A Dominant-negative Form of Mouse SOX2 Induces Trophoderm Differentiation and Progressive Polyploidy in Mouse Embryonic Stem Cells*

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SOX2 plays an important role in early embryogenesis by cooperating with OCT4 in regulating gene expression in fertilized eggs, yet the precise mechanism through which SOX2 accomplishes this important function remains poorly understood. Here, we describe the identification of two nuclear localization signals (NLS) in SOX2 and the generation of a dominant-negative mutant (Dmu-mSox2) by mutating these two NLS in its high mobility group domain. Characterization of this mutant demonstrated that SOX2 shuttles between the cytoplasm and nucleus using these two NLS. The mutant has lost its ability to interact with OCT4, but remains competent to interact with wild-type SOX2. Functionally, Dmu-mSox2 is inactive and unable to cooperate with OCT4 in transactivating target promoters bearing its binding sites. However, Dmu-mSox2 is able to inhibit the activity of wild-type SOX2 and subsequently suppress the activity of downstream genes such as Oct4 and Nanog. When stably expressed in embryonic stem (ES) cells, Dmu-mSox2 triggered progressive doublings of cell ploidy (>8N), leading to differentiation into the trophoderm lineage. Knockdown of Sox2 by small interfering RNA also induced trophoderm differentiation and polyploid formation in mouse ES cells. These results suggest that SOX2 maintains stem cell pluripotency by shuttling between the nucleus and cytoplasm in cooperation with OCT4 to prevent trophoderm differentiation and polyploid formation in ES cells.

Stem cell-based therapies are promising solutions to many current unmet medical needs such as diabetes and Parkinson disease. Given their potential to differentiate into virtually all types of cells in the human body, human embryonic stem (ES) cells may be used to replace any aged or damaged cells under various pathological conditions (1). To accomplish these therapeutic goals, formidable obstacles must be overcome in such areas as the procurement and maintenance of ES cells in pluripotent states and efficient methods to differentiate the ES cells to a specific cell type suitable for transplantation. Although progress has been made in the derivation of human ES cells, the lack of understanding of stem cell pluripotency and differentiation may hamper any serious attempt to harness the potential of stem cell-based therapies (2). Thus, investigations into the molecular and cellular mechanisms of stem cell biology such as self-renewal and pluripotency are essential steps in developing the necessary tools for utilizing ES cells in future therapeutic interventions (2–4).

One of the key properties of ES cells is their ability to undergo self-renewal indefinitely (2). This property appears to be regulated by a network of transcription factors (2, 5). The first such factor recognized to play a critical role in stem cell pluripotency is the homeodomain transcription factor OCT4, a deficiency in which fails to support the development of pluripotent inner cell mass in early embryogenesis (2, 3). Most recently, another homeodomain protein, NANOG, has also been shown to be involved in maintaining the inner cell mass in early embryogenesis and the self-renewal of ES cells in culture (6, 7). Given the common pathway these factors appear to regulate, recent studies have revealed that both factors indeed regulate similar sets of genes by co-occupying adjacent sites within their regulatory regions (5). We (25) and others (8) have demonstrated recently that OCT4, NANOG, and FOXD3 are part of a regulatory network in that they regulate each other’s activity at the transcriptional level. Furthermore, our work suggests that these factors may form a negative feedback loop that limits the activity of OCT4 and ensures the proper expression of these factors in a dynamic fashion in ES cells. (5) This observation is consistent with the hypothesis that transcription factors play a critical role in controlling stem cell self-renewal in a cooperative fashion (2, 3).

SOX2 is a transcription factor with a high mobility group (HMG) domain and has also been implicated in the regulation of stem cell pluripotency by maintaining the expression of Fgf4 in the inner cell mass (9). Gene targeting experiments revealed a cell-autonomous requirement for SOX2 in both the epiblast and extraembryonic ectoderm (10). Mechanistically, SOX2...
functions to regulate downstream genes through cooperation with OCT4 by binding to adjacent sites (8, 10–14). In addition to its role in early embryogenesis, when SOX2 and OCT4 coexpress in pluripotent cells, SOX2 appears to play an additional role in later developmental stages such as neural differentiation by consolidating the neural identity of early neuroectoderm cells in Xenopus and the proliferation and/or maintenance of neural stem cells in mice (15, 16). Indeed, mutations in SOX2 have been identified in individuals with anophthalmia (17, 18). SOX2 is also expressed in pluripotent cells in the extraembryonic ectoderm (10). These observations raise the possibility that SOX2 may be able to mediate gene expression independently of its known partner, OCT4, yet the mechanism through which SOX2 can regulate gene expression and cell differentiation remains poorly understood.

