A). Fifty to 100 embryoid-like bodies were identified in 100-mm culture plates, whereas most remaining cells retained a fibroblast morphology. Smaller aggregates of round (nonmitotic) cells were also detected. In contrast, 3T3 cells exposed to their own extract grew as fibroblasts and were not distinguishable from untreated cells (Figure 9A). The ESC-like phenotype was maintained after passaging cells weekly for at least 10 wk. RT-PCR analysis of the embryoid body-like structures clearly revealed *Oct4* transcripts (see below), whereas *Lmna*, but not *Lmnb1*, was strongly down-regulated (Figure 9B).

Embryoid-like bodies derived from ESC extract-treated cells expressed ALP, another embryonic and ESC marker, after 8 d of culture (Figure 9, C and D; 1 wk after extract exposure). ALP expression was inhibited by a 24-h exposure to 25 μM RNA PolII inhibitor 5,6-dichloro-1-D-ribofuranosyl benzimidazole (DRB) or to 10 μg/ml protein synthesis inhibitor cycloheximide (CHX) (Figure 9D). Thus, ALP expression was endogenous to the extract-treated cells. No expression was detected in control cells (Figure 9, C and D). Furthermore, immunofluorescence analysis showed that ~90% of embryoid-like body-derived cells expressed intranuclear Oct-4, whereas in the same cells lamin A/C expression was reduced to undetectable levels (our unpublished data). Immunoblots confirmed this observation (Figure 9E) and indicated that ESC extract-treated cells expressed Oct4 to levels similar to ESCs. Oct4 not was detected in 3T3 cells incubated in their own extract or in intact (nonpermeabilized) 3T3 cells treated with ESC extract (Figure 9E). This ruled out the detection of an unspecific anti-Oct4 immunoreactive product and of any extract-derived Oct4 protein that would stick to the cell surface. Detection of Oct4 protein in ESC extract-treated cells required a threshold

