

PARP1 Poly(ADP-ribosyl)ates Sox2 to Control Sox2 Protein Levels and *FGF4* Expression during Embryonic Stem Cell Differentiation^{*[5]}

Received for publication, March 16, 2009, and in revised form, June 12, 2009. Published, JBC Papers in Press, June 16, 2009, DOI 10.1074/jbc.M109.033118

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Transcription factors Oct4 and Sox2 are key players in maintaining the pluripotent state of embryonic stem cells (ESCs). Small changes in their levels disrupt normal expression of their target genes. However, it remains elusive how protein levels of Oct4 and Sox2 and expression of their target genes are precisely controlled in ESCs. Here we identify PARP1, a DNA-binding protein with an NAD⁺-dependent enzymatic activity, as a cofactor of Oct4 and Sox2 to regulate expression of their target gene *FGF4*. We demonstrate for the first time that PARP1 binds the *FGF4* enhancer to positively regulate *FGF4* expression. Our data show that PARP1 interacts with and poly(ADP-ribosyl)ates Sox2 directly, which may be a step required for dissociation and degradation of inhibitory Sox2 proteins from the *FGF4* enhancer. When PARP1 activity is inhibited or absent, poly(ADP-ribosyl)ation of Sox2 decreases and association of Sox2 with *FGF4* enhancers increases, accompanied by an elevated level of Sox2 proteins and reduced expression of *FGF4*. Significantly, specific knockdown of *Sox2* expression by RNA interference can considerably abrogate the inhibitory effect of the poly(ADP-ribose) polymerase inhibitor on *FGF4* expression. Interestingly, *PARP1* deficiency does not affect undifferentiated ESCs but compromises cell survival and/or growth when ESCs are induced into differentiation. Addition of *FGF4* can partially rescue the phenotypes caused by *PARP1* deficiency during ESC differentiation. Taken together, this study uncovers new mechanisms through which Sox2 protein levels and *FGF4* expression are dynamically regulated during ESC differentiation and adds a new member to the family of proteins regulating the properties of ESCs.

Embryonic stem cells (ESCs),² derived from the inner cell mass of the blastocyst-stage embryo, are pluripotent. They can differentiate into all cell types of an organism and self-renew indefinitely *in vitro* (1, 2). Intensive research over past decades has demonstrated that transcription factors Oct4 and Sox2 are key players in maintaining the pluripotent state of ESCs (3, 4). Recently, their central position in stem cell biology has been further highlighted by their critical role in the establishment of induced pluripotent stem cells (5–7). It is also clear that Oct4 and Sox2 cooperatively regulate their own expression as well as that of different sets of target genes, such as *FGF4* (8), *Nanog* (9), and *UTF1* (10). Remarkably, small changes in the levels of Oct4 and Sox2 disrupt normal expression of their target genes and alter cell fate determination in ESCs (11–15). Therefore, levels of Oct4 and Sox2 as well as of their target genes must be tightly controlled. However, to date, our knowledge of the molecular mechanisms controlling their expression is limited. Obviously, answers to these questions are not only fundamental to ESC maintenance and differentiation but also have important implications for efficient generation of induced pluripotent stem cells.

As is the case for most other transcription factors, Oct4 and Sox2 are regulated at both transcriptional and post-transcriptional levels. However, past emphasis has been mainly placed on their transcriptional regulation, whereas their post-transcriptional control has been little touched upon. Previous studies in our laboratory demonstrated that Oct4 could be ubiquitinated and sumoylated and that ubiquitination and sumoylation jointly maintain the protein level of Oct4 in a normal range in ESCs (16, 17). Recently, phosphorylation of Oct4 was also reported (18). As for Sox2, one study indicated that Sox2 sumoylation negatively regulates its transcriptional activity, although its role in control of the Sox2 protein level is not known (19). In addition to ubiquitination, sumoylation, and phosphorylation, a wide variety of post-translational modifications, such as glycosylation and poly(ADP-ribosyl)ation, exists, and enzymes mediating these modifications make a great con-

* This work was supported by National Natural Science Foundation of China Grant 30730051, Shanghai Science and Technology Developmental Foundation Grant 08JC1413100, National High Technology Research and Development Program of China Grants 2006CB943901 and 2007CB947904, and Shanghai Leading Academic Discipline Project S30201.

[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Experimental Procedures, Figs. 1 and 2, and Table S1.

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² The abbreviations used are: ESC, embryonic stem cell; EC, embryonic carcinoma; LIF, leukemia inhibitory factor; NE, nuclear extract; RA, retinoic acid; CHIP, chromatin immunoprecipitation; CoIP, coimmunoprecipitation; qPCR, quantitative PCR; PAR, poly(ADP-ribose); PARP, poly(ADP-ribose) polymerase; EGFP, enhanced green fluorescent protein; GST, glutathione S-transferase; RNAi, RNA interference; 3AB, 3-aminobenzamide.

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tribution to modulation of transcription factors. One such enzyme is poly(ADP-ribose) polymerase-1 (PARP1), a 114-kDa, abundant nuclear DNA-binding protein that catalyzes the covalent attachment of poly(ADP-ribose) (PAR) from NAD^+ to itself and other nuclear protein acceptors such as topoisomerase I and II, NF- κ B, p53, and histones (20–25). In contrast to Oct4 and Sox2, which are specifically expressed in pluripotent stem cells, PARP1 is a constitutively expressed protein (26, 27). Although the best studied function of PARP1 is in the maintenance of genomic integrity (28), studies over the past decade have demonstrated its role in the regulation of gene expression (29–32). However, the underlying mechanism responsible for its functions in transcription regulation is not well defined, and its role in ESC proliferation and differentiation has not been explored.

Recent studies indicate that additional factors are involved in the regulation of target genes of Oct4 and Sox2 and that these factors function in a gene-specific manner. For example, Nakatake *et al.* (33) reported that Klf4 cooperates with Oct4 and Sox2 to activate *Lefty1* expression. However, its presence is not required for expression of *Oct4* and *UTF1* under the same conditions (33). Moreover, Esrrb was found to interact with Oct4, positively regulating *Nanog* expression (34). Recruitment of cofactors provides an important mechanism by which transcriptional factors may regulate expression of their target genes precisely and differentially in a tissue-specific and developmentally stage-specific manner. In the search for more such regulatory factors mediating Oct4 and Sox2 functions, the *FGF4* distal enhancer provides a good model. *FGF4* is essential for survival of the postimplantation embryo (35) and plays important roles at multiple stages of development (36). It is expressed in the ICM of the blastocyst *in vivo* and in ESCs as well as embryonal carcinoma (EC) cells *in vitro*. However, its transcription is silent in the adult and down-regulated when ESCs are induced to differentiate (37, 38). Uniquely, *FGF4* transcription is regulated by a powerful distal enhancer located in the 3'-untranslated region, containing the POU and the HMG cassettes (8). Previous studies have demonstrated that Oct4 and Sox2 bind both *in vitro* and *in vivo* to the POU motif and the HMG motif, respectively, to control the expression of *FGF4* (39, 40). However, it is unclear whether other factors, together with Oct4 and Sox2, are implicated in *FGF4* expression in undifferentiated ESCs and during their differentiation.

