Differential Role for Transcription Factor Oct4 Nucleocytoplasmic Dynamics in Somatic Cell Reprogramming and Self-renewal of Embryonic Stem Cells*

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Significance:
The spatiotemporal dynamic behavior of Oct4 remains largely unknown. In the present study we show that Oct4 is a nucleocytoplasmic shuttling protein, and Oct4 mutants with biased nucleocytoplasmic localization show limited potential for cellular reprogramming. An appropriate nuclear retention of Oct4 is critical for cellular reprogramming but not for the self-renewal of ES cells. Our findings will provide novel insight into the role of Oct4 during cellular reprogramming.

Oct4 is a member of the POU family of transcription factors and plays a critical role in both maintenance of the undifferentiated state of embryonic stem (ES) cells and in the reprogramming of somatic cells to induced pluripotent stem cells. Oct4 is imported into the nucleus where it functions as a transcription factor, however, the spatiotemporal dynamic behavior of Oct4 remains largely unknown. In the present study we show that Oct4 is a nucleocytoplasmic shuttling protein. Furthermore, although Oct4 mutants with altered nuclear import/export activity were able to maintain the self-renewal of ES cells, they displayed limited potential for cellular reprogramming. These results indicate that the intracellular localization of Oct4, which is dependent on nucleocytoplasmic shuttling, must be more strictly regulated for cellular reprogramming, suggesting that Oct4 plays differential roles in the self-renewal of ES cells and in somatic cell reprogramming.

Oct4 is a homeodomain transcription factor that belongs to the POU (Pit-Oct-Unc) family (1–3) and is exclusively expressed in totipotent/pluripotent cell lineages, including oocytes, the inner cell mass, primitive ectoderm, primordial germ cells, and stem cell lines derived from the early embryo (4). It has been proposed that Oct4 regulates stem cell pluripotency and differentiation. Indeed, Oct4 is essential for various cellular processes associated with pluripotency, such as formation of the inner cell mass (5), the maintenance of embryonic stem (ES) cells (6), and induction of induced pluripotent cells (7, 8). In ES cells, Oct4 collaborates with Sox2 and Nanog to form a regulatory circuit that maintains ES-cell pluripotency (9, 10). Furthermore, several genomic profiling studies have revealed that Oct4 also maintains the pluripotency of ES cells by regulating genome-wide transcription either positively or negatively (11–14).

The expression level of Oct4 needs to be precisely controlled to sustain undifferentiated proliferation of ES cells (6). Indeed, a decrease in Oct4 expression to <50% of normal levels triggers cell differentiation toward the trophectoderm lineage, whereas a 50% increase in expression promotes cell differentiation into mesoderm or endoderm (6, 15). Although numerous studies have shown that the expression level of Oct4 is transcriptionally regulated, either positively or negatively, by various factors (16), the post-translational regulation of Oct4 is not well characterized. However, there is evidence that Oct4 is modified by ubiquitination (17), sumoylation (18), and phosphorylation (19–21).

The post-translational status of Oct4 is also regulated by its intracellular localization. After translation in the cytoplasm, Oct4 is imported into the nucleus via importin α, which binds to a conserved nuclear localization signal (NLS:RKRKR)3 at the N terminus of the homeobox domain (22–25). However, the dynamic behavior of Oct4 once imported into the nucleus is essentially unknown. In contrast, Oct6, another POU homeodomain transcription factor, is known to shuttle between the nucleus and cytoplasm (26). Thus, in the present study we analyzed the dynamic behavior of Oct4 and its role in the determin-

3 The abbreviations used are: NLS nuclear localization signal; NES, nuclear export signal; LMB, leptomycin B, RanBP Ran-binding protein; DOX, doxycycline; FLIP, fluorescence loss in photobleaching; FRAP, fluorescence recovery after photobleaching; MEF, mouse embryonic fibroblast.


Oct4 Function and Nucleocytoplasmic Shuttling

nuation of cell fate. We demonstrated that Oct4 is a nucleocytoplasmic shuttling protein. Furthermore, we used Oct4 mutants with biased nucleocytoplasmic localization to show that the appropriate nuclear retention of Oct4 is critical for cellular reprogramming but not for the self-renewal of ES cells.