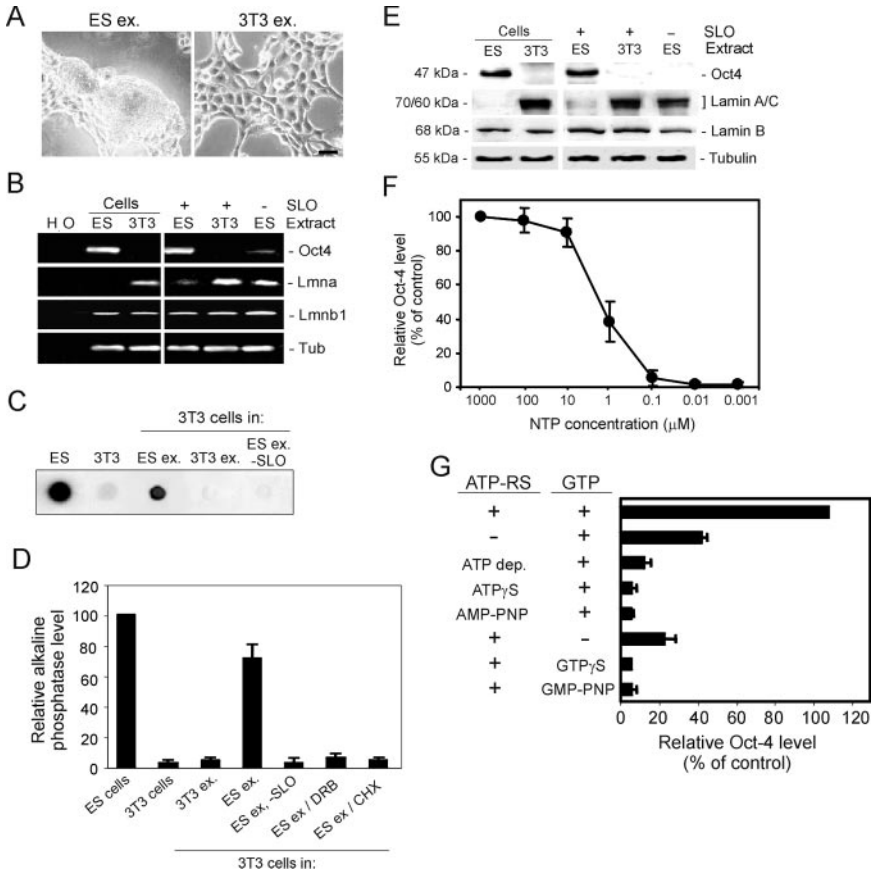

Figure 9. Induction of Oct4 and ALP expression in 3T3 cells exposed to mouse ESC extract. (A) 3T3 cells 10 d after treatment with ESC extract (ES ex.) or 3T3 extract (3T3 ex.). Bar, 20 μm. (B) RT-PCR analysis of expression of indicated genes in ESCs, 3T3 cells and 3T3 cells at 2 wk after treatment with ESC extract. (C) ALP expression in cells treated as described in B (2 wk after extract treatment). (D) Relative ALP level in ESCs, 3T3 cells, and in 3T3 cells treated in 3T3 extract or in ESC extract alone or with 25 μM DRB or 10 μg/ml CHX, as indicated. Cells were analyzed 2 wk after extract treatment. (E) Indicated cell types (as in B) were analyzed by Western blotting for expression of Oct4, lamin A/C, B-type lamins, and γ-tubulin, 2 wk after extract treatment. (F) Relative Oct4 level in 3T3 cells treated with ESC extract containing decreasing concentrations of NTPs (1 wk after extract treatment). (G) Relative Oct4 level in 3T3 cells exposed to ESC extract containing indicated ATP or GTP analogues. ATP-RS, ATP-regenerating system (2 wk after extract treatment).

(10 μM) concentration of NTPs in the extract (Figure 9F), providing additional evidence for the lack of unspecific detection of Oct4 in these cells.

Closer examination of the need for exogenous nucleotides to promote Oct4 detection indicated a requirement for ATP and GTP hydrolysis (Figure 9G). Indeed, ATP depletion from the extract with glucose and hexokinase, replacement of exogenous ATP with adenosine-5′-*O*-(3-thio)triphosphate or adenyl-5′-yl imidodiphosphate, or GTP removal or substitution with guanosine 5′-*O*-(3-thio)triphosphate or guanosine 5′-[β,γ-imido]triphosphate abrogated Oct4 detection (Figure 9G). This is consistent with a role of active nuclear import for transcription in cell extracts (Håkelien *et al*., 2002). Furthermore, heat treatment (95°C; 5 min), trypsinization, or proteinase K treatment of the ESC extract abolished Oct4 detection in 3T3 cells (Figure 10). However, DNAse I (100 mg/ml) or RNAse A (50 mg/ml) did not affect Oct4 levels in extract-treated cells (see below; Figure 10), ruling out a significant contribution of mRNA or DNA of extract origin.

The ESC Extract Induces a Biphasic Wave of Oct4 Transcription and Translation

To gain insight on the dynamics of Oct4 induction in ESC extract-treated cells and evaluate any putative contribution of extract-derived Oct4 protein, we assessed intracellular Oct4 protein levels over time after extract treatment and determined the effect of PolII and protein synthesis inhibitors on Oct4 induction. Relative Oct4 levels in ESC extract-treated cells are shown in Figure 11A. A biphasic response to extract exposure was observed. First, Oct4 was detected as early as 1 h after recovery of the cells from the extract, and the level peaked at 24 h (Figure 11A). This peak was followed by a marked reduction of Oct4 by 36 h to a level barely detectable by 48 h. By 72 h, however, a second wave of Oct4 was detected of amplitude similar to or higher than the first wave, and it persisted for at least 5 d (Figure 11A). Oct4 protein was also detected in these cells 5 and 10 wk after extract treatment (our unpublished data); thus, we anticipate that the second elevation of Oct4 in these cells is long-lasting. Of note, the biphasic Oct4 protein elevation paralleled fluctuations in *Oct4* transcripts, as determined by real-time RT-.PCR (Figure 11D), suggesting a short half-life (a few hours) of Oct4 RNA and protein.

Culture of ESC extract-treated cells with 25 μM DRB from 1 to 24 h after extract treatment dramatically reduced Oct4 levels by 1 h (Figure 11B, top), suggesting that Oct4 detection at this time point resulted primarily from transcription but also to a minor extent from uptake of Oct4 protein from the extract. This hypothesis was verified by a complete double immunodepletion of Oct4 from the ESC extract (Figure 12A), which resulted in a reduced Oct4 level by 1 h in non-DRB-treated 3T3 cells, without affecting subsequent levels (Figure 12, B and 1C). DRB exposure of cells treated with Oct4-depleted extract completely abolished Oct4 detection by 1 h (our unpublished data). We concluded, therefore, that Oct4 detected by the first hour of culture after extract exposure originated from the extract and from RNA PolII-mediated transcription and translation.

The effect of PolII inhibition on the dynamics of Oct4 expression in ESC extract-treated cells differed with timing of drug exposure after extract treatment. A 1- to 24-h DRB treatment almost completely blocked the fist Oct4 elevation