In this study, we identify PARP1 as a novel cofactor of Oct4 and Sox2 and as a regulator of *FGF4* expression. It binds to the *FGF4* enhancer together with Oct4 and Sox2 and is required for appropriate expression of *FGF4* during ESC differentiation. Our data show that PARP1 interacts with Sox2 directly and modifies Sox2 by poly(ADP-ribosylation), which may be a step required for dissociation of excessive Sox2 from the *FGF4* enhancer. When PARP1 activity is absent or inhibited, Sox2 poly(ADP-ribosylation) is reduced, which is associated with an elevated Sox2 protein level and increased binding of Sox2 to the *FGF4* enhancer. Moreover, specific knockdown of *Sox2* expression by RNA interference can rescue reduction in *FGF4* expression caused by inhibition of PARP activity. In addition, we find that *FGF4* can partially rescue *PAPRI* deficiency-associated phenotypes in differentiating ESCs. Therefore, the study estab-

lishes links among PARP activity, Sox2 poly(ADP-ribosylation), and *FGF4* expression, uncovering new mechanisms through which the Sox2 protein level and *FGF4* transcription are dynamically regulated.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Rabbit polyclonal antibodies against GST, Oct4, and Sox2 were raised and affinity-purified in our laboratory using bacterially expressed GST, GST-Oct4N (16), and GST-Sox2C (114-amino acid residues at the C terminus of the Sox2 protein) fusion proteins, respectively. Antibodies against the FLAG (Sigma), tubulin (Sigma), PARP1 (Santa Cruz Biotechnology), and PAR (Alexis Biochemical) were used for immunoblotting. Anti-human *FGF4*-neutralizing antibody (R & D Systems) was reconstituted in 1 \times phosphate-buffered saline and used for neutralization of *FGF4* activity secreted by ESCs. 3AB, *FGF4*, and NAD^+ were purchased from Sigma, and PJ34 was obtained from Calbiochem.

Plasmids and Primers—The cDNA sequences corresponding to the full-length proteins of PARP1, Oct4, and Sox2 were amplified by reverse transcriptase-PCR using the RNA of mouse ESCs as templates, and they were cloned into pET-30a(+) (Novagen) and pGEX-4T-1 (Amersham Biosciences) vectors for expression in bacteria or pPyCAGIP vector (a kind gift of I. Chambers and A. Smith) for expression in mammalian cells. Probes and primers used in this study are listed in [supplemental Table S1](#).

RNA Interference—To make small interfering RNA vectors for Sox2 (*Sox2* RNAi), two independent 19-bp sequences (5'-GGTTGATATCGTTGGTAAT-3' and 5'-CCCTGCAGTCAACTCCAT-3') within the coding region of the murine *Sox2* gene were selected and cloned into the pTER⁺ vectors (41). Small interfering RNA vector for EGFP (*EGFP* RNAi) was made by selecting a 19-bp sequence in the coding region of the *EGFP* gene (5'-GGCTACGTCCAGGAGCGCA-3').

Cell Culture and Transient Transfection—PARP1^{+/+} and PARP1^{-/-} ESCs were grown in media consisting of Dulbecco's modified Eagle's media (Invitrogen) supplemented with 15% (v/v) fetal bovine serum (Hyclone), 2 mM L-glutamine (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 1000 units/ml leukemia inhibitory factor (LIF, Chemicon), 100 units/ml penicillin (Invitrogen), 100 μ g/ml streptomycin (Invitrogen), and 0.1 mM β -mercaptoethanol (Invitrogen). ESCs were maintained on a feeder layer. To induce differentiation by retinoic acid (RA, Fisher), ESCs were cultured on gelatin-coated dishes without a feeder layer at a density of 5×10^5 cells per 35-mm dish for 24 h. Subsequently, RA at a concentration of 1 μ M was added to the culture medium containing LIF. For spontaneous differentiation at low cell density, ESCs were cultured in a gelatin-coated 6-well plate at a density of 3000 per well for 7 days.

P19 and F9 EC cells were maintained in the Dulbecco's modified Eagle's media/F-12 medium supplemented with 10% fetal bovine serum, 1.5% NaHCO_3 (Sigma), 100 units/ml penicillin, and 100 μ g/ml streptomycin. Both ESCs and EC cells were transiently transfected with LipofectamineTM 2000 (Invitrogen) according to manufacturer's instructions. HEK 293 cells were

cultured under standard conditions and transfected using the calcium phosphate method.

For *Sox2* RNAi experiments, P19 cells were transfected with *EGFP* RNAi or *Sox2* RNAi plasmids. Twelve hours after transfection, the cells were incubated in fresh medium and selected by Zeocin at 100 $\mu\text{g}/\text{ml}$ for 12 h. Twenty four hours later, the transfected cells were trypsinized and replated in 24-well plates. After an additional 24 h, 3AB at a concentration of 6 mM was added. The cells were collected 48 h later.

Nuclear Extract Preparation and Protein Purification by Affinity Chromatography—Preparation of nuclear extract (NE), oligonucleotide probes, and protein purification by affinity chromatography were accomplished as described previously (42) with minor changes (details are provided in the [supplementary material](#)).

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays using formaldehyde cross-linking and specific antibodies were performed as described previously (17).

Luciferase Reporter Assays—ESCs were cultured in a gelatin-coated 24-well plate at a density of 1×10^5 per well. After 24 h, cells were cotransfected with 200 ng of the plasmid DNA construct (*FGF4*/pTAL) containing the enhancer fragment of the mouse *FGF4* gene in the pTAL reporter gene or empty vector pTAL and 20 ng of pRL-TK (Promega) as an internal control to normalize the transfection efficiency. Forty eight hours after transfection, samples were collected and analyzed with the dual luciferase reporter assay system (Promega).

Coimmunoprecipitation (CoIP)—The NE of cells was prepared in CoIP buffer (10 mM Hepes, pH 7.6, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride) and incubated with a specific antibody overnight at 4 °C, followed by incubation with protein A-Sepharose beads for 2 h. The samples were analyzed using Western blotting.

GST Pulldown—GST and His fusion proteins were expressed and purified according to the manufacturer's instructions from Amersham Biosciences and Novagen, respectively. GST pull-down experiments were performed as described previously (16).