EXPERIMENTAL PROCEDURES

Cell Culture—NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS). ZHBTc4 ES cells (6) were cultured on gelatin-coated dishes in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids, and 1000 units/ml murine leukemia inhibitory factor.

Plasmids—A retroviral vector expressing mutant Oct4 was generated by modifying the pMXs-Oct4 vector (constructed by the laboratory of Dr. Yamanaka; obtained from Addgene). First, the sequence at the C-terminal extremity of the Oct4 ORF was deleted, which resulted in the inclusion of a multiple cloning site containing the Xhol site at the C terminus of Oct4 (pMXs-Oct4) and an additional 12 amino acids (LERPPAQWSTIK), with deletion of the final asparagine residue of endogenous Oct4. We subsequently inserted oligonucleotides coding for the NLS of the SV40 T-antigen (5’TCCGACAGTACTCTC-CAAAAAAGAAGAGAAAGGTAGAAGACC-3’; 5’TCCA-GGTCTCTTCTTTTCTTTTTTGAGGAGTAC-3’) into the XhoI site of the pMXs vector. To generate pGL4.14-Oct4, the E1B tata sequence was inserted into the multiple cloning site of the pGL4.14 vector. The Tol2-based pT2A-CMH vector containing a multiple cloning site was inserted into the KpnI-Sacl site of pGLA.14-E1Btata. The Tol2-based Oct4 expression vector (pT2AL200R175-CAGGS-Oct4) was generated by first producing pT2A-CMH, a Tol2 transposon-based vector containing a multiple cloning site, by modifying pT2AL200R175G (27). The coding region of Oct4 or mutant Oct4 was subsequently inserted into the multiple cloning site of pT2A-CMH.

Heterokaryon Assay—NIH3T3 cells were infected with the retroviral expression vector for 24 h. Subsequently, 3 × 10⁶ HeLa cells and 3 × 10⁶ infected NIH3T3 cells were mixed and plated onto coverslips (in a 6-well plate) and cultured for another 24 h. Co-cultures of the cells were preincubated with 100 μg/ml cycloheximide and with either leptomycin B (LMB; 10 nm) or vehicle (EtOH) for 60 min. Cell fusion was performed by inverting the coverslips onto a drop of prewarmed (37°C) polyethylene glycol 8000, DMEM for 2 min. After washing the coverslips with PBS, fused cells were further cultured in the presence of 100 μg/ml cycloheximide and with either LMB (10 nm) or vehicle (EtOH) for 3 h. The cells were then fixed, mounted, and observed using a confocal microscope (LSM510).

Oct4 Rescue Experiments—For rescue experiments, 1 × 10⁵ ZHBTc4 ES cells were co-transfected with 200 ng of pCAGGS-mT2TP, an expression plasmid containing the Tol2 transposable cDNA whose codons are optimized for mammals under control of the CAG promoter, and 200 ng of a Tol2 transposon-based pT2A-CMH vector expressing either wild-type or mutant Oct4. One-fifth of the total transfected cells was replated 48 h post-transfection and further cultured with ES medium containing 1 μg/ml doxycycline (DOX). After 7 days, colonies were stained for alkaline phosphatase activity (Sigma) or picked and expanded as clonal cell lines.

Fluorescence Loss in Photobleaching (FLIP)—For FLIP analysis, NIH3T3 cells grown on glass-bottom dishes were transfected with Oct4-EGFP, Oct4 Δ-POUs-EGFP, or H2B-EGFP expression plasmids for 24 h, and the cells were imaged at 37°C with a laser-scanning LSM510 microscope (Carl Zeiss) using a 63 × 1.4 NA oil-immersion objective. A single Z-section image was obtained before and at specific time intervals after each bleaching, which was performed for 100 iterations in a circular region of 6-μm diameter within the cytoplasm using 100% power of an argon laser at 488 nm. Bleaching was repeated every 30 s during the period of the experiment (500 s). After background subtraction, the fluorescent intensities of the regions of interest (nuclei) were determined using LSM software and normalized to those of the pre-bleached images.