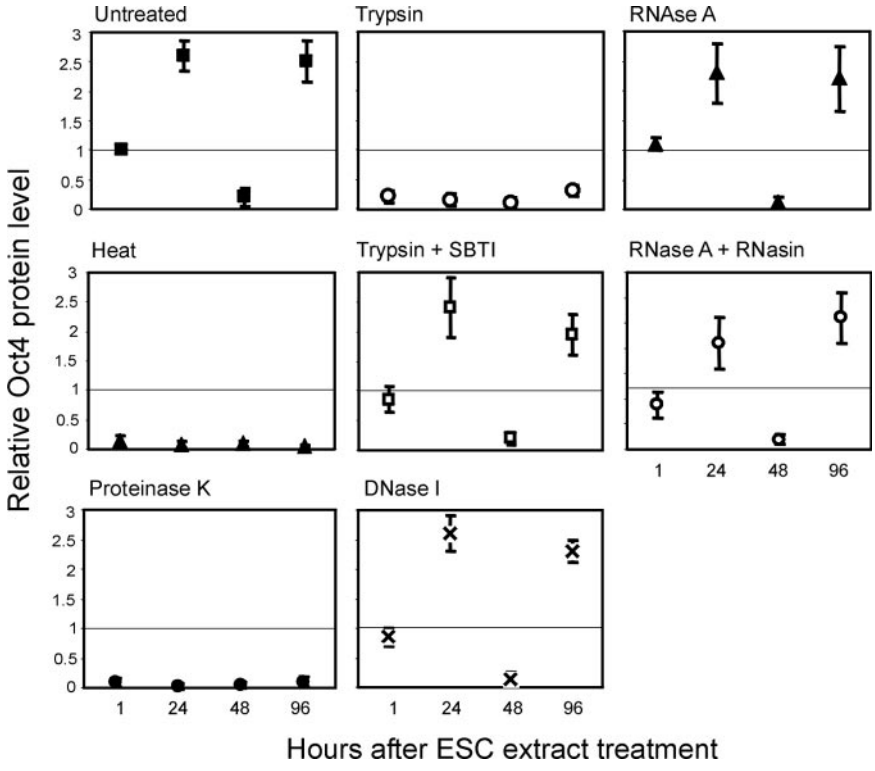

Figure 10. Relative Oct4 protein levels in 3T3 cells exposed to ESC extract pretreated as indicated in each panel. Cells were cultured for 1–96 h after extract treatment, lysed, and analyzed by Western blotting. Oct4 protein levels were determined by densitometric analysis of duplicate blots for each time point under each condition. Level 1 indicates Oct4 level at 1 h after removal of cells from an untreated ESC extract (top left).

(Figure 11B, top), supporting a role of RNA PolII in this process. In addition, and unexpectedly, the second wave of Oct4 induction was also dramatically impaired (Figure 11B, top). Again, these changes paralleled *Oct4* transcript levels: the first up-regulation of *Oct4* mRNA was inhibited, the second rise in *Oct4* transcription was also severely compromised, albeit not fully blocked, such that *Oct4* transcripts were up-regulated five- to sevenfold above the 48-h baseline

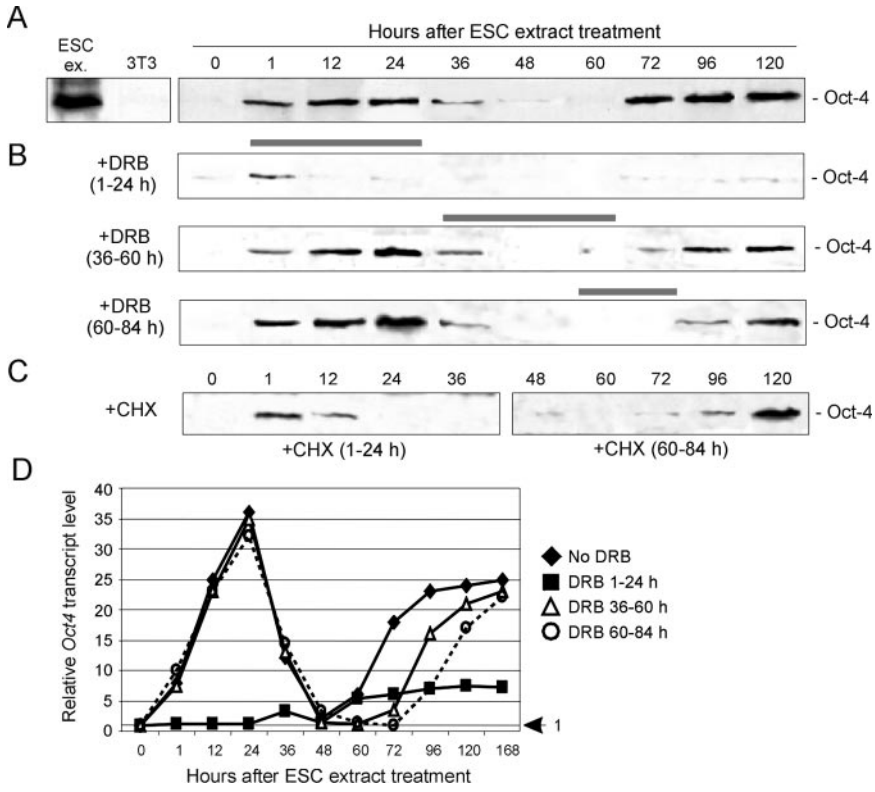

Figure 11. Oct4 expression in ESC extract-treated cells is biphasic and RNA PolII dependent. (A) Immunoblotting analysis of intracellular Oct4 levels in 3T3 cells treated with ESC extract and cultured for indicated time periods. (B) Cells were exposed to 25 μM DRB at 1–24 h (top), 36–60 h (middle), and 60–84 h (bottom) of culture. (C) Cells were exposed to 10 μg/ml CHX at 1–24 h (left) or 60–84 h (right) of culture before immunoblotting. (D) Real-time RT-PCR analysis of *Oct4* expression in 3T3 cells exposed to ESC extract. Cells were cultured with 0 or 25 μM DRB at indicated time periods as described in B. Reference level (level 1) is *Oct4* mRNA level in 3T3 cells immediately upon plating cells after recovery from extract. Data show a representative set from two experiments.