Poly(ADP-ribosyl)ation Assay in Vivo and in Vitro—For the assay *in vivo*, cells were harvested and boiled in the lysis buffer (100 mM Tris-HCl, pH 7.5, 1% SDS) for 10 min to disassociate protein-protein interaction. The lysate was then diluted by 10-fold in the CoIP buffer and sonicated briefly, followed by centrifugation at $14,000 \times g$ for 10 min. The supernatant was immunoprecipitated with specific antibodies overnight at 4 °C, followed by incubation with protein A-Sepharose beads for 2 h. *In vitro* poly(ADP-ribosyl)ation assays were performed as described previously (24, 27). Briefly, purified GST fusion proteins (1 μg) were incubated in the poly(ADP-ribosyl)ation reaction buffer (100 mM Tris-HCl, pH 7.5, 10 mM MgCl_2 , 1 mM dithiothreitol) with purified his-PARP1 fusion proteins (100 ng), 0.5 mM NAD^+ , and 1 μg of sonicated salmon sperm DNA for 40 min at 37 °C. Poly(ADP-ribosyl)ated proteins were detected by Western blot analysis using anti-PAR antibody.

RNA Extraction and Quantitative Real Time PCR (qPCR)—RNAs were extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNAs were synthe-

sized with the ReverTra Ace[®] reverse transcriptase (Toyobo, Osaka, Japan). qPCR was performed using SYBR Green Master Mix on an ABI PRISM 7900 machine as described previously (17). The *Ct* value of each gene was normalized against that of the housekeeping gene *GAPDH*.

Statistical Analysis—Data were analyzed using Student's *t* test. Data are shown as the mean \pm S.D. of at least three experiments. We considered the difference between comparisons to be significant when $p < 0.05$ for all the statistical analysis. All experiments were performed at least three times.

RESULTS

PARP1 Binds Specifically to the FGF4 Enhancer Both in Vitro and in Vivo—To identify proteins associated with control of *FGF4* expression, in addition to its known regulators, Oct4 and Sox2, we conducted affinity chromatography with the NE of F9 mouse EC cells, using synthetic biotinylated oligonucleotides containing the 3'-untranslated region of *FGF4* (biotin-*FGF4* probe). F9 cells were chosen because of their relatively easy and low cost culture as compared with ESCs. The isolated proteins were visualized by Coomassie blue staining (Fig. 1A). Multiple protein bands were seen in the NE column with use of the biotin-*FGF4* probe (Fig. 1A, lane 1). To exclude nonspecific binding proteins, only the protein bands, which vanished when a high concentration of nonbiotinylated *FGF4* probe (non-biotin-*FGF4* probe) was present (compare lanes 1 and 2), were excised and analyzed by mass spectrometry. Among these proteins, the peptides from the 45-kDa protein band matched with Oct4, supporting the validity of the approach used. Interestingly, the peptides derived from the 100-kDa protein band corresponded to PARP1. The implication of PARP1 in transcriptional regulation prompted us to investigate further. We repeated the DNA-protein binding assay described in Fig. 1A and examined the probe-binding proteins by Western blot analysis. Using the probe (2 \times miUR-NF- κ B) known not to bind PARP (43) as a negative control (NC probe), PARP1 was found to bind the *FGF4* probe, in addition to Oct4 and Sox2 (Fig. 1, B and C). Moreover, the binding intensity was dramatically reduced for all three proteins when the non-biotin-*FGF4* probe was included in the reaction mixture (Fig. 1C, lane 3), indicating the specificity of their binding to the *FGF4* enhancer. Next, to examine whether PARP1 was associated with the *FGF4* enhancer under physiological conditions, ChIP assays were conducted. We found that PARP1 was specifically recruited to the *FGF4* enhancer but not to the *Sox2* enhancer or the *FGF4* exon, whereas, as reported previously (12, 39, 40), Oct4 and Sox2 bound to the enhancers of both *FGF4* and *Sox2* (Fig. 1D). In addition, we found that purified His-PARP1 was capable of binding the biotin-*FGF4* probe directly *in vitro* (Fig. 1E). Thus, our data demonstrate for the first time that PARP1 is directly associated with the *FGF4* enhancer, together with Oct4 and Sox2, both *in vitro* and *in vivo*.

PARP1 Is Required for FGF4 Expression and Recombinant FGF4 Can Functionally Rescue Phenotypes of PARP1-deficient Cells—To learn whether PARP1 recruited to the *FGF4* enhancer has any function, we compared mRNA levels of *FGF4* in *PARP1*^{+/+} and *PARP1*^{-/-} ESCs cultured with or without feeder cells for 24 h. As shown in Fig. 2A, the level of *FGF4*

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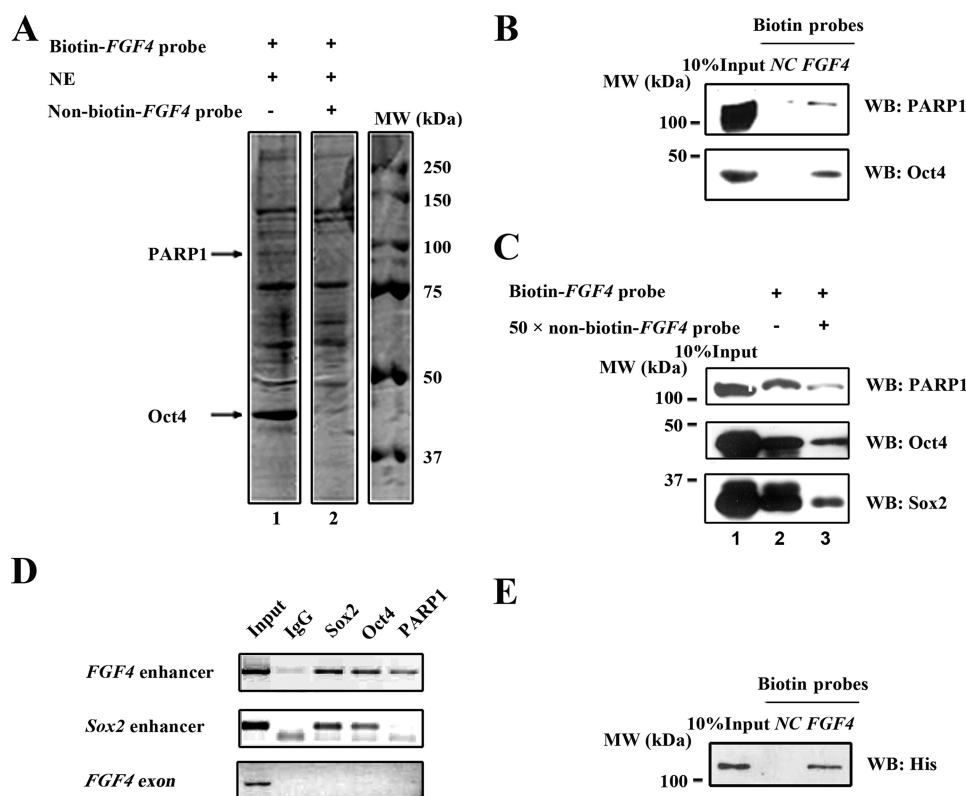