Unlike OCT4, SOX2 appears to be localized in both the nuclei and cytoplasm in pre-implantation embryos (10). This unique subcellular localization pattern suggests that SOX2 shuttles between these two subcellular compartments and that its nuclear localization regulates its transcription activity. Here, we demonstrate that SOX2 contains two nuclear localization signals (NLS). The ablation of these two NLS resulted in a dominant-negative form of SOX2 that we named Dmu-mSox2. When stably expressed in ES cells, this mutant protein triggered a dramatic differentiation of ES cells into the trophoderm lineage and the formation of polyploid (>8N) cells. Our results not only suggest that SOX2 functions to prevent the differentiation of ES cells into the trophoderm lineage, but also demonstrate that Dmu-mSox2 may be a potentially useful tool in controlling stem cell differentiation.

MATERIALS AND METHODS

Plasmid Construction and Reporter Assays—The mouse Sox2 cDNA was amplified by reverse transcription
(RT)-PCR using high fidelity Pfx polymerase (Invitrogen) from mRNA isolated from CGR8 ES cells. The following primers were used: 5′-acccatatcagagacagcccagc-3′ (forward) and 5′-cccttcagctctcttcagcagccggag-3′ (reverse). The SOX2 cDNAs were inserted into the EcorV site of modified pCR3.1 for the mammalian expression vector. SOX2 NLS mutants was generated by site-directed mutagenesis with the following primers: SOX2 NLS1 mu, 5′-gcttgcggagaagatgagggcagacggagc-3′ (forward) and 5′-tctctgcggagcttactcattctccagctgcccggagcagc-3′ (reverse); SOX2 NLS1 KR mu, 5′-cagcgggacgctgatccggcggcggagc-3′ (forward) and 5′-gagggcggcttactcaggggcccgggaccg-3′ (reverse); SOX2 NLS2 KR mu, 5′-ataataactccgcccctgaggggaaacacagagcgcctc-3′ (forward) and 5′-gacgctcttttttttttcacgaggccgtatttata-3′ (reverse).

For mutant SOX2 expression without any tag, primers 5′-tcagatcggcagccccggccagcaggagc-3′ (forward) and 5′-gtcgctgcttctcttcgatcggagc-3′ (reverse) were used. Oct4 and Nanog promoter fragments were amplified by PCR from mouse liver genomic DNA and inserted into the SmaI site of the promoterless luciferase reporter vector pGL-Basic (Promega, Madison, WI). Primers 5′-tgtgagcggctgagctgagggcccgggaccg-3′ (forward) and 5′-tgaaagacggctcacctaggg-3′ (reverse) for the Oct4 promoter (2170 bp) and primers 5′-tgggctctcttcgatcggagc-3′ (forward) and 5′-gtcctcgcggagcggcagc-3′ (reverse) for the Nanog promoter (926 bp) were used. The Oct4 reporter plasmid 6w-Luc and the control vector p37TK-Luc were kindly provided by Dr. Lisa Dailey. For reporter assays, transfection efficiencies were normalized with a Renilla plasmid as internal references, and DNA concentrations were kept constant with an empty expression vector. Cells were harvested 48 h after transfection, and luciferase activity was measured using Dual-Luciferase (Promega).

Cell Culture, Transfection, and Fluorescence-activated Cell Sorter (FACS) Analysis—The mouse embryonic stem cell line CGR8 was used in all experiments. CGR8 ES cells were cultured on 0.1% gelatin-coated substrates in ES medium consisting of Glasgow minimal essential medium (Sigma) supplemented with 20% ES-qualified fetal bovine serum, 100 μg/ml antibiotics (penicillin and streptomycin; Invitrogen), and 1000 units/ml human recombinant leukemia inhibitory factor (Chemicon). 293T cells were transfected by calcium phosphate coprecipitation methods. HeLa cells transfected with the indicated plasmids were lysed and analyzed by Western blotting using anti-FLAG antibody as described under “Materials and Methods.”

Cell Lysate Preparation and Western Blot Analysis—Cell lysates (40 μl) were saved for direct Western blot analysis to determine protein expression levels, and the remaining samples were transferred to a new tube containing 10 μl of anti-FLAG antibody-conjugated agarose beads (Sigma). Cell lysates were then washed twice with phosphate-buffered saline containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, and 1 mM EDTA plus 10 μl of protease inhibitor mixture (Sigma). Cell lysates were then washed three times with Tris-buffered saline. The beads were eluted by boiling for 5 min in 2× SDS loading buffer with 5% β-mercaptoethanol. After centrifugation, supernatants were loaded onto 10% SDS-polyacrylamide gel and blotted onto polyvinylidene difluoride membranes (Millipore). The membranes were then incubated with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, followed by centrifugation at 15,000 rpm for 5 min at 4 °C. Cleared cell lysates were then washed three times with Tris-buffered saline. The beads were then eluted by boiling for 5 min in 2× SDS loading buffer with 5% β-mercaptoethanol. After centrifugation, supernatants were loaded onto 10% SDS-polyacrylamide gel and blotted onto polyvinylidene difluoride membranes for detection.