Fluorescence Recovery after Photobleaching (FRAP)—NIH3T3 cells expressing Oct4-EGFP Oct4 Δ-POUs-EGFP or H2B-EGFP expression vectors grown on glass-bottom dishes were observed using a laser-scanning LSM510 microscope (Carl Zeiss) at 37°C. For FRAP analysis, the cells were photo-bleached using a 488-nm laser at 100% power for 20 iterations over a rectangular region (20 μm²) within the nucleus. A single Z-section image was obtained before and at specific time intervals after each bleaching. Images were recorded every 0.2 s for 30 s. After background subtraction, the fluorescent intensities of the regions of interest were determined using LSM software and normalized to those of the pre-bleached images.
Induction of Cellular Reprogramming—Mouse-induced pluripotent cells were generated essentially as described previously (28) using PMXs retroviruses expressing mouse Sox2, Klf4, and c-Myc (Addgene) together with wild-type Oct4 (Addgene) or a series of mutant Oct4 proteins. Briefly, Plat-E cells were transfected with the retroviral vectors using FuGENE 6 transfection reagent (Roche Applied Science). The medium was changed at 24 h post-transfection, and viral supernatants were harvested 20% knockout serum replacement (Invitrogen). The medium was changed every other day. Alkaline phosphatase staining was performed using the leukocyte alkaline phosphatase kit (Sigma).

Antibodies—The following primary antibodies were used: rabbit polyclonal anti-Nanog (Reprocell), mouse monoclonal anti-Oct4 (BD Biosciences), rabbit polyclonal anti-RanBP1 (Santa Cruz), and mouse monoclonal anti-GAPDH (Ambion).

Immunofluorescence Staining and Confocal Microscopy—Cells were grown on coverslips and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. After permeabilization with 0.5% Triton X-100 in PBS for 5 min, the cells were incubated in blocking buffer (PBS containing 3% skim milk) for 30 min and subsequently incubated overnight with primary antibodies at 4 °C. After 4 washes with PBS, cells were incubated with secondary antibodies for 45 min. The cells were washed again with PBS, cell nuclei were stained with DAPI, and images were acquired using a confocal microscope (LSM 510 META, Carl Zeiss) equipped with a 63 × 1.4 NA oil objective lens (Carl Zeiss) and analyzed using Zeiss LSM software.

Immunoblotting Analysis—Cells were resuspended in a radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitors (leupeptin, aprotinin, and phenylmethylsulfonyl fluoride) and incubated on ice for 15 min. After centrifugation at 16,000 × g for 10 min at 4 °C, the supernatants were collected and used for further analysis. Protein extracts were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking with 3% skim milk in TBST buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.1% Tween 20) for 30 min at room temperature, the membrane was incubated overnight with primary antibodies at 4 °C. After incubation with secondary antibodies conjugated to horseradish peroxidase, bands were visualized using Pierce Western blotting substrate (Thermo Scientific).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Total RNA was extracted from cells using TRIzol reagent (Invitrogen) and used for cDNA synthesis with the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science). PCR amplification was performed using rTaq DNA polymerase (Takara). The primer sequences are detailed in Table 1.

RNA Interference—Cells were transfected with mouse Oct4 siRNA (5′-GUGUCAAGGAUGUUCUGUAdTdT-3′ and 5′-UACAGAAACAUACUGAAcTdT-3′) or control non-targeting siRNA (5′-GGACAGUUUUUUCAcAdTdT-3′ and 5′-UUGUUAAUCAUGUGUAdTdT-3′) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions (10 nM final concentration).

