Cyclin-dependent Kinase-mediated Sox2 Phosphorylation Enhances the Ability of Sox2 to Establish the Pluripotent State

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Sox2 is a key factor in maintaining self-renewal of embryonic stem cells (ESCs) and adult stem cells as well as in reprogramming differentiated cells back into pluripotent or multipotent stem cells. Although previous studies have shown that Sox2 is phosphorylated in human ESCs, the biological significance of Sox2 phosphorylation in ESC maintenance and reprogramming has not been well understood. In this study we have identified new phosphorylation sites on Sox2 and have further demonstrated that Cdk2-mediated Sox2 phosphorylation at Ser-39 and Ser-253 is required for establishing the pluripotent state during reprogramming but is dispensable for ESC maintenance. Mass spectrometry analysis of purified Sox2 protein has identified new phosphorylation sites on two tyrosine and six serine/threonine residues. Cdk2 physically interacts with Sox2 and phosphorylates Sox2 at Ser-39 and Ser-253 in vitro. Surprisingly, Sox2 phosphorylation at Ser-39 and Ser-253 is dispensable for ESC self-renewal and cell cycle progression. In addition, Sox2 phosphorylation enhances its ability to establish the pluripotent state during reprogramming by working with Oct4 and Klf4. Finally, Cdk2 can also modulate the ability of Oct4, Sox2, and Klf4 in reprogramming fibroblasts back into pluripotent stem cells. Therefore, this study has for the first time demonstrated that Sox2 phosphorylation by Cdk2 promotes the establishment but not the maintenance of the pluripotent state. It might also help explain why the inactivation of CDK inhibitors such as p53, p21, and Arf/Ink4 promotes the induction of pluripotent stem cells.

Embryonic stem cells (ESCs) have the ability to differentiate into various cell types in the adult body (1, 2). They have been advocated to be important cellular resources for treating varieties of human degenerative diseases (3–5). Particularly, patient-specific-induced pluripotent stem cells (iPSCs) offer even greater promise in treating human diseases due to the absence of immune rejection (5–8). Moreover, human ESCs and iPSCs have also become important tools for understanding human biology and disease mechanisms and for developing better and more effective drugs for many human diseases (5, 9). Although much progress has been made in understanding the molecular mechanisms underlying ESC self-renewal and cellular reprogramming (10, 11), many important biological questions remain to be answered. A better understanding of such mechanisms will improve our ability to use pluripotent ESCs and iPSCs to treat human diseases.

In human and mouse ESCs, Sox2, Oct4, and Nanog represent core transcription factors for maintaining the pluripotent state by sustaining the expression of pluripotency genes and repressing the expression of lineage differentiation genes (12). Such core transcription factors are capable of re-establishing the pluripotent state during cellular reprogramming (6–8). Mass spectrometry-based proteomics has identified four phosphorylation sites on Sox2, namely Ser-246, Ser-249, Ser-250, and Ser-251, in human ESCs (13, 14). Interestingly, their phosphorylation promotes SUMOylation of Sox2 on the site located upstream of the phosphorylation sites in human ESCs (13). SUMOylation of Sox2 at Lys-247 inhibits the ability of Sox2 to bind to its transcriptional targets and its interaction with Oct4 (15, 16). Likely Sox2 phosphorylation at these sites negatively

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Sox2 phosphorylation regulation in ESC pluripotency. It has recently been shown that Sox2 phosphorylation at Thr-118 by Akt promotes the establishment of the pluripotent state during reprogramming (17). Sox2 monomethylation at Lys-119 by Set7 inhibits its transcriptional activity and induces its ubiquitination and degradation, and Akt-mediated phosphorylation at Thr-118 and Set-mediated Sox2 methylation at Lys-119 mutually inhibit each other (18). Acetylation of Sox2 on a lysine residue in the nuclear localization sequence promotes Sox2 nuclear export, thus decreasing its function in the nucleus (19). In addition, Oct4 and Nanog are also phosphorylated (14, 20). Although the biological function of Nanog phosphorylation remains unknown, Oct4 phosphorylation at Ser-111 by active ERK leads to rapid ubiquitination and protein degradation, explaining why FGF signaling promotes Oct4 degradation and thus ESC differentiation (21). In contrast, Akt-mediated phosphorylation of Oct4 at Ser-235 stabilizes Oct4 protein and facilitates its nuclear localization and interaction with Sox2, indicating that Akt-mediated Oct4 phosphorylation helps sustain ESC self-renewal (22). Therefore, posttranslational modifications of core ESC transcription factors likely play important regulatory roles in pluripotency maintenance and/or establishment.

During reprogramming, Oct4 and Sox2 activate p53 (Trp53) and p21 (Cdkn1a) expression, and inactivation of p53 or p21 can enhance Oct4/Sox2-mediated iPSC induction (23–25). The Ink4a/Arf locus contains the Cdkn2a-Cdkn2b genes encoding p16 (Ink4a), p19 (Arf), and p15 (Ink4b) (26, 27). These Cdk inhibitors are normally repressed in iPSCs and ESCs, and their inactivation in differentiated cells promotes Oct4/Sox2-mediated iPSC reprogramming (25). p21 and p16 function as Cdk inhibitors by directly binding to active Cdk protein complexes and inhibiting kinase activity (27). p53 transcriptionally activates p21 expression, thereby inhibiting cell cycle progression (28). These results suggest that Cdkks play pivotal roles in cellular programming, but it remains unclear how they regulate the functions of core pluripotency factors. In this study we have demonstrated that Cdk2-mediated phosphorylation of mouse Sox2 at Ser-39 and Ser-253 is dispensable for ESC self-renewal but promotes the ability to establish the pluripotent state during reprogramming.