Microscopy and Cell Staining—For confocal microscopy, cells were observed using a Nikon digital camera and then imported into Adobe Photoshop Version 6.0. For immunostaining, cells grown on coverslips were fixed with 2% paraformaldehyde in phosphate-buffered saline; washed; blocked in 10% normal goat serum; and then stained with primary antibodies, including anti-SOX2 (catalog no. sc-20088) and anti-OCT4 (catalog no. sc-5279) antibodies (Santa Cruz Biotechnology, Inc.), anti-SSEA-1 antibody (Chemicon), and anti-GFP antibody (Chemicon), and anti-TROMA-1 antibody. Secondary antibodies, including anti-IgG, enhanced GFP.

FIGURE 1. Identification of two NLSs in mSOX2. A, a schematic presentation of mSOX2 NLS mutants and the two-NLS motif. The mSOX2 coding region and two NLS oligonucleotides were fused to GFP-FLAG (R) at the C terminus. B, Western blot analysis of these constructs. HeLa cells transfected with the indicated plasmids were lysed and analyzed by Western blotting using anti-FLAG antibody as described under “Materials and Methods.”

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resuspended in phosphate-buffered saline; and incubated with anti-TROMA-1 primary antibody (1:100; Institut Pasteur), followed by fluorescein isothiocyanate-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.), using the MoFlo high performance cell sorter (excitation at 488 nm and measured at 530/40 nm with a band-pass green filter).

For mutant SOX2 expression without any tag, primers 5′-tcagatcggcagccccggccagcaggagc-3′ (forward) and 5′-gtcgctgcttctcttcgatcggagc-3′ (reverse) were used. Oc...
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A

WT-mSox2-F
1mu-mSox2-F
2mu-mSox2-F
Dmu-mSox2-F

B

MV (KDa)

CK WT 1mu 2mu Dmu

32.5

3.0

3.5

4.0

4.5



C

Vector
WT-mSox2-F
Dmu-mSox2-F
1mu-mSox2-F
2mu-mSox2-F

D

Fold Activity

1

2

3

4

5

6

7

8



TRITC-conjugated goat anti-mouse IgM (SouthernBiotech) and TRITC-conjugated goat anti-rabbit IgG and fluorescein isothiocyanate-conjugated goat anti-rat IgG (Santa Cruz Biotechnology, Inc.), were used for detection. The images were captured using an Olympus FV500 confocal system.

RT-PCR—Cytoplasmic RNAs were extracted from lysates of cells treated with TRizol reagent (Invitrogen) and quantified by spectrophotometry. First-strand cDNA was synthesized using reverse transcriptase (Invitrogen). PCRs were then performed to amplify the intended genes under the following conditions: 95 °C for 3 min, 94 °C for 30 s, 58 °C for 40 s, and 72 °C for 45 s for 25–32 cycles. The sequences of the primers used are as follows: Oct4, 5'–gagagagagagagagagagc-3' (forward) and 5'–tgtctagtctctctctcagc-3' (reverse); Nanog, 5'–acagttgctcttctgtcagcaccc-3' (forward) and 5'–cagac- cattgtgggaccttctaaccc-3' (reverse); Pi-1, 5'–gactacgctctgccctgagcc-3' (forward) and 5'–gaagacgacgcatc- cct-3' (reverse); Cdx2, 5'–gggagagagagagcttctc-3' (forward) and 5'–gattgcttgctggcgcgcttctcag- acc-3' (reverse); Sox2, 5'–gattgca- cactggagatca-3' (forward) and 5'– gctgtcgactctgtctcgcg-3' (reverse); Gata4, 5'–cttccacagctacagtg-3' (forward) and 5'–acaatgcatgagggg- tgtc-3' (reverse); Gata6, 5'–ggagagt- gtgaggtgaggg-3' (forward) and 5'– tgtgtacgctgcctcagcg-3' (reverse); and glyceraldehyde-3-phosphate dehydrogenase, 5'–aacttgctgagggg-3' (forward) and 5'–acaatgagtggagagaa-3'. The PCR products were electrophoresed on 1.5% agarose gels in the presence of ethidium bromide and then photographed in a UV light box.

