Identification of a Nuclear Localization Signal in OCT4 and Generation of a Dominant Negative Mutant by Its Ablation*

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OCT4 plays a critical role in maintaining stem cell pluripotency in a dose-dependent manner by activating and repressing multiple downstream genes. The precise mechanism by which OCT4 achieves these diverse biological functions remains unknown. In this report, we identify and characterize 190RKRRKRR as a nuclear localization signal responsible for its localization in the nuclei and required for the transactivation of its target genes. Point mutations within this motif yielded a mutant that localizes randomly throughout the cells and is defective in transactivating target genes. However, restoration of nuclear localization with a heterologous nuclear localization signal failed to rescue its transactivation function, suggesting that this 190RKRRKRR motif has additional function in mediating transactivation function. We further demonstrate that this mutant is competent in dimerization with not only itself but also wild type OCT4 and can interfere with the activity of wild type OCT4, thus acting as a dominant negative mutant. Indeed, this mutant can induce the differentiation of P19 cells into trophoblast-like giant cells. These data suggest that this dominant negative form of OCT4 may be a useful tool for modulating the activity of OCT4 in pluripotent cells such as embryonic stem cells to achieve the desired cell types for therapeutic applications.

Stem cells are pluripotent cells capable of producing virtually all cell types in our body, thus possessing great therapeutic potential for many degenerative conditions such as cancer, arthritis, and Parkinson diseases (1). Replacement of aged organs with new ones generated through stem cells and bioengineering would offer enormous hopes for the aging population (1–3). However, there are many conceptual as well as technical obstacles associated with the application of stem cells for medical therapies. One of the most critical issues is about the maintenance and generation of stem cells suitable for transplantation and further engineering (1). Adult stem cells can be isolated from patients and then transplanted back to the same patients directly upon amplification and engineering without the problem of rejection (4). However, adult stem cells may have limited potential in generating useful cell types, thus offering only restricted uses clinically (3, 5). Embryonic stem cells offer almost unlimited regenerative potential and thus should be able to meet most of the demands clinically (3, 5). Yet problems associated with the availability and host rejection of embryonic stem cells have to be solved before any realistic attempt can be mounted. Nevertheless, stem cell biology and regenerative medicine hold such promise that tremendous efforts are currently devoted to a wide spectrum of investigations both basic and applied to harness the potentials of stem cells. One active area of investigation is the cellular and molecular mechanism controlling stem cell pluripotency (6–10).

OCT4 is a POU domain transcription factor known to regulate stem cell pluripotency (6, 9, 10). It is exclusively expressed in preimplantation stage embryos and has become a defining feature for mouse embryonic stem cells (11, 12). OCT4 deficiency hampers the formation of inner cell mass formation in blastocysts, thus leading to embryonic lethality in mice (10, 13, 14). As a transcription factor, OCT4 is known to activate a battery of genes (9, 11, 13), which presumably act to maintain stem cell pluripotency. Although the role of OCT4 in maintaining stem cell pluripotency is not in doubt, few mechanistic details are available regarding the biochemical and cell biological properties of OCT4 in regulating gene expression (9, 10, 13). To this end, we have analyzed the mechanism through which OCT4 is localized to the nuclei and identified its nuclear localization signal (NLS).1 We further demonstrate that the ablation of this nuclear localization signal generates a dominant negative mutant for OCT4, which may be a useful tool for the investigation into the role of OCT4 in stem cell pluripotency both in vitro and in vivo.

MATERIALS AND METHODS

Cell Culture and Reporter Assays—HeLa cells, 293T cells, and P19 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 100 μg/ml antibiotics (penicillin and streptomycin, Invitrogen). For reporter assays, HeLa cells or 293T cells were seeded in 12-well cell culture plates and transfected by calcium phosphate co-precipitation methods with 0.25 μg of p68T-k-luciferase or pTK-luciferase, 0.25–1.5 μg of pNLS(-)OCT4, 0.25 μg of pOCT4. Transfection efficiencies were normalized with Renilla plasmid (0.01 μg/well, Promega, WI) as internal references, and DNA concentrations were kept constant with empty expression vector. Cells were harvested 48 h after transfection, and luciferase activity was measured by using Dual-luciferase Reporter Assay system according to the manufacturer’s instructions (Promega).