Materials and Methods

MudPIT Analysis of Sox2 Protein Phosphorylation Sites—Three biological replicates of FLAG-affinity-purified Sox2 preparations were TCA-precipitated, urea-denatured, reduced, alkylated, and digested with endoproteinase Lys-C (Roche Applied Science) followed by modified trypsin (Promega). Another two affinity-purified fraction was digested with elastase (Calbiochem) or endoproteinase Lys-C followed by endoproteinase Glu-C (Roche Applied Science) as previously described (29). Peptide mixtures were analyzed using fully automated 10-step chromatography run as previously described (29, 30). Full MS spectra were recorded on the peptides over a 400–1600 m/z range followed by fragmentation at 35% collision energy on the 1st to 5th most intense ions. Dynamic exclusion was enabled for 120 s (31). Mass spectrometer scan and HPLC solvent gradients were controlled by the Xcalibur data system (Thermo Fisher Scientific). Tandem mass (MS/MS) spectra were searched using SEQUEST (32) against a database of 61428 sequences consisting of Mouse Sox2 and 30552 Homo sapiens non-redundant proteins (downloaded from NCBI on March 4, 2008), 162 usual contaminants (such as human keratins, IgGs, and proteolytic enzymes), and to estimate false discovery rates, randomized amino acid sequences derived from each non-redundant protein entry. As previously described (33), the MS/MS datasets were searched in a recursive fashion, first for serine, threonine, and tyrosine phosphorylation residues (combined with methionine oxidations) on peptides derived from Mouse Sox2; the next spectra-matching modified peptides were searched again with the same differential options against the complete protein database. Spectra/peptide matches were only retained if they had a DeltCn of at least 0.08 and minimum XCorr of 1.0 for single-., 2.0 for double- and 3.0 for triple-charged spectra. In addition, the peptides had to be at least seven amino acids long, and their ends had to comply with the specificities of the enzymes used in the digestions. All spectra matching phosphorylated peptides were visually assessed. In-house-written software, NSAF version 7, was used to extract total and modified spectral counts for each amino acid within mouse Sox2 and calculate modification levels based on local spectral counts (33).

Sox2, Cdk1–6, and Cdk2 Mutant Constructs—HA-tagged wild-type Cdk1–6 and dominant-negative Cdk2 and -4 were obtained from Addgene. FLAG-tagged Cdkks were PCR-amplified and inserted into the vector of pEF1-IRES GFP, which was modified from pIRES2 GFP (Clontech) by replacing the CMV promoter with EF1 promoter. The Sox2 encoding region was amplified from cDNA by PCR and was cloned into pGEX6p1 (GE Healthcare) for the kinase assay. FLAG-Sox2 and phosphorylation-defective or -mimic Sox2 mutants were cloned into pEF1-IRES GP. Three specific shRNA targeting Sox2 3′-UTRs (Fig. 3D) were cloned into the lentiviral vector of pSicoR-EF1GFP and were further tested for knockdown efficiencies by transfecting mouse ESCs. For the Sox2 RNAi rescue constructs (Fig. 3E), wild-type Sox2 and phosphorylation mutants together with IRES-GFP were cut from pEF1-IRES-GFP vector and put into lentiviral pSicoR-Sox2-i2-EF1GFP.

Cdk2 and constitutively active Cdk2 containing T14A, Y15F, and T16D (34) was subcloned into lentivirus vector pSico-EFα under the EFα-1 promoter. Point mutations in three sites were generated with three oligonucleotides by two-step PCR mutagenesis from a template pSico-EFα-Cdk2 vector.

32P-Labeled Sox2 Protein in 293T Cells and Kinase Assay—293T cells were transfected with FLAG-Sox2 for 2 days, and 5 μCi of [γ-32P]ATP was added for 30 min before the cells were harvested in the RIPA buffer. Anti-FLAG (M2)-agarose beads were used for immunoprecipitation (IP). After the electrophoresis, autoradiography was performed using Kodak BioMax MS film.

For the kinase assay, 293T cells transfected with FLAG-tagged Cdks were lysed in the buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P40, 1 mM dithiothreitol (DTT), and protease and phosphatase inhibitors. Anti-FLAG (M2)-agarose beads were used for IP. The purified FLAG-Cdks were incubated with bacterial GST-Sox2 for 1 h in 20 μl con-
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TABLE 1