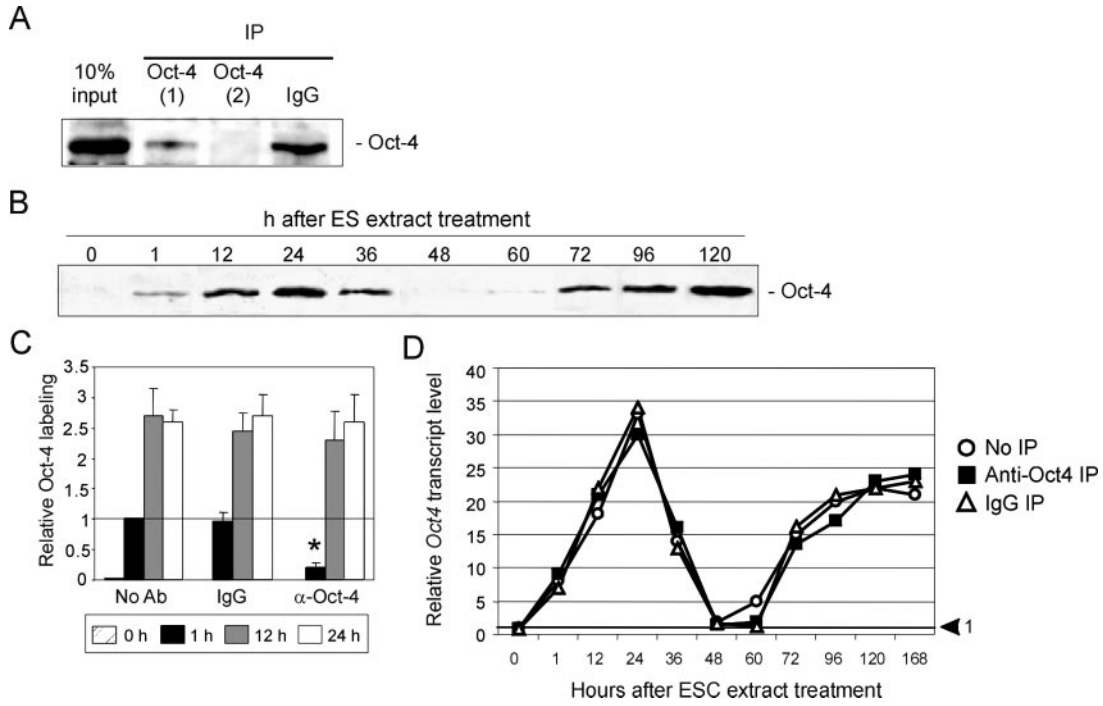

Figure 12. Immunodepletion of Oct4 from ESC extract maintains Oct4 expression profile. (A) Immunoprecipitation of Oct4 from ESC extract. Numbers indicate rounds of immunoprecipitation (IP). Resulting extracts were immunoblotted using anti-Oct4 antibodies. (B) 3T3 cells were exposed to immunodepleted ESC extract, cultured for indicated time periods, and blotted using anti-Oct4 antibodies. (C) Relative intracellular Oct4 protein level in 3T3 cells exposed to control (no antibody), mock-depleted (IgG), or anti-Oct4-depleted ESC extract and cultured for indicated time periods. *$p < 0.001$ relative to the 1-h time point in other treatments (ANOVA). Data from two experiments. (D) Real-time RT-PCR analysis of *Oct4* expression in 3T3 cells exposed to intact, mock-depleted, or Oct4-depleted ESC extract. Reference level (level 1) is Oct4 transcript level in 3T3 cells immediately upon plating cells after recovery from extract. Data show a representative set of two experiments.

instead of the ~25-fold normally observed (Figure 11D). This suggests that the second, long-lasting induction of *Oct4* up-regulation is dependent on an early, short-term boost of *Oct4* transcription and/or translation.