FIGURE 1. PARP1 binds specifically to the FGF4 enhancer in vitro and in vivo. *A*, purification of FGF4 enhancer-binding proteins by affinity chromatography using biotin-labeled oligonucleotide of the FGF4 enhancer containing Oct4 and Sox2 binding sequence (biotin-FGF4 probe). Purified proteins were subjected to SDS-PAGE and visualized with Coomassie Blue staining. *B*, Western blot (WB) analysis of affinity-purified DNA-binding proteins. NC is a negative control probe. *C*, PARP1 bound specifically to the FGF4 enhancer. F9 nuclear proteins were subjected to SDS-PAGE with the biotin-FGF4 probe in the presence or absence of 50-fold excess unlabeled FGF4 probe (non-biotin-FGF4 probe). *D*, ChIP assay was performed in F9 EC cells with antibodies indicated. An antibody against IgG was used as a negative control. *E*, PARP1 bound to the FGF4 enhancer directly. Bacterially expressed fusion protein of his-PARP1 was purified and subjected to SDS-PAGE with the indicated biotin-labeled probes.

transcripts was significantly lower in *PARP1* knock-out cells than in wild type cells under both conditions. Furthermore, the mRNA level of *FGF4* decreased in both wild type and *PARP1*-deficient ESCs during ESC differentiation induced by RA (Fig. 2*B*), consistent with a previous report (38). Significantly, *FGF4* expression was lower in *PARP1*^{-/-} cells than in *PARP1*^{+/+} cells at all time points tested (Fig. 2*B*). In contrast, there was no detectable difference in the mRNA level of *Sox2* between wild type and *PARP1* knock-out cells (Fig. 2, *A* and *B*). The results indicate that PARP1 may play a positive role in maintaining *FGF4* expression in ESCs and during their differentiation. To verify that the effect of PARP1 on *FGF4* expression is mediated through the *FGF4* enhancer, we transfected the *FGF4*/pTAL luciferase reporter containing the *FGF4* enhancer sequence into *PARP1*^{+/+} and *PARP1*^{-/-} ESCs cultured without feeder cells, and we found that the luciferase activity was significantly lower in *PARP1* knock-out cells than in wild type cells (Fig. 2*C*). This result indicates that PARP1 may regulate *FGF4* expression, at least partially, through the *FGF4* enhancer.

We further reasoned that the phenotypes of *PARP1*-deficient cells should resemble those of *FGF4*^{-/-} ESCs to a certain extent if PARP1 is an important regulator of *FGF4* expression. It is known that undifferentiated *FGF4*^{-/-} ESCs proliferate normally *in vitro*. However, the growth and/or survival of RA-

induced differentiated cells is severely compromised in the absence of *FGF4* (44). Similarly, we found that *PARP1*^{-/-} ESCs grew normally as wild type ESCs did when they were cultured under undifferentiated conditions (Fig. 2*D*). We next induced differentiation of *PARP1*^{-/-} ESCs by two culture conditions as follows: spontaneous differentiation at low cell density and RA-induced differentiation, respectively. In both cases, the cells were cultured in the presence of LIF but in the absence of a feeder layer. At low cell density, the majority of colonies formed by wild type ESCs contained differentiated alkaline phosphatase-negative cells surrounding central undifferentiated alkaline phosphatase-positive cells (Fig. 2*E*, upper left panel). A minority of colonies was small and did not have differentiated cells peripherally (Fig. 2*E*, upper right panel). Addition of the anti-human FGF4-neutralizing antibody significantly increased the number of small colonies in wild type ESCs (Fig. 2*E*, bottom left panel). In contrast, *PARP1*^{-/-} ESCs produced more small colonies without differentiated cells than wild type ESCs did. Importantly, addition of recombi-

nant FGF4 significantly decreased the number of small colonies in *PARP1*^{-/-} ESCs in a dosage-dependent manner (Fig. 2*E*, bottom right panel). Next, more dead floating cells, fewer surviving cells, and slower cell growth rates were observed in differentiating *PARP1*^{-/-} cells than in differentiating *PARP1*^{+/+} cells, when RA was used to induce ESCs to differentiate. However, the differences in cell growth and/or survival between wild type and *PARP1*^{-/-} ESCs after RA treatment were diminished when FGF4 was included in the culture media (Fig. 2*F* and data not shown). Intriguingly, the above described phenotypes seen in both low density and RA-treated culture of *PARP1*^{-/-} ESCs were reported, to a great extent, for *FGF4*^{-/-} ESCs (44). Therefore, it is reasonable to propose that a low level of *FGF4* expression is at least partially responsible for the phenotypes observed in *PARP1* deficiency and that PARP1 plays a positive role in *FGF4* expression during ESC differentiation.

PARP1 Physically Interacts with Sox2—The next question involves the manner in which PARP1 positively participates in the regulation of *FGF4* expression. Because it has been well documented that Oct4 and Sox2 are key factors binding to the *FGF4* enhancer and modulating its expression (39, 45), we suspected that PARP1 might associate with Oct4, Sox2, or both. To test this hypothesis, CoIP experiments were conducted in HEK 293 cells expressing FLAG-PARP1, together with HA-Sox2 or