Statistical Analysis—Statistical analysis was carried out using the unpaired Student’s t test. The p values ≤0.05 were considered to be statistically significant difference (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

RESULTS

Oct4 Is a Nucleocytoplasmic Shuttling Protein—An interspecies heterokaryon assay was used to determine whether Oct4 shuttles between the nucleus and cytoplasm. NIH3T3 cells were retrovirally transduced with C-terminal EGFP-tagged Oct4 (Oct4-EGFP) and fused with HeLa cells to form heterokaryons in order to monitor the dynamic behavior of Oct4. DAPI staining allowed discrimination between NIH3T3 nuclei (punctate) and HeLa (homogenous) nuclei. As shown in Fig. 1A, Oct4-EGFP was detected in HeLa nuclei, indicating that Oct4-EGFP shuttles between the nucleus and the cytoplasm. In contrast, the control non-shuttling histone-H2B protein, also C-terminally tagged with EGFP, was not observed in HeLa nuclei of the heterokaryons.

Oct6, another member of POU transcription factor family, is exported from the nucleus by the nuclear export factor Crm1, LMB did not inhibit the shuttling of Oct4-EGFP. Thus, the nuclear export of Oct4 is not primarily mediated by Crm1.

| TABLE 1 | Oligonucleotides used for RT-PCR |
|---------|--------------------------------|
| **Gene** | **Forward primer** | **Reverse primer** |
| Cdx2 | AGGGCGCAACATGAGGATGA | CGAAGTCCTGATATTCCACTCA |
| Egf5 | GGAGGCTCTAGAGACACAGAA | CTGTGAGCCACCAAAGACAGAA |
| Gapdh | GGTGGTTCCTACCCCAATTGTT | AATGGCATACGAGAATAGMG2CTT |
| Oct4 (3-UTR) | CAAGCCAGGGAGGTAGACAA | CAAATGATGAGTGACAGG |
| Sox2 | GAAGCTGAACTTCTAGAGTCCTG | CACACACTGCTTCTGACCCG |
| Nanog | AGGCGCTTCCTAGAGTCTGCCTG | TCACTGCTTTTCTGACCCG |
| Hand1 | AGCGAATCTGCTGGCAGGTA | TCACTGCTTTTCTGACCCG |
Oct4 is Exported from the Nucleus by Passive Diffusion—Oct4 consists of a POU domain, a DNA binding motif comprising a POU-specific domain and a POU homeodomain, and N- and C-terminal transactivation domains (29, 30). To determine whether Oct4 contains an NES, we constructed a series of deletion mutants that were N-terminally tagged with EGFP. As shown in Fig. 2A, Oct4 mutants displayed either diffuse distribution in both the nucleus and cytoplasm (DL1 and DL5) or exclusive nuclear localization (DL3). Moreover, deletion of the NLS present in DL3 (DL4) resulted in diffuse localization throughout the cell, which is consistent with a previous study (22). However, we noticed that a subpopulation of the cells expressing DL2 mutant, which contains the POU-specific domain, displayed predominant cytoplasmic localization. Therefore, additional deletion mutants of the POU-specific domain were generated to determine the relationship between the POU domain and cellular localization. As shown in Fig. 2A, predominant cytoplasmic localization was observed only for those containing the third helix of POU-specific domain (DL6, DL9, and DL11), which is also demonstrated by biochemical fraction-
FIGURE 2. The POU-specific domain is not essential for Oct4 shuttling activity. A, shown is a schematic diagram and cellular localization of various Oct4 deletion mutants N-terminally tagged with EGFP. The regions corresponding to POU, NLS, and POU-HD are indicated. NIH3T3 cells were transfected with the constructs, and cells were fixed, permeabilized, and counterstained with DAPI at 24 h post-transfection. Subcellular localization of EGFP-Oct4 and its mutants was classified into three categories: nuclear (blue), nuclear dominant (red), and cytoplasmic dominant (green). B, shown is a schematic representation of the Oct4 ΔPOUs-EGFP and Oct4-GST-EGFP fusion proteins and heterokaryon assay results. NIH3T3 cells were infected with retroviruses expressing Oct4 ΔPOU-EGFP or Oct4-GST-EGFP, and heterokaryon assays were performed as described in Fig. 1. Arrows indicate the nuclei of HeLa cells.
ation (data not shown), indicating that this portion of the POU-specific domain is essential for the cytoplasmic localization of Oct4.