| Gene        | Sense primers 5'-3'                                              | Antisense primers 5'-3'                  |
|-------------|----------------------------------------------------------------|----------------------------------------|
| Gapdh       | aattttgcagcttggaagggctca                                       | tggagccacgatctgtagcagggg                 |
| Dppa3       | tggggggagaaggggaggaga                                           | aatgaatgacaggggtcgctgtg                 |
| Dmnt3l      | cggaggagggagggagggaggg                                          | gagcagaggagggagggagggg                  |
| Endoct4     | tagtgagggagggagggagggg                                          | gttcagcagcagcagggagggg                  |
| Endo2f54    | aggctggagggagggagggagg                                          | ggtcagcagcagcagggagggg                  |
| Nanog       | ctcacagatcagatcagatcag                                          | cttaccatagaagatcaggtgtg                 |
| Rexl        | cagccagcagcagcagcagcagg                                         | gttcagcagcagcagcagcagcagg               |
| Ezh2        | ttcttttgagggagggagggagg                                         | aagggagggagggagggaggagggg               |
| E-cadherin  | caggcctttttgtgagggagggg                                         | ggttagcagcagcagcaggtggtg                |
| Klf7        | cagccccctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
Western Blot—ICR MEF cells within four passages were seeded at 10,000 cells/cm² in DMEM supplemented with 10% FBS and infected with virus as the iPSC generation procedure. The day that viral supernatants were removed and the test media were added was defined as day 0 post-infection. MEF cells were digested for protein extraction beginning with day 2 until day 4 because the induced pluripotent stem cell (iPSC) colony appeared on day 5 in which endogenous Sox2 begins to express. MEF cells were washed with ice-cold PBS and lysed in radioimmuno precipitation assay buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 2 mM EDTA, 0.5% Triton X-100, 2 mmol/liter PMSE, 1× protein inhibitor (Roche Applied Science)). After 30 min on ice, the lysates were sonicated and centrifuged at 13,000 rpm for 15 min. Lysates (20–40 mg of total protein) were electrophoresed on a 10–12% SDS-PAGE gel and electrophoretically transferred to a PVDF membrane (Millipore). Membranes were blocked in Tris-buffered saline with 5% milk and 0.1% Tween. The blots were probed with primary antibodies overnight and then with horseradish peroxidase-conjugated secondary antibodies. Antibody-antigen complexes were detected using ECL Plus reagent (the Thermo Scientific). The primary and secondary antibodies were anti-FLAG (1:1000; Sigma), anti-HA (1:800; Santa Cruz), anti-Sox2 (1:800, R&D Systems), anti-GAPDH antibody (1:50000, Abcam), peroxidase donkey anti-mouse IgG (1:10000; Jackson Immunoresearch), and peroxidase donkey anti-rabbit IgG (1:5000; Promega).

Co-IP Experiments—HEK293T expressing FLAG-Sox2 and HA-Cdk5 were collected and washed twice with PBS. Nuclear extracts were adjusted to 0.3 M NaCl and 0.5% Triton X-100 and centrifuged at 40,000 rpm for 30 min at 4 °C in a Ti-70 rotor. After centrifugation the supernatants were mixed with anti-FLAG (M2) resin in a ratio of 30 μl of beads/1 ml of nuclear extract and gently rocked for 2 h at 4 °C. The beads were washed 5 times with excess wash buffer containing 50 mM Hepes-NaOH, pH 7.9, 0.25 M KCl, 0.1% Triton X-100, and 10% (v/v) glycerol and then eluted with 30 μl of wash buffer containing 0.5 mg/ml FLAG peptide. Blots were developed in ECL reagent (PerkinElmer) and exposed to x-ray films.

Extracts of 293T cells transfected with plasmids encoding Oct4 and Sox2 wild type or its mutants were prepared in TNE lysis buffer containing 50 mM Tris, pH 8.0, 150 mM sodium chloride, 2 mM EDTA, 1% Nonidet P-40, 2 mM PMSE, 1× protein inhibitor (Roche Applied Science). The cell lysate was incubated with 2 μg of anti-Oct4 antibody (Santa Cruz) at 4 °C for at least 2 h. Subsequently, 25 μl of protein G-agarose and 25 μl of protein A-agarose (Millipore) were added and incubated with rotation at 4 °C overnight. The beads were spun down and washed 5 times with washing buffer containing 50 mM Tris, pH 8.0, 150 mM sodium chloride, 0.5% Triton X-100, 2 mM PMSE, and 1× protein inhibitors (Roche Applied Science). Finally, the proteins on the beads were eluted by boiling in the 2× SDS-PAGE loading buffer.

Generation of Rabbit Anti-Ser(P)-39 and Ser(P)-253 Polyclonal Antibodies—Rabbit polyclonal anti-Ser-39 and Ser-253 phosphorylation-specific antibodies were generated by Covance Inc (now Biologend Inc.) using the keyhole limpet hemocyanin (KLH)-conjugated phosphorylated peptides CKKKGNNQKNPSYFRVKR (Ser(P)-39) and CKKKVKEASSKPSVVTSE (Ser(P)-253). Each of the synthetic peptides was immunized into two rabbits, and the blood samples from the first, second, and third bleeding were tested at a dilution of 1:50 against the phosphorylated FLAG-tagged wild-type and phosphorylation-defective Sox2 proteins for specificity in 293T cells.

Results

Sox2 Is Phosphorylated in Mouse ESCs and Neural Precursor Cells—As previously reported in human ESCs (13, 14), Sox2 is also phosphorylated in mouse ESCs based on two pieces of experimental evidence. First, there are multiple bands migrating slower than the predicted band (Fig. 1A). Second, the slower migrating Sox2 bands disappear after phosphatase treatment, indicating that the slower migrating Sox2 bands indeed represent phosphorylated Sox2 protein (Fig. 1B). In addition we also observed the slower migrating Sox2 protein bands in the extracts of neural progenitors and retinal progenitors, suggesting that Sox2 is also phosphorylated in neural and retinal progenitors (Fig. 1C). To determine if mouse Sox2 is phosphorylated in human cells, we expressed FLAG-tagged Sox2 in 293T cells cultured in the presence of [γ-32P]ATP and pulled down the FLAG-tagged Sox2 protein. Indeed, Sox2 is also phosphorylated in 293T cells (Fig. 1D). Taken together, Sox2 is phosphorylated in many different cell types.

