Cited2 Gene Controls Pluripotency and Cardiomyocyte Differentiation of Murine Embryonic Stem Cells through Oct4 Gene*

Received for publication, May 3, 2012, and in revised form, June 25, 2012. Published, JBC Papers in Press, July 3, 2012, DOI 10.1074/jbc.M112.378034

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**This work was supported, in whole or in part, by National Institutes of Health Grants R01HL091896 (to Y.-C. Y.) and R01HL096597 (to D. R.-B.). This work was also supported by National Health and Medical Research Council Senior Research Fellowship 514900 (to S. L. D.).

†‡PK containing supplemental Figs. 51–57.

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Embryonic stem cell pluripotency circuit is controlled by the fine-tuning of transcription factors, interaction of transcription cofactors with transcription factors, and epigenetic modifications (1–4). Oct4, Klf4, Sox2, and c-Myc are transcription factors crucial for ESC stemness and reprogramming of somatic cells to induced pluripotent stem cells (5–7). Manipulation of the Oct4 gene dosage by either ectopic or knockdown expression results in attenuated ESC pluripotency (8). Recently, it was demonstrated that Oct4 promotes mesendodermal differentiation at the early stage of ESC differentiation (48 h post-removal of leukemia inhibitory factor), emphasizing that Oct4 is a critical determinant for the ESC lineage specification (9).

ESC cell fate decision is also determined by interaction of transcription factors with transcription cofactors (2). One such cofactor, p300, has been mapped in the circuit of ESC stemness through its co-occupancy with OCT4 on target genes by genome-wide chromatin immunoprecipitation sequencing (2). p300 plays crucial roles in ESC differentiation by epigenetic modifications of histones on the Nanog promoter (10). The CBP/p300-interacting transactivator with ED tail-rich (CITED) family has three members in mammalian cells (Cited1, Cited2, and Cited4), and all are functionally active in ESCs (11, 12). Specifically, overexpression of Cited1 induces ESC differentiation in mouse ESCs (mESCs), and knockdown of CITED4 induces human ESC differentiation (11, 12). Cited2, a founding member of the CITED family, was identified by a global gain-of-function approach as one of the genes whose expression can maintain mESCs under an undifferentiated state even in the absence of leukemia inhibitory factor (LIF) (11). In another study, Cited2 mRNA was highly expressed in the Oct4-GFPlow population when mESCs harboring the Oct4-GFP reporter were induced to differentiate (13). Recently, Cited2 was identified as one of the direct target genes of a FoxP1 isoform encoded by an alternatively spliced FoxP1 RNA, which mediates mESC
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pluripotency (14). Cited2 was also found to be up-regulated by the knockdown of crucial mESC stemness-associated genes, BAF250a and Geminin (15, 16). These independent studies strongly indicate that Cited2 is important in mESC identity; however, the mechanisms involved remain elusive.

Cited2 total knock-out mouse models have been generated independently by us and other investigators (17–21). Knock-out of Cited2 is embryonic lethal, and the embryos display multiple developmental defects, including heart, lung, liver, and eyes (17–19, 21–24). Mechanistically, HIF-1, TFAP2, and Nodal-Pitx2 pathways are in part responsible for heart defects in Cited2−/− mice (17, 19, 20, 25). Conditional knock-out of Cited2 in heart and hematopoietic tissues also displayed multiple phenotypes (18, 26–29). In particular, deletion of Cited2 at the epiblast stage using Sox2-Cre phenocopies cardiac defects in Cited2 total knock-out mouse model (28). Depletion of Cited2 in the hematopoietic tissues via Mx1-Cre affects hematopoietic stem cell quiescence and apoptosis (26, 27).

In this study, we generated Cited2 total knock-out ESCs and investigated the roles of Cited2 in ESC pluripotency and differentiation. We demonstrate for the first time that Cited2 knock-out ESCs remain undifferentiated in the presence of LIF. Depletion of Cited2 also causes delayed silenced of Oct4 in part through the impaired recruitment of CITED2 to the Oct4 promoter, which results in defective cardiomyocyte, hematopoietic, and neuronal differentiation.

EXPERIMENTAL PROCEDURES

Embryonic Stem Cell Maintenance and Induced Differentiation—Embryonic stem cell clone ES II20 with one allele of Cited2 flanked by loxP sites (W9.5 background) (30) was maintained on SNL 7c/7 feeder cells cultured in high glucose/DMEM supplemented with 15% FBS (Gemini), 100 µg/ml captoethanol, and 1,000 units/ml ESGRO LIF (Millipore). To generate gelatin-adapted ESCs, cells were weaned off feeder cells by serial passages on 6-well plates coated with 1% gelatin (Sigma) as described elsewhere (31–34).