Transfections of P19 cells were performed by using Effectene transfe-
Fig. 1. Identification of an NLS in mouse OCT4. A, schematic presentation of an NLS mutant of mOCT4. The mouse OCT4 coding region is fused to the GFP tagged with a FLAG (F) tag at its C terminus. The putative NLS, RKKRRR (wt), and its mutant form GSRVD (mut), are indicated. B, Western blot analysis of mOCT4-GFP-F. HeLa cells transfected with control vector (lane 1), wild type mOCT4-GFP-F (lane 2), and the mutant NLS(-)mOCT4-GFP-F (lane 3) were lysed and analyzed by Western blot using anti-FLAG antibody as described under “Materials and Methods.” C, nuclear localization of wild type and mutant mOCT4-GFP-F. Control vectors (a−c), GFP (d−f), wild type mOCT4-GFP-F (g−i), and NLS(-)mOCT4-GFP-F (j−l) were transfected to HeLa cells grown on coverslips. The pictures were taken 36 h after transfection in bright fields (a, d, g, and k) and fluorescent fields (c, f, i, and n). Both fields were combined to show the localization of each protein (b, e, h, and l).

DNA Plasmids and Oligonucleotide Primers—Mouse OCT4 open reading frame was amplified by reverse transcriptase-PCR using high fidelity polymerase PfX (Invitrogen) from mRNA isolated from P19 cells. The primers used are as follows: OCT4U, 5′-ACC ATG GCC GAG ACC GCT GTG CAT CTC TGG TCT-3′; OCT4L, 5′-ACC CCA AAG CTC CAG GTT CTC TTG TCT-3′. The OCT4 cDNAs were inserted into the SmaI site of modified pCR3.1 for mammalian expression and pET15b for prokaryotic expression as described above.

Expression and Purification of mOCT4 and Preparation of Anti-OCT4 Antibody—The mouse OCT4 cDNA was inserted into a Smal site in the modified vector pET15b as described (17), which was subsequently introduced into BL21-DE3 for expression. OCT4 expression was induced by isopropyl-1-thio-β-D-galactopyranoside (final concentration 1 mM) for 5 h at 37 °C. Under these conditions, OCT4 was produced in an inclusion body. Cells were harvested by centrifugation, and the cell pellet was completely resuspended in 10 ml of PBS buffer (50 mM PBS, pH 7.4, 0.5 M NaCl, 20 mM imidazole, 1 mM PMSF, 8 M urea). Eluted fractions were then rapidly diluted to 0.1 M with a nickel resin charged and packed according to the manufacturer’s instruction (Amersham Biosciences). The column was washed under denaturation conditions with column wash buffer (50 mM PBS, pH 7.4, 0.5 M NaCl, 20 mM imidazole, 1 mM PMSF, 8 M urea) until the A280 of the flow-through fractions fell below 0.05. The bound protein was then eluted successively with 5 column volumes of denatured Elution Buffers (50 mM PBS, pH 7.4, 0.5 M NaCl, 250 mM imidazole, 1 mM PMSF, 8 M urea). Eluted fractions were then rapidly diluted to 0.1 mg/ml and dialyzed two times with 2000 ml of PBS buffer (50 mM PBS, pH 7.4, 0.15 M NaCl) for renaturation. OCT4 protein concentration and purity were evaluated by the Bradford assay (Bio-Rad) and confirmed by SDS-PAGE. For antibody preparation, four rabbits were immunized with purified OCT4 protein (500 mg of protein each rabbit) in complete Freund’s adjuvant (Sigma) after immunized serum was collected.
Fig. 2. Expression of mOCT4 in E. coli and generation of anti-mOCT4 antibodies. A, prokaryotic expression of mOCT4. BL21-DE3 cells harboring His-mOCT4 expression vector (lane 1) was induced to express the recombinant protein (lane 2) by using 1 mM isopropyl-β-D-thiogalactopyranoside. Prokaryotically expressed mOCT4 was then purified by using Ni²⁺ affinity chromatography (lane 3). Prokaryotically expressed proteins were analyzed by SDS-PAGE and stained with R-250. B, specificity of anti-OCT4 antibody. Cell lysates of HEK293T cells transfected with control vector (lanes 1, 4, 7, and 10), mOCT4 (lanes 2, 5, 8, and 11), mOCT4-GFP-F (lanes 3, 6, 9, and 12) were fractionated on 10% SDS-PAGE and then electroblotted to PVDF membranes. The proteins were detected by Western blotting using preimmunized serum (lanes 1–3), anti-FLAG antibody described under “antibodies and detected with goat anti-mouse (lanes 4–6), affinity-purified anti-mOCT4 antibody (lanes 7–9), or anti-OCT4 antibody preabsorbed with mOCT4 protein (lanes 10–12) as first antibodies and detected with goat anti-mouse (lanes 4–6) or goat anti-rabbit as secondary antibodies conjugated with alkaline phosphatase as described under “Materials and Methods.”