Real-time RT-PCR Analysis—2 µg of total RNA was reverse-transcribed in a final volume of 20 µl as described previously. PCRs were undertaken using a Real-Time PCR Master Mix (SYBR green) reagent kit (Toyobo) according to the manufacturer's protocol. PCR was performed in a 15-µl total volume for 45 cycles. The primer sequences were as follows: p21, 5'–atggcaccctc- tgggattc-3' (forward) and 5'–aaa- gttcagcttcgctgccc-3' (reverse); p27, 5'–atggccattacctat- agttcagcttcgctgccc-3' (reverse) and 5'–ccctcagcttcgctgccc-3' (reverse); cyclin A, 5'–gagttcctcttcata- aaggctc-3' (forward) and 5'–cagggtc- atctctcagcttcgctgccc-3' (reverse); cyclin B1, 5'–atgggctcctgctcctc-3' (forward) and 5'–attaccatgtctctcctc-3' (reverse); cyclin B2, 5'–atgggctcctgctcctc-3' (forward) and 5'–ccctcagcttcgctgccc-3' (reverse); cyclin D1, 5'–tgctctcgcctctcctcctg-3' (forward) and 5'–ggagagagagagagc-3' (forward) and 5'–gattgcttgctggcgcgcttctcag- acc-3' (reverse); cyclin D2, 5'–gagagagagagagcat- gctgaggg-3' (forward) and 5'–cagacgagagagagctt-3' (reverse); cyclin E, 5'–atgggctcctgctcctc-3' (forward) and 5'–cagacgagagagagctt-3' (forward); actin, 5'–atggcaccctc- tgggattc-3' (forward) and 5'–tgctctcgcctctcctcctg-3' (reverse); Nanog, 5'–tcttcctgctccccacccagtt-3' (forward) and 5'–gacagagagagagctt-3' (reverse); Oct4, 5'–cagacgagagagagctt-3' (forward) and 5'–gattgcttgctggcgcgcttctcag- acc-3' (reverse); Gata4, 5'–cttccacagctacagtg-3' (forward) and 5'–acaatgcatgagggg- tgtc-3' (reverse); Gata6, 5'–ggagagt- gtgaggtgaggg-3' (forward) and 5'– tgtgtacgctgcctcagcg-3' (reverse); Cdx2, 5'–cagggtc- atctctcagcttcgctgccc-3' (reverse) and 5'–gctgtcgactctgtctcgcg-3' (forward); and 5'–gaaagggg-
**RESULTS**

**Mouse (m) SOX2 Contains Two NLS Required for Nuclear Localization and Transcription Activity**—SOX2, a member of the HMG transcription factor family previously shown to be expressed early in embryogenesis and in neural stem cells, interacts with the POU transcription factor OCT4 through the HMG/POU domains to regulate the expression of many downstream genes, including Fgf4, Utf1, Nanog, and pouf5, for maintaining the pluripot- or multipotent states both in vivo and in vitro (8, 10, 14, 19). As a nuclear protein, SOX2 must enter the nucleus to regulate the expression of its downstream genes, yet little is known about how SOX2 is localized in the nucleus. Interestingly, it has been observed that SOX2 appears to be present in the cytoplasm and to shuttle between the cytoplasm and nuclei during early embryogenesis (10), suggesting that it may have a function outside the nucleus. To characterize the cellular distribution of SOX2 further, we fused a green fluorescent protein (GFP)-FLAG tag to its C terminus (Fig. 1A) and expressed the chimera in HeLa cells. As shown in Fig. 1C, although GFP alone diffused randomly in the whole cell (panels d–f), SOX2-GFP localized exclusively to the nuclei (panels g–i), confirming the fact that SOX2 is indeed a nuclear protein. This appears to agree with SOX9, a close relative of SOX2, which was reported to localize in the nuclei by two functional NLS within the HMG domain: a traditional basic amino acid clusters and a bipartite motif (20). Apparently, these two NLS are well conserved in the HMG transcription factor family, as shown in Fig. 1A.

To test the role of these two NLS in SOX2, we performed single or double mutagenesis on these sites (Fig. 1A). Upon transfection into HeLa cells, these mutants were expressed as stable proteins, like the wild-type protein (Fig. 1B). On the other hand, as shown in Fig. 1C, mutations of NLS1 (panels s–u), NLS2 (panels v–x), or both mutations (panels j–l) caused the diffusion of SOX2 into the whole cells, suggesting that each NLS is partially effective in localizing SOX2 to the nuclei. Indeed, when fused to GFP, either NLS could drive GFP to the nuclei only partially (Fig. 1C, panels m–r), further demonstrating the requirement of both NLS for the nuclear localization of SOX2.