To confirm whether the POU-specific domain of Oct4 plays a role in selective nuclear export, a deletion mutant (ΔPOU) was constructed that lacked the second and third helices of the POU-specific domain (amino acids 150–188). The resulting mutant protein was C-terminally fused to EGFP (ΔPOU Oct4-EGFP) and employed in the heterokaryon assay (Fig. 2B). However, the ΔPOU Oct4-EGFP mutant retained shuttling ability, suggesting that the Oct4 POU-specific domain may be important for cytoplasmic retention rather than selective nuclear export and that Oct4 may not possess an active nuclear export signal. Thus, efficient nuclear export of Oct4 is most likely to be mediated by passive diffusion. To confirm this hypothesis, an Oct4-GST-EGFP retroviral expression vector was generated. The predicted molecular mass of Oct4-GST-EGFP was more than 90 kDa, which is large enough to prevent its passive diffusion. As shown in Fig. 2B, the heterokaryon assay revealed that Oct4-GST-EGFP was retained in NIH3T3 cell nuclei, and it did not migrate into HeLa cell nuclei. Therefore, we concluded that the Oct4 protein is primarily exported via passive diffusion.

The Intranuclear Mobility of Oct4 Correlates with Its Export Efficiency—Because the nuclear export of Oct4 may be independent of export factors, it is possible that the intranuclear mobility of Oct4, which is dependent on its interaction with chromatin or nuclear protein complexes, affects Oct4 export efficiency. To examine this possibility, the dynamics of Oct4 in living cells were investigated in more detail. First, FRAP analysis was performed to examine the mobility of Oct4-EGFP within the nucleus. Consistent with a previous report (31), Oct4-EGFP displayed higher mobility compared with that of the control histone-H2B-EGFP protein (Fig. 3A). We also examined the mobility of ΔPOU Oct4-EGFP, which lacks the POU-specific domain required for DNA binding and transcriptional activity (Fig. 3B). FRAP analysis revealed that ΔPOU Oct4-EGFP showed a significant increase in the initial rate of fluorescence recovery compared with that of wild-type Oct4-EGFP (Fig. 3A), indicating that the ΔPOU Oct4 mutant is highly mobile within the nucleus.

FIGURE 3. Intranuclear mobility of Oct4 is a key determinant of its export rate. A, FRAP analysis is shown. NIH3T3 cells were transfected with Oct4-EGFP, Oct4 ΔPOUs-EGFP, or H2B-EGFP, and FRAP experiments were performed with confocal laser scanning microscopy. The fluorescence intensity in the bleached region was measured and expressed as a relative ratio. Bars represent the means ± S.E. (n = 4–7). B, shown is a luciferase assay. NIH3T3 cells were transiently co-transfected with pRL-Renilla, the pGL4.14-Oct4 reporter plasmid, and the indicated Oct4-expressing plasmids. Results are presented as the firefly luciferase/Renilla luciferase ratio and normalized to the value obtained with the control (empty) vector. The results shown represent the mean ± S.D. of three independent experiments. *** indicates p < 0.001. C, shown is FLIP analysis. NIH3T3 cells were transfected with the indicated expression plasmids for 24 h, and the cytoplasm of cells expressing Oct4-EGFP, Oct4 ΔPOUs-EGFP, Oct4-NES-EGFP, Oct4-NLS-EGFP, or H2B-EGFP was irradiated every 30 s for ~20 s with a laser. The fluorescence intensity in the nucleus was monitored after each bleaching and expressed as a relative ratio. Bars represent the means ± S.E. (n = 7–8).
The nucleocytoplasmic shuttling activity of these constructs was investigated in living cells by FLIP analysis. After repeated photobleaching in the cytoplasm, the nuclear fluorescence signal of wild-type Oct4-EGFP showed a slight but significant decrease compared with that of histone-H2B-EGFP (Fig. 3C). Furthermore, the ΔPOU Oct4-EGFP mutant displayed a considerable decrease in nuclear fluorescence intensity compared with wild-type Oct4-EGFP (Fig. 3C). Therefore, the intranuclear mobility of Oct4 correlated with protein export efficiency and is probably dependent on the binding of Oct4 to other nuclear proteins or chromatin. These results are also in good agreement with a previous report using a truncated mutant of Oct4 that lacked the C terminus, including the homeodomain (32).