DRB exposure from 36 to 60 h after extract treatment, during the dip in Oct4 mRNA and protein levels, did not affect early Oct4 transcript and protein levels and only slightly delayed the second rise of Oct4 (Figure 11B, middle). This delay was also noticed at the transcript level, but it did not significantly affect Oct4 mRNA or protein level at 7 d (168 h; Figure 11D). Moreover, DRB applied from 55 to 84 h after extract treatment completely abrogated Oct4 transcription and translation by 72 h, but removal of the drug ultimately restored control Oct4 levels (Figure 11B, bottom, and D). Thus, there is a requirement for PolII activity for de novo transcription of *Oct4* 3 d after extract treatment. Last, both Oct4 elevations were abolished upon incubation with 10 μg/ml CHX, a protein synthesis inhibitor (Figure 11C). We concluded that whereas uptake of limited amounts of short-lived extract-derived Oct4 protein probably occurs, the two phases of Oct4 induction result from transcriptional and translational activity in extract-treated cells.

In summary, this study provides transcriptional and functional evidence that an extract of undifferentiated EC or ES cells can induce markers of dedifferentiation and signs of differentiation plasticity in an otherwise more developmentally restricted cell type. Furthermore, the NCCIT extract induces DNA demethylation of *OCT4*, indicative of an epigenetic reprogramming event at this locus.

DISCUSSION

Target Cell Type-specific Programming of Gene Expression

The induction of a transcriptional profile of, according to our data, uncertain stability, suggests the establishment of a program of gene expression, as opposed to a complete functional reprogramming such as that occurring after nuclear transfer. Of the hundreds of NCCIT-specific genes up- or down-regulated on any given week after NCCIT extract treatment, only 5–7% are altered by Jurkat extract, and most of these genes overlap with genes altered (statistically by chance) in cells exposed to their own extract. This argues for some cell-type specificity in the nature of genes modulated by extract treatment. Despite some stability in the expression profile of specific genes after extract exposure, not all changes seem to be heritable. Genes with unstable expression pattern may include "passive bystanders" that generate a transcriptional noise (Paulsson, 2004) and result from more specific alterations in the transcriptional network. Perturbation in the network, however, would be expected to lead to changes trickling down the network until a transcriptional equilibrium is reached (Miklos and Maleszka, 2004). Fluctuations in the gene expression profile therefore may result from incomplete reprogramming and from heterogeneity in the transcriptional response to extract treatment. Nevertheless, the dynamics of gene expression may also illustrate a temporal compartmentalization, in terms of timing, duration, and periodicity of gene activity required to establish a heritable transcriptional network (Klevecz *et al*., 2004).

Evidence of Induction of Potential for Pluripotency

The gene expression program elicited by NCCIT extract suggests the establishment of a potential for multilineage differentiation in otherwise more developmentally restricted cells. An indicator of dedifferentiation is the down-regulation of genes indicative of a differentiated state. This is exemplified by the down-regulation of many 293T cell genes, including the kidney natriuretic peptide receptor C (*NPR3*), and the repression of lamin A (*LMNA*), a marker of differentiated cells (Hutchison and Worman, 2004). *LMNA* down-regulation seems specific for extracts of undifferentiated cells that do not express lamin A/C. In contrast, cardiomyocyte extracts can up-regulate *LMNA* expression in adipose stem cells, an event that correlates with differentiation toward a cardiomyocyte phenotype (Gaustad *et al*., 2004), and *LMNA* is reactivated upon retinoic acid-mediated differentiation of NCCIT extract-treated cells. Thus, the transcriptional status of *LMNA* provides a direct assessment of (de)differentiation transitions mediated by cell extracts. The mechanism of gene inactivation in extracts is unclear. However, evidence for the down-regulation of many genes by single small interfering RNAs (Mathieu and Bender, 2004), possibly through a control of DNA methylation (Matzke and Birchler, 2005), raises the hypothesis of a contribution of small RNAs in extract-based nuclear (re)programming.

An indicator of pluripotency is the up-regulation of genes characteristic of undifferentiated EC (NCCIT) or ES cells. Several embryonic, germ cell, and stem cell genes are activated to levels similar to those of NCCIT cells. Of note, *Oct4* is expressed in ESCs to maintain pluripotency and acts in cooperation with *SOX2* (Avilion *et al*., 2003). The latter is also expressed in extract-treated cells. The Oct4 transcription factor acts on a subset of target genes, including *UTF1*, *REX1*, *OCT2*, and *NANOG* (see Hoffman and Carpenter, 2005 for an updated review of human embryonic and ESC genes). These were found to be up-regulated by NCCIT extract. Furthermore, because *UTF1* expression requires synergistic activities of Oct4 and Sox2 (Nishimoto *et al*., 1999), our results suggest the formation of a functional transcriptional complex between these factors.

Another feature of NCCIT extract-treated cells is the expression of genes suggestive of a potential for multiple lineage differentiation and acquisition of neurogenic, adipogenic, osteogenic, and endothelial differentiation ability. Differentiation potential toward other lineages was not investigated. Multilineage priming is a hallmark of hematopoietic stem cells (Akashi *et al*., 2003) and mesenchymal stem cells from bone marrow (Woodbury *et al*., 2002) and adipose tissue (Boquest *et al*., 2005). It may reflect their ability to promptly differentiate into a specific cell type in the tissue in which they reside, in response to simulation. Thus, similarly to somatic stem cells, the transcriptional signature of NCCIT extract-treated cells extends across germ layer boundaries. Additionally, because they also express embryonic and ESC markers, these cells display characteristics of a perhaps more precursor cell than the starting epithelial cell type.