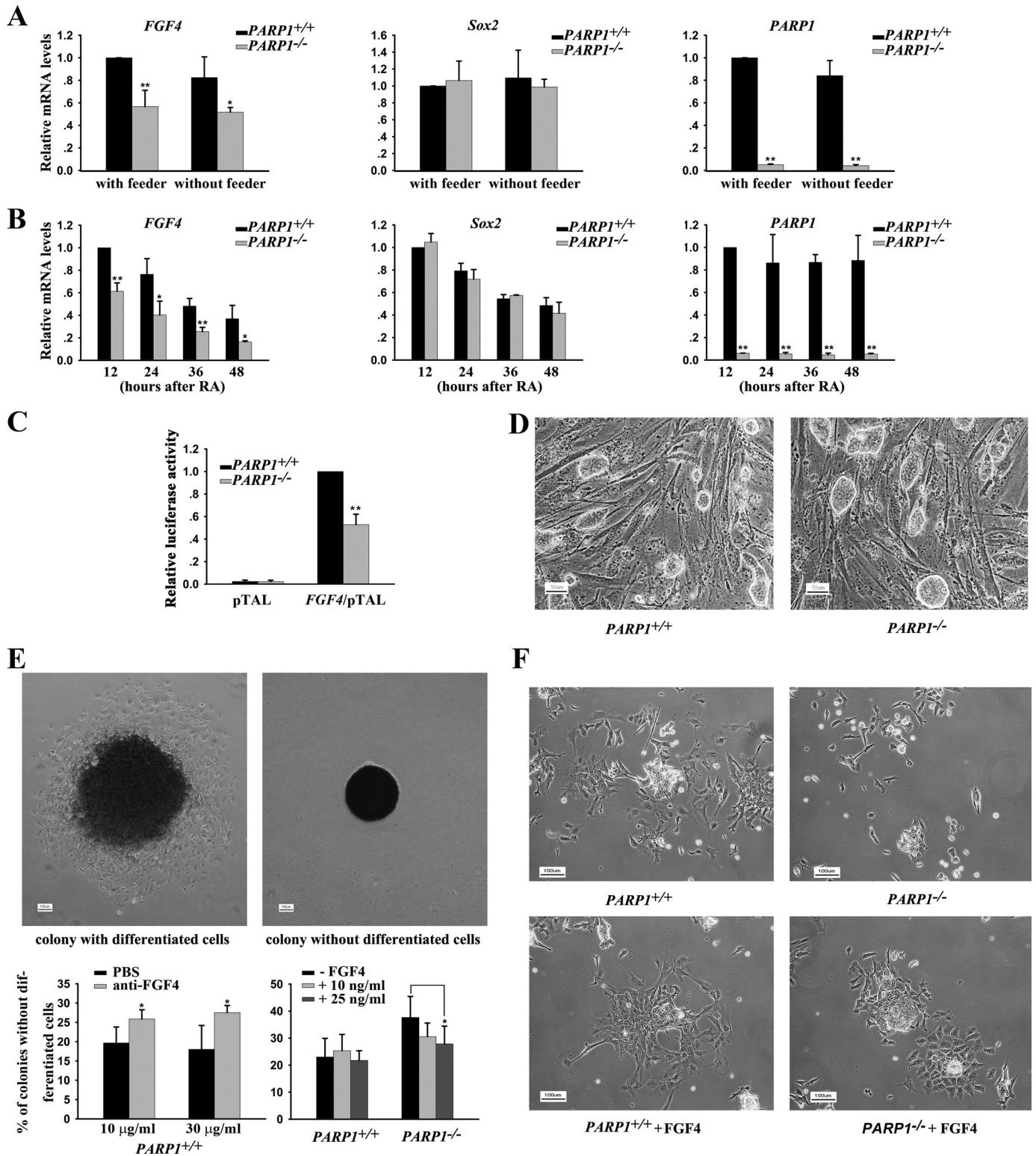


FIGURE 2. **PARP1 is implicated in FGF4 expression.** *A* and *B*, qPCR analysis of gene expression in undifferentiated and differentiating *PARP1*^{+/+} and *PARP1*^{-/-} ESCs cultured without a feeder layer and treated with 1 μM RA. *, *p* < 0.05; **, *p* < 0.01, *n* = 3. *C*, activity of FGF4/pTAL luciferase activity in *PARP1*^{+/+} and *PARP1*^{-/-} ESCs cultured without a feeder layer was compared. **, *p* < 0.01, *n* = 3. *D*, morphology of *PARP1*^{+/+} and *PARP1*^{-/-} ESCs. *PARP1*^{+/+} and *PARP1*^{-/-} ESCs were cultured for several passages on feeder layers. The scale bar is 50 μm. *E*, ESCs were cultured at a low cell density for 7 days without a feeder layer on gelatin-coated surfaces in the presence or absence of an anti-human FGF4-neutralizing antibody or FGF4. Culture media of cells were refed with fresh media every day. Prior to photography, the cells were stained for alkaline phosphatase expression, and colonies were subclassed into colonies with or without differentiated cells on their periphery. The scale bar is 100 μm. *, *p* < 0.05, *n* = 3. *F*, morphology of differentiated cells derived from *PARP1*^{+/+} and *PARP1*^{-/-} ESCs. ESCs were cultured on gelatin-coated surfaces in the medium containing LIF and induced to differentiate with RA (1 μM) for 3 days in the presence or absence of FGF4. The scale bar is 100 μm. The representative results of three independent experiments are shown.

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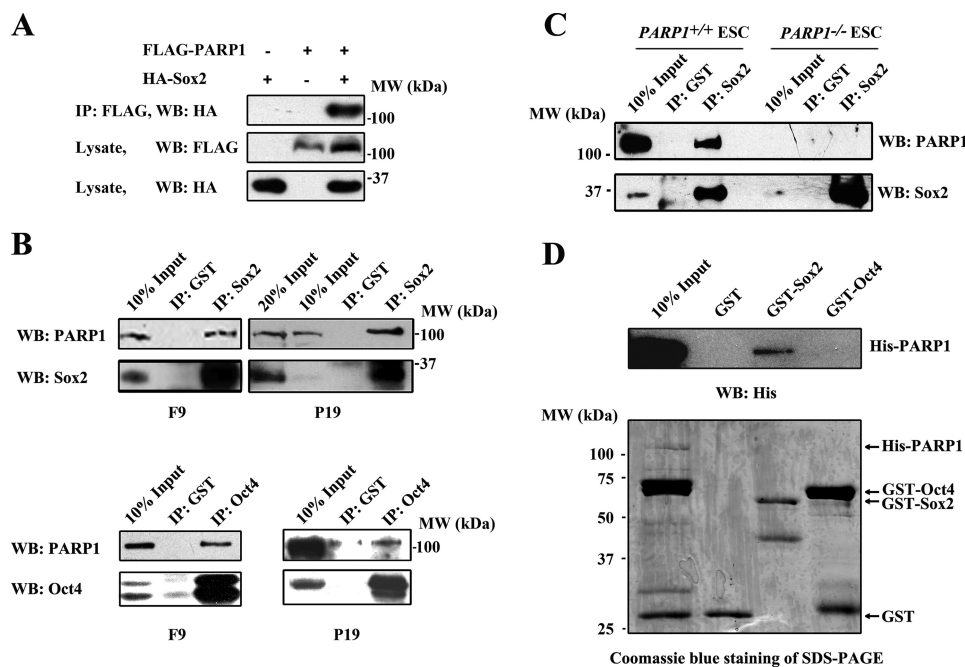


FIGURE 3. Association of PARP1 with Sox2 or Oct4. *A*, CoIP of FLAG-PARP1 and HA-Sox2 in HEK 293 cells, which were transiently cotransfected with FLAG-PARP1 and HA-Sox2 or vector. *B*, association of endogenous PARP1 with Sox2 or Oct4 in F9 and P19 cells. The NE of F9 or P19 cells was subjected to CoIP with anti-Sox2 antibody or anti-Oct4 antibody. Anti-GST antibody was used as a negative control. *C*, association of endogenous PARP1 with Sox2 in *PARP1*^{+/+} and *PARP1*^{-/-} ESCs. *D*, Sox2 but not Oct4 interacts directly with PARP1 *in vitro*. A GST-pull-down assay was performed with purified GST-Sox2, GST-Oct4, and His-PARP1 fusion proteins. The representative results of three independent experiments are shown. *IP*, immunoprecipitation; *WB*, Western blot.