Fig. 3. The ablation of OCT4 NLS blocks its nuclear localization and abolishes its transcription activity. A, Western blot analysis of mouse OCT4 and its NLS− mutant. HeLa cells transfected with control vector (lane 1), wild type mOCT4 (wt, lane 2) and NLS(−) mOCT4 (mut., lane 3) were lysed and analyzed by Western blot using anti-OCT4 antibody. B, the NLS mutation is localized in the cytosol. HeLa cells were transfected with wild type OCT4 (a–f) or NLS mutant OCT4 (g–i) and subsequently stained with preimmune serum (a–c), anti-OCT4 antibodies (d–i) first, followed by TRITC-conjugated goat anti-rabbit antibody (a–i). The cells were then scanned by confocal microscopy in the fluorescent field (c, f, and i) or bright field (a, d, and g). The signals are combined (h, e, and k) to show that wild type mOCT4 (without GFP and FLAG tag) is localized in the nucleus (e), whereas the NLS mutant is in the cytosol (k). C, the ablation of the NLS destroys its transcription activity. The reporter, p6w-luciferase (0.25 µg each), was co-transfected with control vector (0.25 µg, lane 4), mOCT4 (0.25 µg, lane 5), mOCT4-GFP-F (0.25 µg, lane 6), the NLS mutant (0.25 µg, lane 7), or the NLS mutant OCT4-GFP-F (0.25 µg, lane 8) to HEK293T cells cultured in a 12-well plate. pTK-luciferase (0.25 µg each) was co-transfected with equal volume of control vector (lane 2) or mOCT4 (lane 3) as control. Lane 1 was cell blank with no plasmids transfected. Each transfection was carried out in duplicate, and the luciferase activity was assayed 36 h after transfection by using the dual-reporter assay system as described under “Materials and Methods.” Transfection efficiency was normalized by co-transfection of Renilla plasmids (0.01 µg/well). The DNA plasmid concentrations were normalized by adding appropriate volumes of control vector.

The rabbits were then boosted three times with mOCT4 protein in incomplete Freund’s adjuvant (Sigma). The titer and specificity of the resulting sera were tested by enzyme-linked immunosorbent assay by using purified OCT4 protein as antigen and by Western blotting against mOCT4 expressed in 293T cells. After a final boost, those sera with high titer and specificity were purified through an affinity column with OCT4 coupled to CNBr-activated Sepharose 4B (Amersham Biosciences) according to the manufacturer’s instructions.