Chromatin Remodeling Associated with Nuclear Reprogramming

The NCCIT extract retains the ability to elicit epigenetic reprogramming of *OCT4* in 293T cells. Our data illustrate the demethylation of six of eight cytosines in CpG dinucleotides between CR2 and CR3 in the *OCT4* promoter (Nordhoff *et al*., 2001). *Oct4* DNA demethylation in thymocyte nuclei has been reported after fusion with EG or ES cells (Tada *et al*., 1997, 2001) and is required for *Oct4* transcription after nuclear transplantation into *Xenopus* oocytes (Simonsson and Gurdon, 2004). The process driving *OCT4* DNA demethylation remains unclear but seems to require deproteinization (Simonsson and Gurdon, 2004), and it may involve cleavage of methyl groups (Ramchandani *et al*., 1999) or cytosine deamination (Morgan *et al*., 2004). The ability to induce DNA demethylation in bulk cells or nuclei incubated in extracts raises the possibility of isolating the DNA demethylation activity involved.

A transient induction of *Oct4* transcription and translation—independent of uptake of residual Oct4 protein from the extract—is triggered within the first hours after extract exposure. This early *Oct4* up-regulation may be explained by nuclear uptake of extract-derived transcription factors and chromatin remodelers that target the Oct4 promoter (Nordhoff *et al*., 2001). This possibility is supported by the inhibitory effect of removing proteins from the extract (Figure 10) and of immunodepleting BRG1 from mouse ESC extracts on *Oct4* transcription in 3T3 cells (our unpublished data). The transient (24- to 48-h) nature of this first wave of *Oct4* activation presumably results from depletion of factors (most transcription factors have a half-life of hours). This suggests that transcription factor synthesis and targeting are not optimally sustained during the first hours after extract treatment. The second wave of *Oct4* up-regulation, however, is sustained for several days and weeks. Long-term *Oct4* expression is consistent with DNA demethylation taking place in our system. Timing of long-term *Oct4* activation by ESC extract is consistent with the time interval observed between introduction of nuclei into oocytes and *Oct4* demethylation in *Xenopus* oocytes (Simonsson and Gurdon, 2004). How early Oct4 demethylation occurs after extract exposure, however, remains undetermined, Nevertheless, if *Oct4* demethylation is required for expression of the gene (Simonsson and Gurdon, 2004), the rapid induction of RNA PolII-dependent *Oct4* transcription in ESC extract (Figure 11) also suggests that demethylation (at least of *Oct4*) is very rapidly triggered upon extract treatment.

Alteration of gene expression in extract-treated cells implies a global and locus-specific remodeling of chromatin. Remodeling of mammalian chromatin by *Xenopus* egg extract depends on ATPase activity of a chromatin remodeling complex (Kikyo *et al*., 2000), and in a similar system BRG1 was shown to be involved in *OCT4* activation (Hansis *et al*., 2004). We have to date no evidence for ATP-dependent chromatin remodeling in our system. However, *OCT4* activation requires ATP hydrolysis, and immunodepletion of BRG1 from mouse ESC extracts abolishes *OCT4* transcription (our unpublished data). *OCT4* promoter DNA demethylation (this study), and hyperacetylation of histone H4 at the *IL2* locus in cells treated with Jurkat extract (Håkelien *et al*., 2002) provide evidence that locus-specific chromatin remodeling takes place in our system. Conceivably, controlled manipulations of epigenetic alterations may enhance the heritability of gene expression in (re)programmed cells and may prove beneficial for reprogramming cell fate in a therapeutic context.

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REFERENCES

Akashi, K., He, X., Chen, J., Iwasaki, H., Niu, C., Steenhard, B., Zhang, J., Haug, J., and Li, L. (2003). Transcriptional accessibility for genes of multiple tissues and hematopoietic lineages is hierarchically controlled during early hematopoiesis. Blood *101*, 383–389.

Avilion, A. A., Nicolis, S. K., Pevny, L. H., Perez, L., Vivian, N., and Lovell-Badge, R. (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev. *17*, 126–140.

Blelloch, R. H., Hochedlinger, K., Yamada, Y., Brennan, C., Kim, M., Mintz, B., Chin, L., and Jaenisch, R. (2004). Nuclear cloning of embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA *101*, 13985–13990.

Boquest, A. C., Shahdadfar, A., Fronsdal, K., Sigurjonsson, O., Tunheim, S. H., Collas, P., and Brinchmann, J. E. (2005). Isolation and transcription profiling of purified uncultured human stromal stem cells: alteration of gene expression following in vitro cell culture. Mol. Biol. Cell *16*, 1131–1141.