The Addition of Import/Export Signals Alters the Nucleocytoplasmic Localization of Oct4—We subsequently investigated whether the intracellular dynamic behavior of Oct4 affects its function. To disturb the spatiotemporal regulation of Oct4, a
A typical basic-type NLS derived from SV40 T antigen or a typical leucine-rich NES derived from the HIV Rev protein was fused to the C terminus of Oct4 to create Oct4-NLS or Oct4-NES mutants (Fig. 4A). In addition, constructs containing tandem alanine residues (AAA), an export-deficient NES mutant (M10), and two tandem leucine-rich NESs (NES-NES) were also generated. Confocal microscopy revealed that wild-type Oct4 was exclusively localized in the nucleus of NIH3T3 cells. In addition, Oct4-NLS and Oct4-M10 mutants displayed similar localization patterns to wild-type Oct4 (Fig. 4B). In contrast,

FIGURE 5. Transgene expression of Oct4 mutants sustains the self-renewal of ES cells. Oct4 rescue experiments are shown. ZHBTc4 cells were transfected with a Tol2-based transposon expressing Oct4 transgenes. After 3 days, the cells were passaged in fresh media containing DOX to suppress tet-regulated wild-type Oct4. Eleven days after transfection, colonies were stained using an alkaline phosphatase staining kit. Representative images of the plates (A) and statistical analysis (B) are shown. Bars represent the mean ± S.D. of three independent experiments. C, shown is morphology of ZHBTc4 cells or isolated Tol2-Oct4-expressing ZHBTc4 clones cultured in the presence of DOX. D, shown are protein expression levels of wild-type or mutant Oct4 in isolated Tol2-Oct4-expressing ES clones. Cell lysates (20 μg) were used for Western blotting, and GAPDH was used as a loading control. E, shown are RT-PCR analyses of isolated ES cell clones. Total RNA was isolated from Tol2-Oct4-expressing ES clones (two clones each). Note that the primers specific for Oct4 (3′-UTR) detect expression of endogenous Oct4 but not Oct4 expression from transgenes. GAPDH was used as a loading control. F, shown is differentiation of Tol2-Oct4-expressing clones by Oct4 knockdown. Tol2-Oct4-expressing clones (WT, NLS, NES-NES) were transfected with siRNA against Oct4 or control siRNA. Left, shown are phase contrast images of clones at 4 days after transfection. Right top, shown is RT-PCR analysis of Cdx2, Hand1, and β-actin gene expression in control- or Oct4-siRNA-treated cell (4 days after transfection). Right bottom, shown is immunoblotting analysis of Oct4 and β-actin in control- or Oct4-siRNA-treated cell lysates (2 days after transfection).
Oct4-NES showed weak diffuse cytoplasmic localization as well as nuclear localization. FLIP analysis using EGFP fusion proteins revealed that the rate of nuclear export of Oct4-NES-EGFP was higher than that of ΔPOU Oct4-EGFP, a DNA binding-deficient mutant with a higher export rate than wild-type Oct4 (Fig. 3C). Furthermore, Oct4-NES was either evenly distributed between the nucleus and cytoplasm or predominantly localized in the cytoplasm, indicating that its nuclear export efficiency is much higher than that of Oct4-NES. The addition of LMB to the culture medium led to the accumulation of cytoplasmic Oct4 mutant proteins in the nucleus (Fig. 4C), confirming that Oct4-NES and Oct4-NES-NES are first imported into the nucleus and then selectively exported by Crm1.