Co-immunoprecipitation—For co-immunoprecipitation, 3 µg of pNLS(−)OCT4 (with GFP and FLAG) or pWTOCT4 (with GFP FLAG)
Identification of an NLS in mOCT4 Required for Its Transactivation Activity—As a transcription factor, OCT4 must be transported to the nuclei where it binds to its target octamer motif and transactivates its target genes (9, 12). The mechanism by which OCT4 is transported to nuclei remains undefined. We have analyzed the sequence of mouse OCT4 and identified a conserved nuclear localization signal RKRKR at the N-terminal portion of the homeobox domain. To test the possibility that this is the signal responsible for OCT4 localization to the nuclei, we mutated this sequence into GSRVD by site-directed mutagenesis as shown in Fig. 1A. To monitor its localization, we fused the wild type and the mutated OCT4 with GFP at their C termini as shown in Fig. 1A. Upon transfection into HeLa cells, the fusion proteins migrated as a single species around ~58 kDa by Western blotting analysis (Fig. 1B, lanes 2 and 3). Confocal analysis of their subcellular localization revealed that the mutant OCT4 is present in a diffuse manner throughout the cells, whereas the wild type OCT4 is localized exclusively in the nuclei (Fig. 1C, lanes 1 and 2), suggesting that the RKRKR motif is required for the nuclear localization of OCT4. To confirm this finding further without GFP tagging, we generated a polyclonal antibody against OCT4 expressed in Escherichia coli (Fig. 2A, lane 3). Upon affinity purification against the recombinant protein coupled to agarose beads, the antibody can detect both OCT4 protein with or without the GFP tag (Fig. 2B, lanes 8 and 9). To demonstrate further the specificity of the antibody, we preincubated the antibody with the purified OCT4 protein prior to being used for Western blotting. As shown in Fig. 2B, preabsorption of the antibody with the purified OCT4 protein eliminated the signal completely (Fig. 2B, lanes 11 and 12). This antibody was then used to detect OCT4 proteins without any tag. In Fig. 3A, the antibody was able to react specifically with wild type and mutant OCT4 proteins migrating at 44 kDa (lanes 2 and 3). To examine the subcellular localization of the untagged wild type and mutant OCT4 proteins, we expressed both proteins by transfections in HeLa cells and analyzed their cellular localization by immunostaining using the affinity-purified antibody. Confocal images obtained shown in Fig. 3B reveal that wild type OCT4 localizes exclusively in nucleus, whereas mutant OCT4 is in the cytoplasm.
(g–i versus d–f). To see if nuclear localization is required for OCT4 activity, we analyzed the transcription activities of NLS mutant mOCT4 by using the p6w-luciferase reporter (11). As shown in Fig 3C, both untagged (lane 7) and tagged (lane 8) NLS mutant mOCT4 failed to transactivate the reporter p6w-luciferase, which contains six copies of octamer motif, whereas the wild type OCT4 is highly active (lanes 5 and 6). However, the transcription activity of wild type mOCT4-tagged C-terminally with GFP is significantly lower than that of the untagged form (Fig. 3C, lanes 6 versus 5). Because both the N- and C-terminal domains of mOCT4 have transcription activities (18), it is highly likely that C-terminally tagged GFFP (where GFFP is GFP tagged with a FLAG (F) tag at its C terminus) may interfere with the function of the C-terminal domain, thus diminishing its activity.

Heterologous NLS Can Rescue the Nuclear Localization but Not the Transcription Activity of NLS Mutant OCT4—The loss of transcription activity for the NLS mutant may be explained by its mislocalization to the cytoplasm as shown in Fig. 3 of the localization process. To test this possibility, we fused a known NLS identified in the PSR protein (19), RRKKRR, to either the C or N terminus of the mutant OCT4. As shown in Fig. 4A, the fusions produced protein species of the expected size (lanes 3 and 4). Both fusions localized to the nuclei (Fig. 4B, lanes c–f versus a and b), confirming that the heterologous NLS can rescue the localization of mutant OCT4. However, neither fusions possess any transactivation function when co-transfected with the reporter plasmid (Fig. 4C, lanes 4 and 5 versus 2 and 3), suggesting that the RRKKRR motif have a function other than nuclear localization.