To further determine the phosphorylation sites on Sox2, we expressed the FLAG-tagged mouse Sox2 protein in human 293T cells and subjected the affinity-purified proteins to MudPIT analysis for the identification of phosphorylation sites (Fig. 1E). Combining three different protein digestion methods, we obtained high sequence coverage (>95%) and identified phosphorylation sites on 5 serine residues (Ser-18, Ser-39, Ser-140, Ser-143, Ser-253), one threonine residue (Thr-128), and two tyrosine residues (Tyr-Y2 and Tyr-127) (Fig. 1F). Mouse Sox2 has two glycine residues inserted after the 22nd amino acid residue compared with the human sequence; hence, Ser-251, Ser-252, and Ser-253 in mouse Sox2 correspond to Ser-249, Ser-250, and Ser-251 in human Sox2, respectively. This study uncovered seven new and conserved phosphorylation sites on 5 serine residues (Fig. 1G). Predicted by the consensus sequence (SP), Ser-39 and Ser-253 are likely Cdk kinase targets (37). These phosphorylation sites and their surrounding amino residues are conserved from fish to human, indicating that the phosphorylation of those sites on Sox2 might have evolutionarily conserved biological functions (Fig. 1H).

Cdk2 Directly Phosphorylates Sox2 at Ser-39 and Ser-253 Sites in Vitro—To determine if Sox2 is phosphorylated at Ser-39 and Ser-253 by Cdk2, we generated and purified rabbit polyclonal antibodies against the peptides containing either the phosphorylated Ser-39 or Ser-253 site. When expressed in human 293T cells, FLAG-Sox2 exhibits multiple forms as in mouse ESCs, retinal progenitors, and neural progenitors (Fig. 2A). Interestingly, FLAG-Sox2 (3S-A)
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only shows one major isoform but specifically lacks the slower migrating forms (the phosphorylated forms) (Fig. 2A). Because Ser-222 phosphorylation is not detected in human 293T cells by MudPIT, Ser-39 and Ser-253 likely represent major phosphorylation sites. Both anti-phosphorylated Ser-39 (anti-Ser(P)-39)- or Ser-253 (anti-Ser(P)-253)-specific antibodies can recognize FLAG-Sox2, but not FLAG-Sox2 (3S-A), which is expressed in human 293T cells, indicating that mouse Sox2 is phosphorylated at both Ser-39 and Ser-253 sites (Fig. 2A). These results have confirmed that mouse Sox2 is phosphorylated at Ser-39 and Ser-253 sites and have also verified the specificity of these two phosphorylation-specific antibodies.

To determine which Cdk proteins might interact with Sox2 and phosphorylate Ser-39 and Ser-253, we co-expressed a HA-tagged version of Cdk1, -2, -3, -4, -5, or -6 with FLAG-Sox2 in human 293T cells and performed co-IP experiments. Based on the pulldown FLAG-Sox2 levels, FLAG-Sox2 should interact with HA-Cdk2 and HA-Cdk4 but could also interact with the other HA-tagged Cdk proteins to a lesser extent (Fig. 2B). Based on the pulldown FLAG-Sox2 levels, Sox2 is expected to interact with Cdk4 stronger than Cdk2. When HA-Cdk2 and HA-Cdk4 proteins are expressed at similar levels, our results show that FLAG-Sox2 can indeed pull down HA-Cdk4 more efficiently than HA-Cdk2 (Fig. 2C). It is also worth noting that co-expression of FLAG-Sox2 with HA-Cdk2, but not HA-Cdk4, increases the ratio of the slow migrating phosphorylated form of Sox2 protein to the nonphosphorylated form of Sox2 protein, suggesting an interesting possibility that Cdk2, but not Cdk4, might phosphorylate Sox2 (Fig. 2C). To directly test that possibility, we generated FLAG-tagged versions of wild-type and dominant-negative Cdk2 and Cdk4 and expressed them in human 293T cells and further purified wild-type and mutant Cdk2- or Cdk4-containing protein complexes. The freshly purified wild-type Cdk2- and Cdk4-containing protein complexes should be protein kinase active because of direct association with their Cyclin partners, whereas the purified mutant Cdk2- and
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Cdk4-containing protein complexes should lack the protein kinase activity because of their mutated active site. Surprisingly, only the FLAG-Cdk2-containing protein complexes, but not the FLAG-Cdk4-containing protein complexes, are capable of phosphorylating the bacterially expressed GST-Sox2 (1–111 amino acids (aa)) at Ser-39 and GST-Sox2 (224–319 aa) at Ser-253 (Fig. 2D). Furthermore, the FLAG-Cdk2 (DN)-containing proteins fail to phosphorylate Sox2 at either the Ser-39 or Ser-253 site (Fig. 2D). These results have demonstrated that Cdk2 can directly phosphorylate Sox2 at Ser-39 and Ser-253 in vitro.

We then examined Sox2 phosphorylation status in mouse ESCs using the anti-Ser(P)-39- and anti-Ser(P)-253-specific antibodies. In the control, rabbit polyclonal anti-Sox2 antibodies recognize different forms of Sox2 protein (Fig. 2E). As expected, anti-Ser(P)-39 and anti-Ser(P)-253 antibodies can recognize the slow migrating phosphorylated Sox2 proteins forms of Sox2 protein (Fig. 2E). Interestingly, the two anti-Ser(P)-39 and anti-Ser(P)-253 antibodies also recognize extremely slow migrating large molecular weight Sox2 isoforms, which most likely represent SUMOylated forms of Sox2, whereas arrowheads denote the nonphosphorylated Sox2 protein.