Byrne, J. A., Simonsson, S., Western, P. S., and Gurdon, J. B. (2003). Nuclei of adult mammalian somatic cells are directly reprogrammed to oct-4 stem cell gene expression by amphibian oocytes. Curr. Biol. *13*, 1206–1213.

Cattaneo, E., and McKay, R. (1990). Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. Nature *347*, 762–765.

Chambers, I., and Smith, A. (2004). Self-renewal of teratocarcinoma and embryonic stem cells. Oncogene *23*, 7150–7160.

Chaudhary, N., and Courvalin, J.-C. (1993). Stepwise reassembly of the nuclear envelope at the end of mitosis. J. Cell Biol. *122*, 295–306.

Cibelli, J. B., Stice, S. L., Golueke, P. J., Kane, J. J., Jerry, J., Blackwell, C., Ponce, d. L. F., and Robl, J. M. (1998). Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells. Nat. Biotechnol. *16*, 642–646.

Cowan, C. A., Atienza, J., Melton, D. A., and Eggan, K. (2005). Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science *309*, 1369–1373.

Damjanov, I., Horvat, B., and Gibas, Z. (1993). Retinoic acid-induced differentiation of the developmentally pluripotent human germ cell tumor-derived cell line, NCCIT. Lab. Investig. *68*, 220–232.

Flasza, M., Shering, A. F., Smith, K., Andrews, P. W., Talley, P., and Johnson, P. A. (2003). Reprogramming in inter-species embryonal carcinoma-somatic cell hybrids induces expression of pluripotency and differentiation markers. Cloning Stem Cells *5*, 339–354.

Fortunel, N. O., *et al*. (2003). Comment on "'Stemness': transcriptional profiling of embryonic and adult stem cells" and "a stem cell molecular signature". Science *302*, 393

Gaustad, K. G., Boquest, A. C., Anderson, B. E., Gerdes, A. M., and Collas, P. (2004). Differentiation of human adipose tissue stem cells using extracts of rat cardiomyocytes. Biochem. Biophys. Res. Commun. *314*, 420–427.

Gurdon, J. B., and Byrne, J. A. (2003). The first half-century of nuclear transplantation. Proc. Natl. Acad. Sci. USA *100*, 8048–8052.

Håkelien, A. M., Gaustad, K. G., and Collas, P. (2004). Transient alteration of cell fate using a nuclear and cytoplasmic extract of an insulinoma cell line. Biochem. Biophys. Res. Commun. *316*, 834–841.

Håkelien, A. M., Gaustad, K. G., Taranger, C. K., Skålhegg, B. S., Kuntziger, T., and Collas, P. (2005). Long-term in vitro, cell-type-specific genome-wide reprogramming of gene expression. Exp. Cell Res. *309*, 32–47.

Håkelien, A. M., Landsverk, H. B., Robl, J. M., Skålhegg, B. S., and Collas, P. (2002). Reprogramming fibroblasts to express T-cell functions using cell extracts. Nat. Biotechnol. *20*, 460–466.

Hansis, C., Barreto, G., Maltry, N., and Niehrs, C. (2004). Nuclear reprogramming of human somatic cells by xenopus egg extract requires BRG1. Curr. Biol. *14*, 1475–1480.

Hoffman, L. M., and Carpenter, M. K. (2005). Characterization and culture of human embryonic stem cells. Nat. Biotechnol. *23*, 699–708.

Hosler, B. A., LaRosa, G. J., Grippo, J. F., and Gudas, L. J. (1989). Expression of REX-1, a gene containing zinc finger motifs, is rapidly reduced by retinoic acid in F9 teratocarcinoma cells. Mol. Cell Biol. *9*, 5623–5629.

Hutchison, C. J., and Worman, H. J. (2004). A-type lamins: guardians of the soma? Nat. Cell Biol. *6*, 1062–1067.

Hwang, W. S., *et al*. (2004). Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst. Science *303*, 1669–1674.

Ji, W., Zhou, W., Gregg, K., Lindpaintner, K., Davis, S., and Davis, S. (2004). A method for gene expression analysis by oligonucleotide arrays from minute biological materials. Anal. Biochem. *331*, 329–339.

Kikyo, N., Wade, P. A., Guschin, D., Ge, H., and Wolffe, A. P. (2000). Active remodeling of somatic nuclei in egg cytoplasm by the nucleosomal ATPase ISWI. Science *289*, 2360–2362.

Klevecz, R. R., Bolen, J., Forrest, G., and Murray, D. B. (2004). A genomewide oscillation in transcription gates DNA replication and cell cycle. Proc. Natl. Acad. Sci. USA *101*, 1200–1205.

Landsverk, H. B., Håkelien, A. M., Küntziger, T., Robl, J. M., Skålhegg, B. S., and Collas, P. (2002). Reprogrammed gene expression in a somatic cell-free extract. EMBO Rep. *3*, 384–389.