NLS Mutant OCT4 Behaves as a Dominant Negative Mutant in Suppressing the Activity of Both Exogenous and Endogenous mOCT4 through Dimerization—It had been reported that OCT4 regulates the expression of target genes through dimerization. Therefore, the failure of the NLS mutant OCT4 to activate target genes may be due to its failure to dimerize. To test this possibility, we co-transfected wild type mOCT4-GFPF with untagged wild type mOCT4 to 293T cells and then performed co-immunoprecipitations by using anti-FLAG resin and detection with anti-OCT4 antibodies as described under “Materials and Methods.” As shown in Fig. 5A, untagged mOCT4 (lane 8, lower band) was co-precipitated with mOCT4-GFPF (lane 8, upper band), confirming OCT4 dimerization. Similarly, as shown in Fig. 5B, the NLS mutant mOCT4 can also dimerize (lane 8, upper and lower bands), demonstrating that the mutation in the RKKRR motif did not interfere with the dimerization process. Furthermore, as shown in Fig. 5, C and D, heterodimerization between wild type and NLS mutant mOCT4 proteins can be detected (Fig. 5C, lane 8 and D, lane 8). These data demonstrate that the NLS mutation does not affect the dimerization of OCT4.

The apparent dimerization between wild type and NLS mutant OCT4 proteins suggests that the NLS mutant may behave as a dominant negative mutant in suppressing the activity of co-expressed wild type OCT4. To test this idea, we co-transfected the NLS mutant with wild type OCT4 in addition to the OCT4 reporter plasmid in HeLa cells. As shown in Fig. 6, the NLS mutant suppressed the activity of wild type OCT4 in a dose-dependent manner, up to ~50% (lanes 4–9), suggesting that the NLS mutant does behave as a dominant negative mutant. We also co-transfected wild type mOCT4-GFPF with the untagged NLS mutant to see if the NLS mutant affects the nuclear localization of the wild type molecule through dimerization. As shown in Fig. 6B, wild type mOCT4-GFPF when transfected alone into HeLa cells is localized exclusively in the nuclei as expected (d–f) but is shifted to the cytoplasm when co-expressed with untagged NLS mutant mOCT4 (g–i), presumably through dimerization. This observation is consistent with the heterodimerization between wild type and NLS-OCT4 proteins as shown in Fig. 5, C and D. These data also confirm the heterodimerization microscopically.

Dominant negative mutants have been useful tools in delineating signaling transduction pathways. The NLS mutant of OCT4 thus may be used to inhibit the activity of endogenous OCT4. To this end, we performed transient co-transfection experiments with NLS mutant mOCT4 and the OCT4 reporter genes in P19 embryonic carcinoma cells. Widely used for differentiation into several cell types, P19 cells express relatively...
high levels of mOCT4 as demonstrated by Western blotting analysis by using the antibody we developed (Fig. 7A, lane 1) and thus could be a good model for the investigation of OCT4 function. Indeed, endogenous OCT4 activates the OCT4 reporter gene efficiently (Fig. 7B, lanes 2 versus 1). When co-transfected with increased amounts of NLS mutant mOCT4, the luciferase activity activated by endogenous mOCT4 became progressively lower (Fig. 7B, lanes 3–6), approaching ~70% of inhibition for the endogenous OCT4. Together, we conclude that the NLS mutant OCT4 acts as a dominant negative mutant capable of suppressing the activity of both exogenous and endogenous OCT4.

Constitutive Expression of NLS Mutant mOCT4 GFPF Leads to Differentiation of P19 Cells—One of the known functions for OCT4 is to regulate stem cell pluripotency in a quantitative manner. Differentiation of P19 cells induced by retinoic acid or Me2SO is accompanied by the reduction of mOCT4 expression. It is possible that these inducers trigger differentiation by down-regulating the levels of OCT4 and/or other related transcription factors (e.g. Nanog) inside the stem cells. To test this possibility, we generated stable cell lines expressing NLS mutant mOCT4-EGFP from P19 cells. Control P19 clones expressing only the GFP gene were also obtained in a similar fashion. As shown in Fig. 8, cells from GFP-transfected colonies (c and d) were similar to parental P19 cells (a and b) in morphology. In contrast, cells expressing NLS mutant mOCT4 (Fig. 8, e and f) have a distinct morphology compared with GFP-transfected or parental cells. In general, the cells expressing NLS mutant mOCT4-EGFP grow much larger and become more spread out with expansive cytoplasm (Fig. 8, g and h), a clear sign of differentiation into trophoblastic giant cells. To confirm the differentiated properties, we analyzed the expression of SSEA-1, a marker for stem cell pluripotency, in these transfected cells (Fig. 9). As expected, the pluripotency marker, SSEA-1, was readily detected in P19 parental cells (Fig. 9, a–c) and GFP control-transfected P19 cells (Fig. 9, d–f). On the other hand, the differentiated giant cells expressing NLS mutant OCT4EGFP apparently lost the expression of SSEA-1 as shown by immunostaining (Fig. 9, g–i). These data confirmed that constitutive expression of NLS mutant mOCT4 can induce the differentiation of P19 cells by reducing the function of OCT4.
DISCUSSION