Sox2 Phosphorylation at Ser-39 and Ser-253 Is Dispensable for ESC Pluripotency and Cell Cycle Progression—Mouse and human ES cells proliferate with a short G1 phase and maintain constant high Cdk activity (38). Inactivation of Cdk2 leads to the prolonged G1 phase and eventually differentiation of mouse and human ESCs (38–40), whereas shRNA-mediated Cdk1 knockdown also results in the loss of proliferation and self-renewal of mouse ESCs (41). Thus, we then examined the expression of Sox2 phosphorylation at Ser-39 and Ser-253 in mouse ESCs. In the control, Sox2 protein is uniformly expressed in mouse ESCs, including mitotic cells (Fig. 3A). Interestingly, both phosphorylated Sox2 at Ser-39 and Ser-253 show low levels of general expression in the nuclei of ESCs, but the highest level of expression in mitotic ESCs (Fig. 3, B and C). These results indicate that phosphorylated Sox2 at Ser-39 and Ser-253 is constantly expressed in ESCs with the highest level in mitotic ESCS and further support the idea that the peak active kinase activity of Cdk proteins is in the mitotic phase of the cell cycle.

To investigate the function of Sox2 phosphorylation in the regulation of ESC pluripotency, we first needed to remove endogenous Sox2 proteins in ESCs by RNAi-mediated knockdown. To achieve this goal we designed three shRNA constructs, Sox2-i1, Sox2-i2, and Sox2-i3, to target different regions of the Sox2 3′-UTR, and further tested their knockdown efficiencies in mouse ESCs by Western blotting (Fig. 3D). Although these three shRNA constructs work efficiently to knock down Sox2 protein in ESCs, Sox2-i2 is the most efficient one and is used to remove endogenous Sox2 protein in the following experiments (Fig. 3D). Then we expressed different versions of mutant Sox2 protein in which Ser-39 and Ser-253 have been mutated to either alanine (S2A; phosphorylation-defective) or glutamic acid (S2E; phosphorylation-mimicking) in the Sox2-i2 knockdown ESCs (Fig. 3E). In the lentivirus vector, GFP expression driven by the elongation factor 1α (EF1) gene promoter is used to identify the infected ESCs (Fig. 3E). In the control, GFP-positive ESCs are positive for Oct4, a pluripotency marker (Fig. 3, D and F). Indeed, GFP-positive Sox2-i2 knockdown ESCs lose the expression of Oct4 and also exhibit flat cell morphology indicative of differentiation, demonstrating that it is effective to knock down Sox2 expression and inactivate its function in ESCs.
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To determine if S2A and S2E affect the cell cycle of ESCs, we use fluorescence-activated cell sorter (FACS) to quantify cell numbers in different phases of the cell cycle based on DNA content. Interestingly, the Sox2 knockout ESCs expressing wild-type, S2A, and S2E Sox2 proteins exhibit similar cell cycle profiles (Fig. 3K). These results indicate that Sox2 phosphorylation at Ser-39 and Ser-253 does not affect the cell cycle progression in ESCs.

Phosphorylation at Ser-39 and Ser-253 Enhances Sox2 Function in Pluripotency Establishment during Reprogramming—Previous studies have shown that inactivating p21 and p16, which are known to bind to Cdk2 complexes and inhibit their activities, promotes the efficiency of establishing pluripotency during reprogramming (23–25). Thus, we postulate that Cdk2-mediated Sox2 phosphorylation might be involved in the establishment of pluripotency during reprogramming. In the previous studies (42–44) the Oct4–GFP reporter was used to monitor the establishment of pluripotency, whereas Oct4, Sox2, and Klf4 (OSK) were introduced into the Oct4–GFP fibroblasts to produce iPSC colonies. In this study, we used Oct4-GFP expression and colony formation as indicators of iPSC formation. To facilitate the quantification of Oct4–GFP-positive colonies, we started with the same numbers of Oct4-GFP-positive iPSC colonies 10 days after their expression in the iCD1 media (Fig. 4A). The phosphorylation-defective mutant Sox2 (S39A or S253A) at either Ser-39 or Ser-253 in combination with Oct4 and Klf4 generates the number of GFP-positive iPSC colonies 10 days after their expression in the iCD1 media (Fig. 4A). The phosphorylation-defective mutant Sox2 at both Ser-39 and Ser-253 (S2A) sites in combination with Oct4 and Klf4 significantly decreases GFP-positive iPSC colonies compared with the wild-type Sox2 (Fig. 4, A, B, and E). These results indicate that phosphorylation at both Ser-39 and Ser-253 promotes the ability of Sox2 to establish the pluripotent state in conjunction with Oct4 and Klf4.
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Then we determined if phosphorylation-mimicking Sox2 mutants sufficiently promote the establishment of the pluripotent state. The phosphorylation-mimicking Sox2 mutants S39E and S253E do not show any significant differences in the production of iPSC colonies from the wild-type Sox2, indicating that phosphorylation at either Ser-39 or Ser-253 does not affect Sox2 function in iPSC reprogramming (Fig. 4, B and F). In contrast, the phosphorylation-mimicking Sox2 mutant S2E induces significantly more GFP-positive iPSC colonies than the wild-type Sox2 (Fig. 4, B and G). Therefore, Sox2 phosphorylation in both Ser-39 and Ser-253 is necessary and sufficient to promote iPSC formation in conjunction with Oct4 and Klf4.