**FIGURE 3.** Wild-type mSOX2 interacts with mOCT4 and enhances mOCT4 transcription activity, whereas NLS mutant mSOX2 has lost this function. A, formation of a heterodimer between wild-type mSOX2 and wild-type mOCT4, but not between NLS mutant mSOX2 and wild-type mOCT4. After transfection with mSOX2-FLAG (F), Dmu-mSOX2-FLAG, and untagged OCT4, cell lysates were analyzed for protein expression (lanes 1–6) and dimerization by FLAG resin immunoprecipitation (IP; lanes 7–12). B, wild-type mSOX2 enhances mOCT4 transcription activity, whereas NLS mutant mSOX2 has lost this function in the 6× O/S reporter. C, wild-type mSOX2 enhances mOCT4 transcription activity, whereas NLS mutant mSOX2 has lost this function in the on 6w-Luc reporter.
Our previous work on OCT4 demonstrated that the NLS is essential not only to its nuclear localization, but also to its transcription activity (22). To test whether the same applies to SOX2, we analyzed the transcription activity of wild-type SOX2 or its mutants in HeLa cells with the reporter construct 6w/H11003O/S, which harbors six copies of the OCT4/SOX2-binding site from the Fgf4 promoter (21). As shown in Fig. 2D, SOX2 activated the 6w/H11003O/S reporter strongly but had no effect on the control reporter p37TK (bar 5 versus bar 3). However, SOX2 mutants with either a single or NLS double mutation lost some or most of the transcription activity (Fig. 2D, bars 6–8). These data demonstrate that the two NLS of SOX2 play dual roles in SOX2 function: nuclear localization and transcription activity, as observed in OCT4 (22).

Ablation of the NLS in SOX2 Also Impairs Its Interaction with OCT4—
It is well established that SOX2 interacts with OCT4 to regulate downstream genes. To investigate whether the NLS in SOX2 also play a role in its interaction with OCT4, FLAG-tagged wild-type or NLS double mutant SOX2 was expressed either alone or with untagged OCT4 in 293T cells. The cell lysates were immunoprecipitated by anti-FLAG antibody and probed with anti-FLAG (Fig. 3A, panel a) or anti-OCT4 (panel b) antibody, respectively. As shown in Fig. 3A, wild-type SOX2 interacted with OCT4 as expected in this co-immunoprecipitation experiment (panel b, lane 5 versus lane 11), whereas double mutant SOX2 did not (lane 6 versus lane 12). These data demonstrate that SOX2 interacts with OCT4 in an NLS-dependent fashion.

We then performed reporter assays to determine the consequence of the impaired SOX2/OCT4 interaction on OCT4 activity. Two different reporters were used: 6w-Luc, with six copies of a conventional OCT4-binding site that can be bound only by OCT4 itself, and 6w/H11003O/S-Luc, as mentioned above with six copies of the OCT4/SOX2-binding site capable of binding both OCT4 and SOX2. Both reporters were transfected with OCT4 alone or with either wild-type or NLS double mutant SOX2 into HeLa cells. The reporter activities were evaluated by luciferase assay as described previously (22). As shown in Fig. 3 (B and C), wild-type SOX2 strongly increased the transcription activity of OCT4 in both 6w-Luc and 6w/O/S-Luc (bars 3, 5, and 6), suggesting that SOX2 can cooperate with OCT4 in gene regulation regardless of its binding context. On the other hand, NLS mutant SOX2 failed to enhance the activity of OCT4 in both reporters (Fig. 3, B and C, bars 3, 7, and 8), consistent with the co-immunoprecipitation results. These results demonstrate that the NLS of SOX2 are also required for its ability to cooperate with OCT4 in regulating downstream genes.
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NLS Mutant mSOX2 Forms Complexes with Wild-type SOX2, Suppresses Its Activity, and Down-regulates Its Target Genes—We have shown previously that ablation of the NLS in OCT4 generated a dominant-negative mutant that could suppress the activity of its wild-type protein and induce the differentiation of pluripotent cells (22). The data presented so far on the NLS mutant of SOX2 are also consistent with the idea that it may behave as a dominant-negative mutant. To test this hypothesis, we performed co-immunoprecipitation experiments to demonstrate that the mutant is capable of forming a complex with wild-type SOX2. GFP-FLAG-tagged wild-type SOX2 was transfected with the untagged NLS mutant of SOX2 into 293T cells, and the proteins were immunoprecipitated with anti-FLAG antibody and probed with anti-SOX2 antibody. As shown in Fig. 4A, the untagged NLS double mutant of SOX2 precipitated with wild-type SOX2 tagged with GFP-FLAG (lane 8), suggesting that the NLS double mutant of SOX2 remains competent in forming complexes with the wild-type molecule. Given that the same NLS mutant of SOX2 failed to interact with OCT4 (Fig. 3), we concluded that SOX2 forms homodimers not only independently of its NLS, but also differently from its ability to interact with OCT4.

We then tested whether the NLS mutant of SOX2 can suppress the activity of wild-type SOX2. SOX2 was cotransfected with the NLS mutant, and the effects on the reporter were analyzed as shown in Fig. 4C. The NLS mutant suppressed the activity of wild-type SOX2 in the absence and presence of its cofactor OCT4 in a dose-dependent manner (Fig. 4, C–E). To probe the mechanism of suppression further, we then cotransfected GFP-tagged SOX2 with the FLAG-tagged mutant in HeLa cells and observed that the SOX2 mutant blocked the nuclear localization of wild-type molecules (Fig. 4B), suggesting that the NLS mutant inhibits the wild-type molecules by sequestering them in the cytoplasm.