HA-Oct4. Western blot analysis indicated that FLAG-PARP1 associated with HA-Sox2 (Fig. 3A). However, we were not able to detect an interaction between HA-Oct4 and FLAG-PARP1 (data not shown). Surprisingly, we detected the interaction not only between endogenous PARP1 and Sox2, but also between PARP1 and Oct4, when CoIP experiments were performed in F9 or P19 EC cells (Fig. 3B). We also conducted such experiments in wild type ESCs and found that Sox2 interacted with PARP1 as it did in EC cells. As expected, such interaction was not observed in *PARP1* knock-out ESCs, arguing for a specific association between endogenous Sox2 and PARP1 *in vivo* (Fig. 3C). The reason for our inability to detect an association between PARP1 and Oct4 in HEK 293 cells could be explained by the possibility that PARP1 may interact with Oct4 indirectly in F9/P19 EC cells and that the factor(s) mediating PARP1 and Oct4 association in EC cells are not present in HEK 293 cells. We therefore further examined whether PARP1 directly interacts with Sox2 or Oct4 using an *in vitro* GST pull-down assay. The results show that His-PARP1 could be pulled down by GST-Sox2, but not by GST-Oct4 or GST alone, indicating that Sox2, but not Oct4, directly interacts with PARP1 (Fig. 3D). Thus, it appears that PARP1 controls *FGF4* expression through its direct association with Sox2 and post-translational modification of Sox2.

Poly(ADP-ribosylation) of Sox2 by PARP1—Before examining whether PARP1 could modify Sox2 post-translationally, we assessed the PARP enzymatic activity in ESCs and differentiating ESCs cultured without feeder cells for 24 h and further induced by RA treatment. Assessment was accomplished using Western blot analysis of the total PAR level. As shown in Fig.

4A, PARP enzymatic activity existed in *PARP1*^{+/+} cells but was not detectable in *PARP1*^{-/-} cells. There was a drastic drop of the activity at the onset of ESC differentiation induced by withdrawal of feeder cells. However, the activity recovered after addition of RA and relatively high levels of the PARP activity were detected in *PARP1*^{+/+} cells treated with RA for 24–48 h (Fig. 4A). We termed the culture condition the “differentiation condition” (feeder-free and RA treatment for 24–48 h). Subsequently, we found that Sox2 was modified by PAR in differentiating wild type ESCs (Fig. 4B, lane 2). The modification was substantially weak in *PARP1* knock-out cells (Fig. 4B, lane 4). The residual modification could be catalyzed by other members of the PARP family (46). As in differentiating ESCs, PAR-modified Sox2, but not PAR-modified Oct4, existed in F9 and P19 EC cells (Fig. 4C, left). Both cell lines are considered to be in a differentiated state as compared with

ESCs, with P19 cells being further differentiated than F9 cells, and present a good *in vitro* model of an early differentiation stage of pluripotent embryonic cells (47–49). Remarkably, a stronger signal for PAR-modified Sox2 was detected in P19 cells (Fig. 4C, left) than in F9 cells, consistent with our finding that a higher PARP activity is present in P19 cells as compared with that in F9 cells (Fig. 4D, left). Furthermore, we tested whether the PARP inhibitors (3AB and PJ34) could block the Sox2 and PARP1 PAR modification detected in P19 EC cells. As expected, inhibition of PARP enzymatic activity by PJ34 (Fig. 4, C, right, and D, right) or 3AB (data not shown) decreased the poly(ADP-ribosylation) level of Sox2 and PARP1 evidently. Finally, an *in vitro* poly(ADP-ribosylation) assay with purified GST fusion proteins and His-PARP1 provided biochemical evidence that Sox2 is a direct substrate of PARP1 (Fig. 4E). Collectively, our studies clearly demonstrate that PARP1 can efficiently modify Sox2 both *in vivo* and *in vitro*.

PARP1 Modulates Sox2 Protein Levels to Control FGF4 Expression—To investigate the functional consequences of Sox2 modification by PAR, we compared the protein levels of Oct4 and Sox2 in *PARP1*^{+/+} and *PARP1*^{-/-} ESCs under both undifferentiated and differentiating conditions. Western blot analysis showed that the Sox2 protein level in differentiating *PARP1*^{-/-} cells was markedly higher than that in differentiating *PARP1*^{+/+} cells at each time point after RA treatment. In contrast, a similar Oct4 protein level was found between these two cell types (Fig. 5A). However, there was no distinctive difference in the Sox2 protein level between the two types of undifferentiated ESCs. The obviously higher steady-state level of Sox2 proteins in differentiating *PARP1*^{-/-} cells implies an