Both Phosphorylation-defective and -mimicking Sox2 Mutants Can Generate Germ Line-competent iPSCs—To determine if phosphorylation-defective and phosphorylation-mimicking Sox2 mutants indeed induce functional iPSCs as the wild-type Sox2 in conjunction with Oct4 and Klf4, we examined the expression of other pluripotency markers. As expected, the wild-type Sox2 along with Oct4 and Klf4 is capable of converting fibroblasts into iPSC colonies reactivating Nanog and Rex1 proteins in addition to Oct4-GFP (Fig. 5A). Similarly, the iPSC colonies induced by the phosphorylation-defective mutant S2A or the phosphorylation-mimicking mutant S2E also express Nanog and Rex1 proteins in addition to Oct4-GFP (Fig. 5, B and C). Then we further used RT-PCR to compare the expression levels of pluripotency genes between mouse ESCs and iPSCs. As expected, iPSCs induced by the wild-type Sox2 in combination with Oct4 and Klf4 express pluripotent genes, Sox2, Oct4, Nanog, Dapp3, Essrb, Dmnt3l, and Rex1 at comparable levels to mESCs. For Sox2 and Oct4, the expression levels of endogenous transcripts are measured (Fig. 5D). Similarly, the iPSCs induced by the phosphorylation-defective mutant S2A or the phosphorylation-mimicking mutant S2E in combination with Oct4 and Klf4 also express these pluripotent genes at comparable levels to mESCs (Fig. 5D). These results indicate that both phosphorylation-defective and -mimicking Sox2 mutants are capable of inducing the pluripotent state.

To determine if phosphorylation-defective mutant S2A and phosphorylation-mimicking mutant S2E are capable of inducing germ line-competent iPSCs, we injected the iPSCs induced by wild-type and S2A and S2E mutant Sox2 proteins plus Oct4 and Klf4 into mouse blastocysts to test their ability to form chimera and contribute to the germ line (10). In these experiments only the iPSCs with a normal karyotype were selected to generate chimeric mice. As expected, the wild-type Sox2-induced iPSCs are capable of efficiently generating chimeric mice when injected into blastocysts and contributing germ line transmission by breeding chimeras (Fig. 5E). Similarly, the iPSCs induced by S2A and S2E mutants are also capable of forming chimeric mice and germ line transmission (Fig. 5, F and G). Regarding the rates for chimeric mouse formation and germ line transmission, the iPSCs induced by wild-type Sox2 and S2E mutant Sox2 behave similarly (Fig. 5H). However, it appears that the iPSCs induced by the S2A mutant Sox2 protein have lower chimeric mouse rates than those induced by the wild-type Sox2 (Fig. 5H). Additionally, only one of five iPSC lines induced by the S2A mutant Sox2 is capable of germ line transmission. Therefore, phosphorylation at Ser-39 and Ser-253 might enhance the ability of Sox2 to produce germ line-competent iPSCs along with Oct4 and Klf4.

Phosphorylation-defective and -mimicking Mutant Sox2 Proteins Behave Like Wild-type Sox2 Protein in Protein Stability, Oct4 Interaction, and Gene Activation during Reprograming—To gain insight into how Sox2 phosphorylation at Ser-39 and Ser-253 affects pluripotency establishment during reprogramming, we compared S2A and S2E mutant Sox2 proteins with...
wild-type Sox2 for their stability and physical interaction with Oct4 and target gene activation in fibroblast cells. Based on Western blotting results, the S2A mutant and S2E mutant Sox2 proteins appear to be as stable as wild-type Sox2 proteins in fibroblast cells 2, 3, and 4 days after being expressed (Fig. 6A).

Oct4 and Sox2 form a protein complex that binds to the promoters of pluripotency genes to control their transcription in ESCs (12, 45–47). Based on the co-IP experimental results, the S2A mutant and the S2E mutant Sox2 proteins interact similarly with Oct4 as wild-type Sox2 protein (Fig. 6B). These results suggest that Sox2 phosphorylation at Ser-39 and Ser-253 does not appear to affect its stability and interaction with Oct4.

By following the reprogramming kinetics, S2A or S2E mutant Sox2 protein does not significantly change the time for the emergence of Oct4-GFP+ colonies in comparison to wild-type Sox2 protein. The S2A and S2E mutant Sox2 proteins could potentially affect fibroblast cell proliferation, timely activation of pluripotency genes, or both. To test the first possibility, we quantified cell numbers of the infected fibroblasts expressing wild-type and mutant Sox2 proteins on days 1, 3, 5, 7, and 9. Based on the fibroblast cell numbers at different time points after lentivirus infection, both the S2A and the S2E mutants behave similarly to the wild-type Sox2 in promoting fibroblast cell proliferation, further supporting the idea that phosphorylation at Ser-39 and Ser-253 does not affect cell cycle progression and thus cell proliferation (Fig. 6C). To test the second possibility, we used qRT-PCR to examine the induction of pluripotency genes at different time points. For the activation of endogenous Sox2, Oct4, and Klf4 expression, wild-type and S2A and S2E mutant Sox2 genes show no differences in their initial activation time and dynamic expression levels at different time points in conjunction with Oct4 and Klf4 (Fig. 6D). Similarly, they also do not exhibit any differences in the initial activation time and dynamic expression levels of pluripotent genes Nanog, Essrb, and Dppa3 at different time points (Fig. 6D). Taken together, these results suggest that Sox2 phosphorylation at Ser-39 and Ser-253 does not affect the ability of Sox2 in promoting fibroblast proliferation and in activating the expression of pluripotency genes during reprogramming.