Stem cells have enormous potential in the future of medicine based on their abilities to regenerate virtually all organs and tissues, i.e., pluripotency (2). However, the cellular and molecular mechanism controlling stem cell pluripotency remains largely unknown (9, 10). OCT4 protein has attracted considerable attention as a key regulator of stem cell pluripotency and cell differentiation (6, 9, 10, 12, 13, 20). OCT4 functions to maintain the inner cell mass/epiblast lineage and establish the extraembryonic ectoderm through fgf4 expression (14, 21). As a transcription factor, OCT4 has been shown to activate the expression of several downstream genes involved in cell proliferation and differentiation (6, 9, 11, 13, 21). Mechanistically, OCT4 binds to its cognate octamer motif through the cooperation of the POU and homeobox domains and activates downstream genes through transactivation domains located between the N and C termini to the POU/homeobox domains (for review see Ref. 9). Despite the fact that OCT4 is a nuclear protein, the manner in which OCT4 is transported to the nuclei upon synthesis in the cytoplasm remains unclear. Here we report the identification of a nuclear localization signal in the homeobox of the stem cell pluripotency factor OCT4, and we demonstrated that mutation of this motif results in a dominant negative mutant capable of inhibiting OCT4 activity expressed both exogenously and endogenously. Furthermore, we also show that expression of this NLS mutant leads to differentiation of the pluripotent embryonic carcinoma cell line P19, thus providing a useful tool for the manipulation of stem cell pluripotency and differentiation at the molecular level.

The NLS mutant of OCT4 serves as a dominant negative mutant. When co-expressed with wild type OCT4, the NLS mutant can inhibit the activity of wild type OCT4 in a dose-dependent fashion. This dominant negative effect is likely to...
mediated through dimerization with the wild type molecule as demonstrated in Fig. 5. However, it is not clear at this moment how the NLS mutant poisons the wild type molecule. Based on data obtained in Fig. 6B, one may conclude that the dominant negative effect is due to the trapping of wild type OCT4 by the NLS mutant in the cytosol, thus preventing it from transactivating its target. Paradoxically, the NLS mutant can be restored as a nuclear protein by the insertion of an unrelated NLS, RRKKRR, at its C or N termini (Fig. 4), whereas these nucleary restored NLS mutants remain inactive transcriptionally. One potential explanation is that the original mutation changing RRKKRR into GSRVD altered the conformation of OCT4 in such a way that it can no longer bind to DNA and mediate transactivation function. Alternatively, this RRKKRR motif serves a dual role as an NLS and some unknown function for transactivation. Further studies are required to distinguish these two possibilities.

Technically, the ablation of an NLS in a transcription factor may represent a general strategy to generate dominant negative mutants for transcription factors. To our knowledge, this is the first such report that alteration in subcellular localization of a transcription factor results in a dominant negative mutant. Most transcription factors contain a single NLS and thus are readily available for mutagenesis and the generation of a specific dominant negative mutant. These dominant negative mutants would be invaluable tools as we demonstrated in Figs. 8 and 9 for the assignment of biological functions in complex biological processes such as stem cell pluripotency. Further work in vitro and in vivo should allow us to demonstrate the application of this general strategy that allows for the manipulation of biological functions at the molecular level with precision.

Acknowledgments—We acknowledge the strong support from the laboratories of Prof. Yeguang Chen and Zijie Chang and the generous assistance from the members of the Pei laboratory during this study.

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J. Biol. Chem. 2004, 279:37013-37020.
doi: 10.1074/jbc.M405117200 originally published online June 24, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405117200

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