Mathieu, O., and Bender, J. (2004). RNA-directed DNA methylation. J. Cell Sci. *117*, 4881–4888.

Matzke, M. A., and Birchler, J. A. (2005). RNAi-mediated pathways in the nucleus. Nat. Rev. Genet. *6*, 24–35.

McGann, C. J., Odelberg, S. J., and Keating, M. T. (2001). Mammalian myotube dedifferentiation induced by newt regeneration extract. Proc. Natl. Acad. Sci. USA *98*, 13699–13704.

Miklos, G. L., and Maleszka, R. (2004). Microarray reality checks in the context of a complex disease. Nat. Biotechnol. *22*, 615–621.

Morgan, H. D., Dean, W., Coker, H. A., Reik, W., and Petersen-Mahrt, S. K. (2004). Activation-induced cytidine deaminase deaminates 5-methylcytosine in DNA and is expressed in pluripotent tissues: implications for epigenetic reprogramming. J. Biol. Chem. *279*, 52353–52360.

Munsie, M. J., Michalska, A. E., O'Brien, C. M., Trounson, A. O., Pera, M. F., and Mountford, P. S. (2000). Isolation of pluripotent embryonic stem cells from reprogrammed adult mouse somatic cell nuclei. Curr. Biol. *10*, 989–992.

Nishimoto, M., Fukushima, A., Okuda, A., and Muramatsu, M. (1999). The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2. Mol. Cell. Biol. *19*, 5453–5465.

Nordhoff, V., Hubner, K., Bauer, A., Orlova, I., Malapetsa, A., and Scholer, H. R. (2001). Comparative analysis of human, bovine, and murine Oct-4 upstream promoter sequences. Mamm. Genome *12*, 309–317.

Okuda, A., *et al*. (1998). UTF1, a novel transcriptional coactivator expressed in pluripotent embryonic stem cells and extra-embryonic cells. EMBO J *17*, 2019–2032.

Paulsson, J. (2004). Summing up the noise in gene networks. Nature *427*, 415–418.

Pells, S., Di Domenico, A .I., Callagher, E. J., and McWhir, J. (2002). Multipotentiality of neuronal cells after spontaneous fusion with embryonic stem cells and nuclear reprogramming in vitro. Cloning Stem Cells *4*, 331–338.

Planat-Benard, V., *et al*. (2004). Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation *109*, 656–663.

Qin, M., Tai, G., Collas, P., Polak, J. M., and Bishop, A. E. (2005). Cell extract-derived differentiation of embryonic stem cells. Stem Cells *23*, 712–718.

Ramchandani, S., Bhattacharya, S. K., Cervoni, N., and Szyf, M. (1999). DNA methylation is a reversible biological signal. Proc. Natl. Acad. Sci. USA *96*, 6107–6112.

Simonsson, S., and Gurdon, J. (2004). DNA demethylation is necessary for the epigenetic reprogramming of somatic cell nuclei. Nat. Cell Biol. *6*, 984–990.

Stewart, R., Christie, V. B., and Przyborski, S. A. (2003). Manipulation of human pluripotent embryonal carcinoma stem cells and the development of neural subtypes. Stem Cells *21*, 248–256.

Tada, M., Tada, T., Lefebvre, L., Barton, S. C., and Surani, M. A. (1997). Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. EMBO J. *16*, 6510–6520.

Tada, M., Takahama, Y., Abe, K., Nakastuji, N., and Tada, T. (2001). Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. Curr. Biol. *11*, 1553–1558.

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D. M., Nakano, Y., Meyer, E. M., Morel, L., Petersen, B. E., and Scott, E. W. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature *416*, 542–545.

Teshima, S., Shimosato, Y., Hirohashi, S., Tome, Y., Hayashi, I., Kanazawa, H., and Kakizoe, T. (1988). Four new human germ cell tumor cell lines. Lab. Invest. *59*, 328–336.

Wakayama, T., Tabar, V., Rodriguez, I., Perry, A. C., Studer, L., and Mombaerts, P. (2001). Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. Science *292*, 740–743.

Wilmut, I., Beaujean, N., De Sousa, P. A., Dinnyes, A., King, T. J., Paterson, L. A., Wells, D. N., and Young, L. E. (2002). Somatic cell nuclear transfer. Nature *419*, 583–586.

Woodbury, D., Reynolds, K., and Black, I. B. (2002). Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. J. Neurosci. Res. *69*, 908–917.

Wu, X., Avni, D., Chiba, T., Yan, F., Zhao, Q., Lin, Y., Heng, H., and Livingston, D. (2004). SV40 T antigen interacts with Nbs1 to disrupt DNA replication control. Genes Dev. *18*, 1305–1316.

Ying, Q. L., Nichols, J., Evans, E. P., and Smith, A. G. (2002). Changing potency by spontaneous fusion. Nature *416*, 545–548.