**Mutant Oct4 Proteins with Biased Nucleocytoplasmic Localization Maintain Self-renewal of ES Cells**—We examined whether the mutant Oct4 proteins can replace the function of wild-type Oct4 by using ZHBTc4 ES cells (6) that contain a tetracycline-regulated (Tet-off) wild-type Oct4 transgene and in which both alleles of endogenous Oct4 were inactivated by gene targeting. As a result, in the presence of tetracycline or DOX, the expression of Oct4 is repressed, and the cells differentiate toward trophectoderm lineages (6). We used the Tol2 transposon system (33) to stably introduce the wild-type Oct4 transgene into ZHBTc4 cells. In contrast to untransfected or control EGFP-expressing vector-transduced cells, stable expression of the wild-type Oct4 transgene enabled self-renewal of ZHBTc4 cells, which proliferated in an undifferentiated state (alkaline phosphatase positive) in the presence of DOX (Figs. 5, A and B). Unexpectedly, all of the Oct4 mutants retained the ability to form alkaline phosphatase-positive colonies in the absence of wild-type Oct4. Notably, the Oct4-NES-NES mutant, which was mainly localized in the cytoplasm, was also able to efficiently rescue the self-renewal of ZHBTc4 ES cells. Next, we isolated stable clones expressing Tol2-transposon-based mutant Oct4 in the presence of DOX. These clones were morphologically similar to the wild-type Oct4-expressing clone (Fig. 5C) and could be maintained for ≥10 passages. Immunoblotting analysis confirmed that the tetracycline-regulated wild-type Oct4 was efficiently suppressed in these clones in the presence of DOX. Furthermore, the expression of Cdx2, a trophectoderm marker, was also suppressed in these clones. Oct4 knockdown in those clones caused drastic morphological changes of ES cells concomitant with up-regulation of trophectoderm marker genes, Cdx2 and Hand1 (Fig. 5F), demonstrating that those clones at
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A

B

C

D

E

F

G

OSKM  O(NLS)SKM  O(AAA)SKM
O(NES)SKM  O(M10)SKM  O(NES-NES)SKM

Number of colonies

WT  NLS  AAA  NES  M10  NES-NES

Fold induction

Fold induction

pMX-Oct4 (WT)  pMX-Oct4 (NLS)  pMX-Oct4 (AAA)  pMX-Oct4 (NES)  pMX-Oct4 (M10)  pMX-Oct4 (NES-NES)

Relative luciferase activity

ZHBTc4 WT-1  ZHBTc4 WT-2  ZHBTc4 NES-1  ZHBTc4 NESNES-2
least possess differentiation potential into trophoderm lineages. Confocal imaging using an anti-Oct4 antibody further confirmed the altered localization of Oct4-NES and Oct4-NES-NES. Moreover, in contrast to endogenous Nanog, the majority of Oct4-NES-NES was not accumulated in the nucleus (Fig. 6A). Thus, although Oct4-NES-NES enters the nucleus via its own NLS, it is rapidly and actively exported from the nucleus by the addition of two NES. Furthermore, the addition of LMB to the culture medium led to the accumulation of cytoplasmic Oct4-NES-NES proteins into the nucleus (Fig. 6B). Therefore, these results confirm that Oct4-NES-NES indeed exists at a low concentration in the nucleus due to its enhanced Crm1-mediated nuclear export yet is able to maintain the self-renewal of ES cells. This indicates that Oct4 maintains the pluripotent state of ES cells regardless of the length of time that Oct4 is present in the nucleus; transient localization in the nucleus is sufficient for Oct4 to maintain self-renewal of ES cells.

**Mutant Oct4 Proteins with Biased Nucleocytoplasmic Localization Show Limited Potential for Cellular Reprogramming**—The ability of the aforementioned Oct4 mutants to reprogram fibroblasts into induced pluripotent cells was compared. To this aim, induced pluripotent cells were generated by transduction of MEFs with retroviral vectors expressing Sox2, Klf4, c-Myc, and either wild-type or a series of mutant Oct4 proteins. Immunoblotting analysis of infected MEF cell lysates revealed that comparable levels of expression were achieved for each mutant Oct4 protein (Fig. 7C). As shown in Figs. 7, A and B, the Oct4-NLS and Oct4-NES-NES mutants demonstrated a significantly reduced ability to reprogram cells to a pluripotent state compared with wild-type Oct4. Because the Oct4-NLS mutant contains an additional SV40 T-NLS, it is likely that Oct4-NLS is constitutively transported into the nucleus in an unregulated manner. These results suggest that both the appropriate nuclear entry and adequate intranuclear retention of Oct4 are crucial for efficient cellular reprogramming.