During the iPSC reprogramming process, fibroblast cells undergo the mesenchyme-to-epithelium transition by turning off mesenchyme-specific genes and turning on epithelium-specific genes. As previously reported (43, 48), mesenchyme-specific genes Krt7 and Snail are quickly down-regulated after introduction of the reprogramming actors, whereas epithelium-specific genes Epcam and Cdh1 are gradually up-regulated (Fig. 6, E and F). The S2A and S2E mutant Sox2 genes show no
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FIGURE 6. Sox2 phosphorylation at Ser-39 and Ser-253 does not affect protein stability, interaction with Oct4 and gene activation during reprogramming. A, mutant Sox2 proteins, S2A and S2E, show similar stability to wild-type Sox2 in fibroblast cells 2, 3, and 4 days after infection. B, co-IP results indicate that mutant Sox2 proteins, S2A and S2E, show similar interaction affinity with Oct4 protein to wild-type Sox2 in fibroblast cells. WB, Western blot. C, Sox2 mutants, S2A and S2E, affect fibroblast proliferation along with Oct4 and Klf4 to the same extent as the wild-type Sox2. D, qRT-PCR results show that the two Sox2 mutants and the wild-type Sox2 (along with Oct4 and Klf4) are capable of initiating the timely activation of the pluripotent genes (Nanog, Esr2b, and Dppa3 as well as endogenous Sox2, Oct4, and Klf4) and maintaining their dynamic expression levels during the period of initial 12 days (D0 – D12). E–F, qRT-PCR results show that the phosphorylation-defective and phosphorylation-mimicking Sox2 mutants can effectively repress the expression of Snail (E) and Krt7 (F’) and activate the expression of Epcam (F) and Cdh1 (F’) as the wild-type Sox2 by working with Oct4 and Klf4.

obvious differences from the wild-type Sox2 gene in repressing the expression of mesenchyme-specific genes and in activating the expression of epithelium-specific genes during reprogramming (Fig. 6, E–F). These results indicate that Sox2 phosphorylation at Ser-39 and Ser-253 is not critical for repressing mesenchyme-specific genes and activating epithelium-specific genes during reprogramming.

Proper Modulation of the Cdk2 Activity Is Critical for OSK-mediated iPSC Induction—Our earlier result that Cdk2 directly phosphorylates Sox2 at Ser-39 and Ser-253 led us to investigate if exogenous Cdk2 facilitates OSK-mediated iPSC generation. In comparison with the control (the empty vector), the overexpression of the wild-type Cdk2 gene along with OSK increases the number of the Oct4-GFP+ iPSC colonies, suggesting that Cdk2 enhances OSK-mediated iPSC generation (Fig. 7, A–C). To determine whether the kinase activity of Cdk2 is required for its enhancement of OSK-mediated iPSC induction, we also tested if overexpressing the dominant-negative kinase-dead Cdk2 (Cdk2DN) and constitutively kinase-active Cdk2 (Cdk2CA) affects the efficiency of the iPSC colony formation induced by OSK. Surprisingly, both Cdk2DN overexpression and Cdk2CA overexpression drastically and significantly decrease the iPSC colony formation efficiency induced by OSK (Fig. 7, C–E). These results have demonstrated that the regulate-able Cdk2 kinase activity is important for OSK-induced pluripotency establishment.

To determine if there are any differences existing among the iPSCs induced by OSK in combination with Cdk2, Cdk2DN, and Cdk2CA, we used qRT-PCR to quantify the mRNA expression levels of pluripotency genes, including Rex1, Dppa3, and Nanog as well as endogenous Sox2, Oct4, and Klf4. Interestingly, the iPSCs derived from Cdk2, Cdk2DN, and Cdk2CA overexpression in combination with OSK show similar expression levels of pluripotency genes in comparison with those iPSCs derived from OSK alone (Fig. 7F). These results indicate that the modulation of Cdk2 kinase activity affects the OSK-induced iPSC efficiency but does not appear to affect the expression of pluripotency genes in iPSCs.

Because Cdk2 is involved in cell cycle regulation, its effect on OSK-mediated iPSC generation could come from either accelerating the reprogramming process or increasing the number of successfully reprogrammed cells or both. To determine by which way Cdk2 facilitates iPSC generation, we examined the total cell number of changes with time after Cdk2, Cdk2DN, and Cdk2CA overexpression along with OSK. Surprisingly, Cdk2, Cdk2DN, and Cdk2CA all attenuate the increase in total cell number in comparison with the control with similar kinetics before the appearance of iPSC colonies on Day 5 (Fig. 7G). Along with the earlier result that Cdk2 facilitates iPSC generation, this result suggests that Cdk2 promotes OSK-mediated iPSC induction likely by accelerating reprogramming kinetics. Taken together, these results suggest that Cdk2 accelerates the kinetics of OSK-mediated reprogramming at least partly through Sox2 phosphorylation but not by regulating cell proliferation.
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FIGURE 7. Wild-type Cdk2, but not constitutively active and dominant-negative forms, promote iPSC formation. A, OSK factors reprogram fibroblasts into Oct4-GFP+ iPSC colonies along with the empty vector (EV). B, wild-type Cdk2 can enhance the formation of Oct4-GFP+ iPSC colonies induced by OSK. C, quantitative results on iPSC colony formation induced by OSK along with the empty vector (control), Cdk2, constitutively active Cdk2CA, and dominant-negative Cdk2DN. D and E, Cdk2DN (D) and Cdk2CA (E) decrease iPSC colony formation induced by OSK. F, the quantitative effect of OSK alone and OSK in combination with Cdk2, Cdk2DN, and Cdk2CA on cell number during the first 7 days of iPSC induction. G, using qRT-PCR to compare the effect of Cdk2, Cdk2DN, and Cdk2CA on the expression levels of pluripotency genes in the iPSCs induced by OSK. Bars: 2 mm.