To further investigate whether the NLS mutant of SOX2 can down-regulate the expression of SOX2 target genes, we analyze its impact on the promoters of Oct4 and Nanog, both reported to be downstream targets of the SOX2-OCT4 complex (8, 14). As shown in Fig. 5A, both promoter regions contain a SOX2-binding site. The activities of these two promoters were then evaluated in pluripotent and non-pluripotent cells. As shown in Fig. 5B, consistent with the endogenous expression levels of Nanog and Oct4, reporters bearing these two promoters were much more active in pluripotent cells (ES and F9) than in non-pluripotent NIH3T3 cells. Used as a control, the reporter bearing the Fgf4 minimal promoter plus six copies of the OCT4/SOX2-binding site had much higher activity in pluripotent cells (ES and F9) than in non-pluripotent NIH3T3 cells. As shown in Fig. 5C, the NLS mutant suppressed the activities of all three promoters in a dose-dependent manner in ES cells. Taken together, our data demonstrate that the NLS mutant behaves as a dominant-negative mutant capable of suppressing the activity of both exogenous and endogenous SOX2, presumably by forming protein complexes, and subsequently suppressing its downstream targets such as Fgf4, Oct4, and Nanog.

Constitutive Expression of the NLS Mutant of mSOX2 Induces the Differentiation of ES Cells into Trophoderm—SOX2 is known to cooperate with OCT4 in a combinatorial fashion to specify the three embryonic lineages in pre-implantation embryos (10). The absence of either factor in early embryos leads to outgrowth of trophoderm from isolated embryos and the complete absence of pluripotent stem cells, suggesting that SOX2 may function to prevent the differentiation of ES cells into trophoderm during early embryogenesis (2, 10). However, very little is known about its role in maintaining stem cell fate and trophoderm differentiation, as previous analysis with SOX2 knock-out mice was potentially complicated by the presence of maternal SOX2 proteins in the cytoplasm of mature oocytes and stromal cells (10). To this end, we took advantage of the apparent dominant-negative effect of the NLS mutant of mSOX2 and analyzed the consequence of its expression in ES cells. The NLS mutant of SOX2 or a control vector was then transfected into mouse ES cells, which were selected in the
Constitutive expression of Dmu-mSox2 in ES cells leads to trophectoderm differentiation and polyploid formation. A, Western blot analysis of Dmu-mSox2 by anti-FLAG antibody. CK, blank. B, cell localization of mSOX2 in pCR3.1 and Dmu-mSox2-FLAG (F) stable clones. C, morphology and DNA contents of stable clones transfected with pCR3.1 and Dmu-mSox2-FLAG. pCR3.1 and Dmu-mSox2-FLAG stable cells (1 x 10^7) were harvested, fixed in 70% ethanol, and stained with propidium iodide. DNA contents were analyzed by FACS. Diploid (2N), tetraploid (4N), octoploid (8N), and multiploid DNA contents are indicated. D, expression of cell cycle regulators in Dmu-mSox2-FLAG-transfected cells. The relative mRNA level was generated by comparison with the expression of actin as the internal control. E, growth curves of the indicated cells. Cells were cultured with or without G418 for 6 days, and cell numbers were counted. F, embryonic body formation of pCR3.1 and Dmu-mSox2-FLAG stable cells. Stable cells (1 x 10^7) were cultured with differentiation medium in a 6-cm bacteria cell dish. G, immunostaining of markers in pCR3.1 and Dmu-mSox2-FLAG stable cells. Dmu-mSox2-FLAG stable cells lost the expression of SSEA-1 and OCT4 compared with pCR3.1 stable cells. Dmu-mSox2-FLAG stable cells express the trophectoderm marker Troma-1. H, FACS analysis of Troma-1-positive cells in Dmu-mSox2-FLAG stable clones. 50.85% of the Dmu-mSox2-FLAG stable cells were detected as Troma antibody staining-positive cells. I, expression of markers associated with the trophectoderm in Dmu-mSox2-FLAG stable cells. cDNAs were prepared from mouse ES cells transfected with control pCR3.1 and Dmu-mSox2-FLAG and from embryonic bodies (EB) derived from wild-type ES cells. The cDNAs were analyzed by real-time PCR with -fold differences measured against pCR3.1 control cells. Note that Dmu-mSox2-FLAG stable cells express very little Gata4 and Gata6, but high levels of Cdx2 and Fgrf2.
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presence of G418. After 2 weeks of selection, ES cell lines transfected with the NLS mutant of SOX2 or the control vector were obtained, and the expression level of SOX2 was confirmed by Western blotting using anti-FLAG antibody (Fig. 6A, lane 3) and immunostaining (Fig. 6B, lower panels). We observed a morphological difference between ES cells expressing the NLS mutant and those transfected with the control vector or the parental ES cells. First, the cells expressing the NLS mutant were much more spread out and larger than the control ES cells (Fig. 6C), indicating a differentiated phenotype. FACS analysis of the DNA content showed that these cells displayed progressive polyploidy compared with the control cells, which were mostly diploid or tetraploid (presumably prior to cell division) (Fig. 6C), indicating that these cells may have a reduced proliferation rate or reduced cell divisions. Indeed, cell proliferation analysis demonstrated that the cells expressing NLS mutant SOX2 grew at a much slower rate than the control cells (Fig. 6E). To determine whether these cells are pluripotent, we examined their capacity to form embryonic bodies. As shown in Fig. 6F, when cultured in suspension, the control cells formed typical embryonic bodies (panels a–d), whereas the NLS mutant-expressing cells did not (panels e–h). These data demonstrate that ES cells expressing the NLS mutant of SOX2 are no longer pluripotent and have undergone differentiation.