Regulation of Sox2 Proteins and FGF4 Levels by PARP1

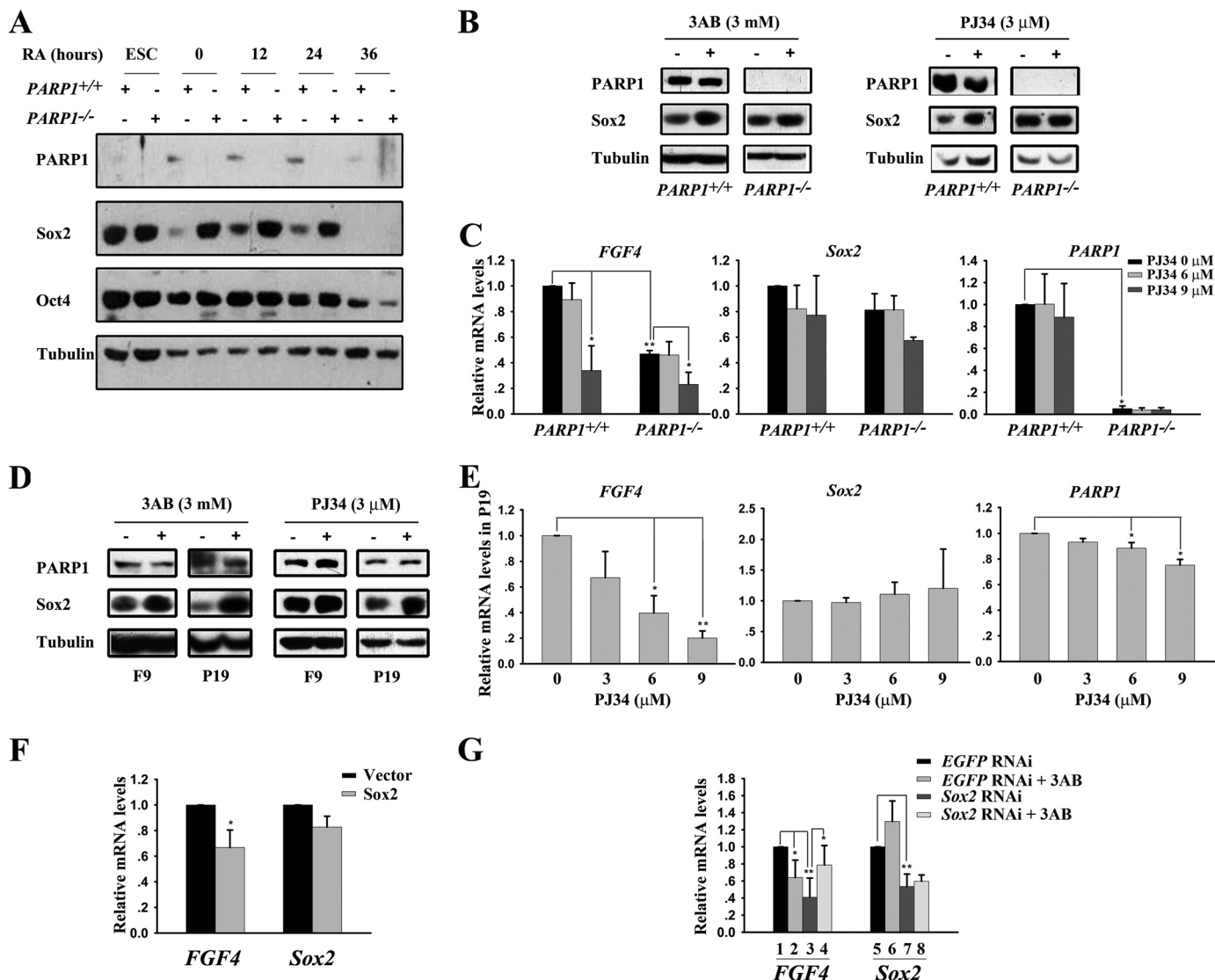


FIGURE 5. PARP1 modulates the Sox2 protein level to control FGF4 expression. A, Sox2 protein level is markedly elevated in differentiating PARP1^{-/-} ESCs. ESCs were cultured under the differentiation condition for different lengths of time, as indicated, and the levels of various proteins were evaluated by Western blot analysis. B, ESCs were cultured under the differentiation condition and treated with the PARP inhibitor 3AB or PJ34 for 48 h. The Sox2 protein level was determined by Western blot analysis. C, ESCs were cultured under the differentiation condition and treated with PJ34 at different concentrations for 48 h. Gene expression levels were determined by qPCR. *, $p < 0.05$; **, $p < 0.01$, $n = 3$. D, inhibition of PARP enzymatic activity by 3AB or PJ34 for 48 h increases the Sox2 protein level in F9 and P19 cells as determined by Western blot analysis. E, qPCR analysis of gene expression in P19 EC cells treated with PJ34 at different concentrations for 48 h. *, $p < 0.05$; **, $p < 0.01$, $n = 3$. F, expression of FGF4 and Sox2 in P19 cells is inhibited by transient overexpression of Sox2. Transcript levels of FGF4 and Sox2 were measured using qPCR. *, $p < 0.05$, $n = 3$. G, Sox2 RNAi blocks the inhibitory effect of the PARP inhibitor on FGF4 expression in P19 cells. P19 cells expressing EGFP or Sox2 RNAi were treated with 3AB at 6 mM for 48 h. Data were obtained from qPCR analysis. *, $p < 0.05$; **, $p < 0.01$, $n = 4$.

Sox2 and PARP1 expression in P19 cells, suggesting a specific role for PARP activity in regulation of FGF4 transcription. Based upon the above results, we came to the conclusion that PARP1 is implicated in the maintenance of an appropriate level of Sox2 proteins and FGF4 expression in an enzymatic activity-dependent manner.

To provide a clear answer to the question of whether the increased Sox2 protein level is responsible for the reduction in FGF4 expression, we transiently transfected P19 EC cells with a Sox2 expressing construct and examined transcript levels of FGF4 and Sox2 (Fig. 5F). P19 cells were used here because they have a constitutively high PARP activity (Fig. 4D). As expected and consistent with previous reports (11, 14), overexpression of Sox2 reduced mRNA levels of FGF4 and also of Sox2 (Fig. 5F).

Overexpression of Sox2 in P19 cells was verified by Western blot analysis (data not shown). Thus, our results further confirmed the inhibitory effect of excessive Sox2 on expression of FGF4 and of itself. We then examined whether knockdown of Sox2 could rescue the reduced expression of FGF4 observed in PARP activity-inhibited P19 cells. As shown in Fig. 5G, 3AB treatment (column 2) significantly reduced FGF4 expression. Notably, this inhibitory effect of 3AB on FGF4 expression was considerably abrogated when Sox2 expression was knocked down by Sox2-specific RNAi (Fig. 5G, column 4), supporting the notion that the elevated Sox2 protein level is a major cause for reduced FGF4 expression in the case of low or absent PARP activity. Furthermore, an evidently low level of FGF4 mRNA observed in cells expressing Sox2 RNAi alone (Fig. 5G, column

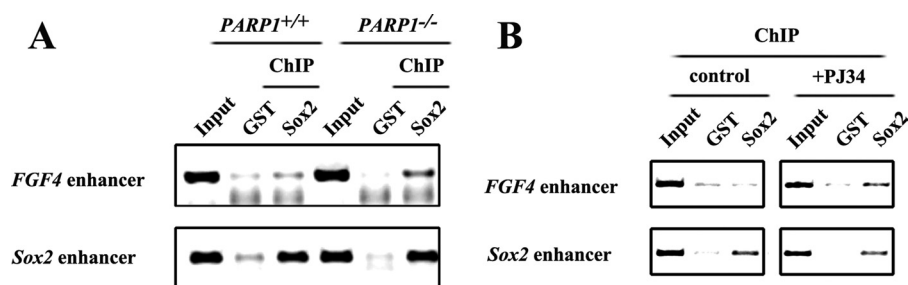


FIGURE 6. **A putative mechanism for PARP1 in control of FGF4 expression.** *A*, ChIP analysis of association of Sox2 with the *FGF4* or *Sox2* enhancer in *PARP1*^{+/+} and *PARP1*^{-/-} ESCs cultured under the differentiation condition. *B*, association of Sox2 with the *FGF4* enhancer in P19 cells was determined by ChIP assay in the presence or absence of PJ34 (3 μ M). The representative results of four independent experiments are shown.

3) highlights the fact that an appropriate expression level of Sox2 is essential for *FGF4* expression. The efficiency of *Sox2* RNAi was confirmed by a simultaneous measurement of the *Sox2* mRNA level (Fig. 5G, columns 5–8). These findings clearly indicate that the inhibitory effect of 3AB on *FGF4* expression is secondary to an elevated Sox2 protein level and that an abnormal level of Sox2 would disrupt normal expression of *FGF4*.