Discussion

Generation of patient-specific pluripotent stem cells via reprogramming holds great promise in treating various devastating degenerative diseases and studying the pathogenesis mechanisms of human diseases. Previous studies have shown that Cdk inhibitors represent a barrier for iPSC reprogramming, but it remains unclear how Cdk activity affects iPSC reprogramming (23–25). In this study we have identified two Cdk phosphorylation sites on Sox2, which can be phosphorylated by Cdk2 in vitro and in 293T cells and have further shown that Cdk-mediated Sox2 phosphorylation enhances the ability of Sox2 to establish the pluripotent state but is dispensable for maintaining the pluripotent state. In addition, regulate-able Cdk2 kinase activity helps OSK-mediated iPSC reprogramming. Our findings in this study could also potentially explain why inactivation of Cdk inhibitors promotes iPSC reprogramming. Sox2 and Cdk2 are also required for maintaining the self-renewal of neural stem cells (36, 49). Because this study shows that Sox2 is phosphorylated in neural precursor cells, our findings suggest that the same regulatory mechanism could also work in neural stem cells. Therefore, this study represents one important step forward toward the understanding of how Cdk-mediated phosphorylation is involved in iPSC reprogramming.

In mouse and human ESCs, Sox2 is phosphorylated, but how phosphorylation affects Sox2 function in maintaining or establishing the pluripotent state has just begun to be revealed. In mouse ESCs, LIF-dependent activation of Akt kinase causes Sox2 phosphorylation at Thr-118, increasing Sox2 protein stability and enhancing its transcriptional activities (17). Moreover, Akt-mediated Sox2 phosphorylation is dispensable for ESC self-renewal in the presence of LIF but enhances iPSC reprogramming from fibroblasts (17). Interestingly, Akt-mediated Sox2 phosphorylation can also inhibit Set7-mediated Sox2 monomethylation at Lys-119, which promotes ubiquitination and degradation (18). In human ESCs, four phosphorylation sites on Sox2 protein, Ser-246, Ser-249, Ser-250, and Ser-251, have been identified by two independent proteomic studies (13, 14). Interestingly, phosphorylation at Ser-249–Ser-251 residues leads to the SUMOylation of Lys-K245 (13). Sox2 SUMOylation decreases its ability to interact with its partner Oct4, resulting in Nanog repression (15, 16). Based on these findings, phosphorylation at Ser-249–Ser-251 should decrease Sox2 function and thus compromise ESC self-renewal. However, it has not been determined if this prediction is true in human ESCs. In this study we have confirmed the phosphorylation on Ser-253 (corresponding to Ser-251 on human Sox2) and have also identified eight new phosphorylation sites in mouse Sox2, five serine residues, one threonine residue, and two tyrosine residues. Based on the phosphorylated peptide counts, Ser-39 and Ser-253 represent the most abundantly phosphorylated residues on the mouse Sox2. Interestingly, Cdk2 directly interacts with Sox2 and phosphorylates Ser-39 and Ser-253 residues of Sox2 in vitro and in human 293T cells. Because Sox2 is also capable of interacting with other Cdk proteins in variable degrees in vitro, we could not rule out the possibility that other Cdk proteins could also phosphorylate Sox2 at these two canonical Cdk phosphorylation sites in vivo. Surprisingly, in mouse ESCs, phosphorylation-defective (S24) and phosphorylation-mimicking (S2E) Sox2 mutants can fully rescue the self-renewal defect of Sox2 knockdown ESCs to the same extent as the wild-type Sox2. These results indicate that Cdk-mediated Sox2 phosphorylation at Ser-39 and Ser-253 is dispensable for ESC self-renewal. Interestingly, the phosphorylation-defective Sox2 mutant decreases, whereas the phosphorylation-mimicking Sox2 enhances its ability with Oct4 and Kif4 to reprogram fibroblasts into pluripotent stem cells, indicating...
that Sox2 phosphorylation promotes Sox2 function in the establishment of the pluripotent state. Consistently, overexpressing the wild-type Cdk2 can enhance the OSK-mediated iPSC reprogramming efficiency, indicating that Cdk2 kinase activity is important for the establishment of the pluripotent state during OSK-mediated iPSC reprogramming. Surprisingly, overexpressing a constitutively active or dominant-negative Cdk2 mutant significantly decreases the OSK-mediated reprogramming efficiency, suggesting that the regulate-able Cdk2 kinase is critical for reprogramming. Taken together, our findings argue strongly that Cdk-mediated Sox2 phosphorylation is important for the establishment of the pluripotent state. Our findings could help explain why inactivation of cell cycle inhibitors enhances iPSC reprogramming (23–25).

In this study we also investigated the potential mechanisms of how Cdk-mediated Sox2 phosphorylation affects the establishment of the pluripotent state during reprogramming. This study has shown that wild-type, phosphorylation-defective, and phosphorylation-mimicking mutant Sox2 proteins have similar abilities to promote fibroblast proliferation, the mesenchymal-to-epithelial transition, and the expression of pluripotent genes in conjunction with Oct4 and Klf4. However, the iPSCs induced by wild-type and mutant Sox2 proteins might have differences in the formation of chimeric mice and germ line transmission, suggesting that Cdk-mediated Sox2 phosphorylation could potentially help generate high quality and germ line-competent iPSCs. One potential mechanism for Sox2 phosphorylation at Ser-39 and Ser-253 to enhance iPSC induction is to increase its ability to modulate chromatin accessibility by recruit epigenetic factors. Although it has not revealed the detailed mechanisms of how Cdk-mediated Sox2 phosphorylation contributes to iPSC reprogramming, this study has provided the entry point into studying how Sox2 phosphorylation regulates the establishment of the pluripotent state.

Author Contributions—J. O., W. Y., J. L., D. P., and T. X. designed the study, and T. X. wrote the paper. W. Y. and N. Z. performed and analyzed the experiments shown in Figs. 1–3. L. F. and M. W. performed the mass spectrometry analysis of phospho-proteins. J. O., J. L., J. C., and H. L. performed and analyzed experiments in Figs. 4–7. All the authors reviewed the results and approved the final version of the manuscript.

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