We then tested the expression of several cell cycle regulators in the Dmu-mSox2-transfected cells by real-time PCR. Among the regulators that we tested, cyclins D1 and D2 were significantly up-regulated; p21 and p27 were slightly up-regulated; and the others remained unchanged (Fig. 6D). ES cells have a unique cell cycle property with a long S phase and a short gap phase (G1 or G2). Consistent with this unique cell cycle property, ES cells have much lower expression levels of G1 phase regulators (cyclins D1 and D2) and cyclin-dependent kinase inhibitors (p21 and p27) compared with differentiated cells. Thus, up-regulation of these factors in Dmu-mSox2-transfected cells suggests that Dmu-mSox2 leads to the formation of progressive polyploidy by releasing the suppression of these factors in ES cells.

To further define the lineage of these differentiated cells, we analyzed the expression of pluripotent and differentiated markers by immunostaining and real-time PCR. As shown in Fig. 6G, pluripotent markers such as Ssea-1 and Oct4 were virtually absent in cells expressing the NLS mutant, but were highly expressed by the control ES cells. We then determined the differentiated state of the NLS mutant transfectants by examining the expression of Troma-1, a trophectoderm-specific marker. As shown in Fig. 6G, these cells expressed a relatively high level of Troma-1, whereas the control cells did not. FACS analysis demonstrated that >50% of the cells transfected with the NLS mutant were positive for Troma-1 in contrast to only a few that were positive among the control cells (Fig. 6H). These data clearly demonstrate that the NLS mutant of SOX2 triggers the differentiation of ES cells into the trophectoderm lineage. Finally, we further confirmed this observation by analyzing more markers by real-time PCR. As shown in Fig. 6I, ES cells transfected with NLS mutant SOX2 had much reduced levels of pluripotency markers (including Oct4 and Nanog), yet had significantly elevated expression levels of trophectoderm markers (Pt-1, Cdx2, and Fgf2) compared with the control ES cells, but no endoderm markers (Gata4 and Gata6) compared with the embryonic bodies derived from untransfected ES cells. We conclude that the NLS mutant of SOX2 induces the differentiation of ES cells into the trophectoderm lineage.

Knockdown of Endogenous Sox2 by Small Interfering RNA (siRNA) Also Induces Trophectoderm Differentiation and Polyploid Formation in Mouse ES Cells—Oct4 has been shown to maintain ES cell pluripotency by preventing trophectoderm differentiation (3). In this study, we have shown that impairment of its partner, Sox2, also caused trophectoderm differentiation, suggesting that both are required to prevent trophectoderm differentiation. To further confirm the role of Sox2 in ES cells, we constructed the Sox2 siRNA expression vector as shown in Fig. 7A. This vector contains a U6 promoter that drives siRNA
expression. A GFP expression cassette was included in this vector to monitor the transfected cells. First, we tested the efficiency of Sox2 siRNA by reporter assays. We cotransfected the Sox2 reporter 6-O/S-Luc with wild-type and mutant (muSox2) Sox2 siRNA constructs. GFP-positive cells were sorted out 1, 3, 5, and 7 days (d) after transfection. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control. C, transcription activity of mSOX2 in its reporter gene can be suppressed by Sox2 siRNA, D, morphology of ES cells transfected with wild-type (panels a and b) and mutant (panels c and d) Sox2 siRNAs. GFP-positive ES cells were sorted out 3 days after transfection and further cultured in normal ES cell medium. The DNA contents of ES cells transfected with wild-type and mutant Sox2 siRNAs were determined by FACS analysis. E, changes in gene expression patterns of ES cells transfected with wild-type and mutant Sox2 siRNAs. The results from RT-PCR analyses of RNAs isolated from ES cells transfected with wild-type (lane 2) and mutant (lane 3) Sox2 siRNAs and from embryonic bodies (EB; lane 4) illustrate gene expression. CK, blank.