Putative Mechanism for PARP1 in Control of FGF4 Expression—As a transcription factor, Sox2 exerts its function primarily via binding to regulatory sequences of its target genes. Therefore, to understand how PARP1-mediated Sox2 post-translational modification is involved in regulation of *FGF4* expression, we compared the Sox2 association with the *FGF4* enhancer in differentiating *PARP1*^{+/+} and *PARP1*^{-/-} cells. Data from ChIP assays revealed that more *FGF4* enhancers were associated with Sox2 in differentiating *PARP1*^{-/-} cells than in differentiating *PARP1*^{+/+} cells (Fig. 6A, top row), whereas there was little difference between these two cell types in Sox2 association with its own enhancer (Fig. 6A, bottom row). Furthermore, Sox2 recruitment to the *FGF4* enhancer was detectable when P19 cells were treated with PJ34 for 48 h but not in untreated control cells (Fig. 6B, top row), although the recruitment to its own enhancer was detected in both control and PJ34-treated P19 cells (Fig. 6B, bottom row). A similar result was obtained when P19 cells were treated with 3AB (data not shown). These findings indicate that more *FGF4* enhancers, but not *Sox2* enhancers, were associated with Sox2 proteins when Sox2 proteins are not modified by PAR, leading to inhibition of *FGF4* expression. In other words, PARP1 may modify Sox2 and promote its dissociation from *FGF4* enhancers, relieving its inhibition of *FGF4* expression in normally differentiating cells.

DISCUSSION

Despite intensive studies, little is known about how other transcriptional coregulators act in concert with Oct4 and Sox2 to precisely control *FGF4* expression during development. Here we bring a new member, PARP1, into the *FGF4* expression regulation network. Our study demonstrates that PARP1 binds to *FGF4* enhancer directly and maintains a normal level of *FGF4* through poly(ADP-ribosyl)ating Sox2. Moreover, we find that differentiating *PARP1*^{-/-} ESCs display some phenotypes, similar to those seen in *FGF4*-deficient ESCs (44), such as fewer surviving cells (Fig. 2, E and F). Functional rescue of the phenotypes by recombinant *FGF4* further supports the functional link between PARP1 and *FGF4* expression (Fig. 2, E and F). Never-

theless, the phenotypes caused by their deletion in animal models are quite different (35, 50). This could be explained by functional redundancy among members of the PARP family during development. The fact that single null mutant *PARP1* or *PARP2* mice are viable but double null mutant *PARP1* and *PARP2* embryos die early in development at the onset of gastrulation (28) clearly demonstrates the essential role of poly(ADP-ribosyl)ation and redun-

dant functions of PARP1 and PARP2 during early embryonic development. Our observation that a small inhibitory effect of PARP inhibitors on *FGF4* expression (Fig. 5C and supplemental Fig. 1A) and a weak signal of Sox2 poly(ADP-ribosyl)ation (Fig. 4B) exist in *PARP1* knock-out cells provides further evidence to support the existence of functional redundancy among members of this family, whereas PARP1 is the major enzyme responsible for poly(ADP-ribosyl)ation of cellular proteins (46). In addition, our results reveal that PARP1 activity is dynamically regulated by ESC differentiation processes, instead of DNA breaks. This is in agreement with previous reports showing that PARP1 can be activated by signals such as increased activity of calmodulin kinase II and ERK2 independently of DNA damage (51, 52). It is interesting to know why PARP activities declined initially after withdrawal of feeder cells but is reactivated during further differentiation induced by RA treatment. Taken together, the establishment of physical and functional links among ESC differentiation, PARP1 activity, and *FGF4* expression provides new evidence for an important role of PARP1 in regulating gene expression and early development.

PARP1 regulates gene expression through diverse mechanisms that are likely to vary in an activator-specific and gene-specific manner (53). In some cases, PARP1 enzymatic activity is not required for its coregulatory activity, e.g. with NF- κ B (30, 54), whereas in others it is required, e.g. with HES1 and NFAT (51, 55). In many of the latter cases, DNA-binding factors or other components of the coregulatory complex are targets for PARP1-dependent poly(ADP-ribosyl)ation (51, 55, 56). For example, PARP1 was reported to poly(ADP-ribosyl)ate several components of a TLA (transducin-like enhancer of split) corepressor complex, resulting in dismissal of the complex from the *MASH1* promoter and neuronal differentiation (51). In this study, we found that PARP1 is recruited to the *FGF4* enhancer together with Oct4 and Sox2, and poly(ADP-ribosyl)ates Sox2 directly. It is possible that PARP1-mediated addition of an anionic polymer on the Sox2 protein causes dismissal of Sox2 from the *FGF4* enhancer, because of repulsion among anionic molecules as proposed previously by other researchers (46, 51, 57). Furthermore, we found that *PARP1* deficiency or inhibition of the PARP activity specifically affects *FGF4* but little, if any, Sox2 transcription (Fig. 2, A and B, and Fig. 5, C and E). It is probably explained by our observations that binding of Sox2 to its own enhancer was not affected by the absent or relatively low PARP1 activity (Fig. 6, A and B) and that PARP1 was not recruited to the Sox2 enhancer (Fig. 1D). However, expression

of Sox2 itself and its other target genes, to which PARP1 is not recruited, could be reduced when the Sox2 protein level is dramatically elevated, such as in the case of overexpression of Sox2 (Fig. 5F) (11). On the other hand, Sox2 is also essential for expression of its target genes (33, 58, 59) (Fig. 5G). Thus, a dynamic and fine regulation is required for normal functions of Sox2. In addition, the mechanism by which PARP1 regulates FGF4 expression in undifferentiated ESCs may be different from that in differentiating ESCs as PARP1 deficiency did not affect the Sox2 protein level (Fig. 5A) but reduced FGF4 expression in undifferentiated ESCs (Fig. 2A). The manner by which PARP1 regulates FGF4 expression in undifferentiated ESCs needs further investigation.

In summary, we have identified PARP1 as a coregulator of Oct4 and Sox2 in FGF4 expression and demonstrated that poly-(ADP-ribosyl)ation of Sox2 plays an important and direct role in maintaining an appropriate level of Sox2 proteins as well as FGF4 expression during differentiation of ESCs. Our work provides the first evidence for the importance of post-translational modifications in control of Sox2 protein levels and opens up a new way to address the critical issue of how the Sox2 expression level is precisely and dynamically controlled during development. Nevertheless, it remains unanswered as to how the accumulated Sox2 at the FGF4 enhancer inhibits FGF4 expression. It is possible that excessive Sox2 disrupts the equilibrium among factors functioning in FGF4 expression, such as enhanced recruitment of transcription repressors. Identification of other proteins potentially implicated in FGF4 expression will help us to understand why a precise level of Sox2 protein is critical for its function.

Acknowledgments—We thank Dr. Zhaoqi Wang for providing PARP1^{+/+} and PARP1^{-/-} ESCs and Dr. Naihe Jing for providing P19 EC cells. We also thank Chunliang Li and Junjie Gu for preparing mouse embryonic fibroblast feeder cells.

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