**Oct4-NES-NES Has Similar Transcriptional Activity to Wild-type Oct4**—Finally, we performed a luciferase assay to determine the effect of Oct4 mutation on transcriptional activity. Reporter constructs were generated by inserting either five tandem copies of an octamer binding motif (pGL4.14-Oct4-luc) (34) or three tandem copies of an Oct4/Sox2 binding motif (pGL4.14-OS-nanog-luc) (35) upstream of the firefly luciferase gene containing the adenovirus E1B TATA-box minimal promoter (pGL4.14-E1B-tata-luc). As shown in Fig. 7D, both reporter genes were selectively activated in ES cells. We subsequently monitored the activation of pGL4.14-Oct4-luc by each Oct4 mutant in NIH3T3 cells. As shown in Fig. 7E, the transcriptional activities of Oct4-NLS was significantly reduced (about 50% compared with wild-type Oct4), which could explain the reduced ability of this mutant to reprogram somatic cells into induced pluripotent cells. However, Oct4-NES-NES, which also demonstrated significantly reduced reprogramming efficiency (~10% compared with wild-type Oct4), showed comparable transcriptional activity to wild-type Oct4. Therefore, these results indicate that transcriptional activity does not necessarily correlate with the ability of Oct4 mutants to reprogram cells.

To further examine the relationship between the transcriptional activity and reprogramming ability of Oct4 mutants, we subsequently employed the pGL4.14-OS-nanog-luc plasmid (Fig. 7F). Co-transfection of Sox2 with Oct4 resulted in about a 4fold increase in luciferase activity, which is consistent with a previous report (Ref. 35 and data not shown). The transcriptional activities of Oct4 mutants, including Oct4-NES-NES, were comparable with those of wild-type Oct4. Finally, we compared the activity of pGL4.14-Oct4-luc in Tol2-Oct4 (wild type)- or Oct4-NES-NES-expressing ZBHTc4 clones. As shown in Fig. 7G, these clones showed a similar activation of a reporter. Thus, these results confirmed that cell reprogramming is not solely dependent on transcriptional activity of Oct4. Collectively, these findings indicate that post-translational, spatiotemporal regulation of Oct4 is critical for reprogramming cells.

**DISCUSSION**

Oct4 is functionally involved in two distinct processes: ES cell self-renewal and cellular reprogramming. However, it is currently unknown whether Oct4 functions in the same manner to mediate both activities. In this study we aimed to analyze the dynamic behavior of Oct4 and examine its role in the determination of cell fate. Oct4 mutants with biased nucleocytoplasmic distribution were able to maintain the self-renewal of ES cells; however, they displayed significantly reduced potential for cellular reprogramming compared with wild-type Oct4. Therefore, although the appropriate spatiotemporal regulation of Oct4 is essential for cellular reprogramming, it is not required for maintenance of the undifferentiated state of ES cells.

Notably, an Oct4-NES-NES mutant, which was primarily localized in the cytoplasm, was also capable of maintaining the undifferentiated state of ES cells. This suggests that despite its rapid export from the nucleus, Oct4 can still act as a transcriptional regulator.
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In summary, Oct4 shuttles between the nucleus and cytoplasm. To function as a transcription factor that maintains the undifferentiated state of ES cells, Oct4 only has to stay transiently in the nucleus. In contrast, to induce cellular reprogramming, Oct4 must stay in the nucleus for a sufficient length of time to induce essential transcription-independent reactions, which may involve chromatin remodeling and/or epigenetic changes. Thus, Oct4 plays distinct roles in the self-renewal of ES cells and in somatic cell reprogramming. Future studies focused on the characterization of cellular reprogramming processes induced by the Oct4-NES-NES mutant may shed light on how nuclear Oct4 retention functions to induce somatic cell reprogramming.

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