FIGURE 7. Knockdown of Sox2 triggers trophectoderm differentiation in mouse ES cells. A, Sox2 hairpin siRNA and GFP expression constructs. RNAi, RNA interference. B, RT-PCR analyses of Sox2 transcript levels in cells transfected with wild-type and mutant (muSox2) Sox2 siRNA constructs. GFP-positive cells were sorted at 1, 3, 5, and 7 days after transfection. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control. C, transcription activity of Sox2 in its reporter gene can be suppressed by Sox2 siRNA, D, morphology of ES cells transfected with wild-type (panels a and b) and mutant (panels c and d) Sox2 siRNAs. GFP-positive ES cells were sorted out 3 days after transfection and further cultured in normal ES cell medium. The DNA contents of ES cells transfected with wild-type and mutant Sox2 siRNAs were determined by FACS analysis. E, changes in gene expression patterns of ES cells transfected with wild-type and mutant Sox2 siRNAs. The results from RT-PCR analyses of RNAs isolated from ES cells transfected with wild-type (lane 2) and mutant (lane 3) Sox2 siRNAs and from embryonic bodies (EB; lane 4) illustrate gene expression. CK, blank.
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FIGURE 8. Proposed model for the role of Dmu-mSox2 in ES cell differentiation.

Together, these data demonstrate that Sox2 functions together with its partner, Oct4, to prevent trophoderm differentiation and polyploid formation in mouse ES cells.

DISCUSSION

Our observation that a dominant-negative form of SOX2 is able to trigger the differentiation of ES cells into the trophoderm lineage and to generate trophoblast-like cells further confirms a general strategy we proposed for the transcription factors involved in the pluripotency of ES cells (22). In contrast to the results obtained with the dominant-negative form of OCT4, we obtained stable clones constitutively expressing Dmu-mSox2. These cells assumed a pattern of gradual differentiation toward trophoblast-like cells, reflected by the gradual shift from 2N/4N to 8N/nN in ploidy. These cells may be a good model to further investigate the uncoupling of chromosomal duplication and cell/nuclear division known for trophoblasts. This mutant may also be used to probe the role of SOX2 in neural function in late developmental stages using a transgenic approach.

Transcription factors that regulate the expression of gene programs associated with stem cell pluripotency or differentiation have recently become a focal point of interest (5, 23, 24). OCT4, SOX2, and NANOG have been implicated in stem cell pluripotency initially through knock-out studies and have been recently proposed to regulate overlapping sets of genes by chromatin immunoprecipitation analysis (5–7, 10). On the other hand, overexpression of Cdx2 has identified it as a key factor specifying the trophoderm lineage by reciprocally suppressing the expression of target genes regulated by the pluripotent factor OCT4 (23). One may argue that stem cell self-renewal or differentiation is regulated by a network of transcription factors such as OCT4, NANOG, and CDX2; yet, the precise role of these factors remains very poorly understood. In this study, we have focused on SOX2, a transcription factor that has been implicated in maintaining stem cell pluripotency through its interactions with OCT4. Our results demonstrate that it contains two distinct NLSs that are required for SOX2 to function as a transcription factor. Furthermore, we have generated a dominant-negative form of SOX2. This SOX2 mutant can interfere with endogenous SOX2 expressed in ES cells and triggers the differentiation of these ES cells into the trophoderm lineage. This observation is consistent with data obtained in SOX2 knock-out experiments demonstrating failure to derive SOX2−/− ES cells, the lack of epiblasts, and the presence of trophoblast giant cells and extraembryonic endoderm (10). However, because of the lack of SOX2−/− ES cells, the precise role of SOX2 in cell fate determination has not been analyzed at the molecular level. Our results recapitulate a portion of the phenotype generated in the knock-out embryos, i.e. trophoderm differentiation of ES cells transfected with Dmu-mSox2, especially formation of the trophoblast giant cells (Fig. 6). Because the trophoderm is the first differentiated cell lineage of mammalian embryogenesis and forms the placenta, the molecular mechanisms that control this differentiation event have attracted considerable attention (23). The apparent role of Cdx2 in trophoderm differentiation observed by Niwa et al. (23) suggests that multiple transcription factors are involved in the cell fate decision during the first cell lineage differentiation. Oct4 has been shown to maintain ES cell pluripotency by preventing EC cell differentiation into the trophoderm lineage (3). Here, we demonstrated that impairment of its partner (Sox2) also triggers trophoderm differentiation, suggesting that the cooperation of Oct4 and Sox2 is required in preventing trophoderm differentiation, as in the model proposed in Fig. 8. Consistent with Oct4 down-regulation, Dmu-mSox2 and Sox2 siRNA can also induce the expression of Cdx2 (Fig. 6), suggesting that they trigger trophoderm differentiation by inducing Cdx2. Thus, Sox2 may participate in the reciprocal inhibition between lineage-specific transcription factors as observed by Niwa et al. (23).

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