To induce ESC differentiation to cardiomyocytes, we used a hanging-drop method with minor modifications (35). Briefly, 400 gelatin-adapted cells in 20 µl of ESC differentiation medium (Iscove’s modified Dulbecco’s medium containing 15% FBS (Hyclone), 1× insulin/transferrin/selenium (Invitrogen), 20 µg/ml ascorbic acid (Sigma), and 450 µM α-monothioglycerol (Sigma)) were spotted on a Petri dish lid. After 72 h, hanging drops were transferred to 1% gelatin-coated 96-well plates or 10-cm tissue culture dishes. Media were then changed every other day, and the occurrence of beating embryoid bodies (EBs) was monitored under an inverted microscope. EBs were harvested and lysed by TRIZol reagent (Invitrogen) and RIPA buffer for RNA and protein analysis, respectively.

For hematopoietic differentiation, a two-step differentiation method was used as described (31). For primary differentiation, gelatin-adapted ESC (3,000 cells) were seeded on methylcellulose-based semi-solid media containing Iscove’s modified Dulbecco’s medium, 15% FBS (STEMCELL Technologies), IL-3, and stem cell factor (R&D Systems) as described previously (31). Medium was replenished on EB day 6. Day 9 EBs were harvested and dissociated into single cell suspension, and the cell numbers corresponding to 50 WT EBs were re-plated in complete medium (HSC007, R&D Systems) to support hematopoietic differentiation. Resultant colony-forming units (CFU) were counted under a microscope.

Neuronal differentiation was carried out as described previously (36). In brief, 2 × 10^4 ES cells were seeded on ultra low cluster plates (Costar) in EpiSC basal medium and 100 ng/ml Noggin (R&D Systems). At EB day 2, cells were replenished with 50% EpiSC basal medium and 50% neural base medium supplemented with 100 ng/ml noggin. At EB day 4, cells were fed with neural base medium plus 100 ng/ml noggin. At EB day 6, cells were dissociated into single cell suspension and replated on poly-L-ornithine and laminin-coated plates for further differentiation.

Plasmids, ESC Electroporation, and Genotyping—Cag-cre plasmid was used to delete lox-P-flanked Cited2 allele in ES II20 clone. Cited2 targeting vector used for generating Cited2 knock-out mice (19) was used for targeting the nonfloxed Cited2 allele in ES II20 clone. Oct4-GFP-Puro’ targeting vector was a gift from Dr. Austin Smith (37, 38). pcDNA3.1 expression vector with hygromycin resistance was used for subcloning the Cited2-FLAG fragment. DNA transfection was performed by electroporation using Gene-Pulser II (Bio-Rad). The parameters for electroporation were set at 240 V/500 microfarads for the initial pulse followed by a 230-V/500-microfarad pulse. ESC clones were screened by genomic PCR using primer sets illustrated in Fig. 2, B and C.

Alkaline Phosphatase (AP) Staining—AP staining of ESCs was performed using the AP staining kit as described by the vendor (Stemgent).

RNA Isolation and Quantitative RT-PCR—Total RNA was extracted using the TRIZol reagent (Invitrogen)-based method. RNA was treated with DNase (Invitrogen) and reverse-transcribed into cDNA. Quantitative RT-PCR was performed using SYBR Green master mix (Roche Applied Science)-based platform. Sequences of primers used for RT-PCR are available upon request. RT-PCR was performed in triplicate on independent clones. β-Actin was used as an internal control, and relative gene expression was calculated as 2^−ΔΔCt. Results were normalized to control WT for comparison.

Western Blot—Protein lysates were prepared in RIPA buffer supplemented with complete protease inhibitors (Roche Applied Science), and protein concentrations were measured by the Bradford protein assay kit (Thermo Scientific). 20 µg of protein lysate was separated by SDS-PAGE and transferred to PVDF membrane (Bio-Rad). Blots were probed with primary antibody and subsequently incubated with the corresponding secondary antibody. HRP substrate (Fisher) was then added to blots, and films were developed using Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific). The following antibodies were used: anti-CITED2 (R&D Systems), anti-CITED2 clone JA22X (Santa Cruz Biotechnology), anti-NFATc4 (Santa Cruz Biotechnology), anti-E-cadherin (BD Biosciences), anti-N-cadherin (BD Biosciences), anti-SM-actin (Sigma), anti-cardiac troponin T (clone CT3, Developmental Hybridoma Deposit Bank), and anti-FLAG-M2 (Sigma). Densitometry of specific bands on Western blot film was analyzed by ImageJ software.
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Flow Cytometry—Cell cycle analysis was performed by a propidium iodide staining-based method. Briefly, $1 \times 10^6$ cells cultured for 24 h were fixed with 70% ethanol overnight at $-20$ °C and treated with 10 μg/ml RNase (Sigma). Cells were resuspended in 42 μg/ml propidium iodide solution, and cell cycle analysis was performed on EPIC-XL cytometer. For apoptosis assay, cells were harvested and stained with FITC-conjugated annexin V and 7-aminoactinomycin D according to the instructions provided in the annexin V apoptosis detection kit (BD Biosciences). For Oct4-GFP flow cytometry, EBs at indicated time points were dissociated into single cells, fixed with 0.4% formaldehyde in PBS, and subjected to flow cytometry.

Immunostaining—EBs induced to cardiomyocyte differentiation at EB day 3 were transferred to 1% gelatin-coated chamber slides. Day 8 whole-mount EBs were fixed with 4% paraformaldehyde in PBS, blocked with 1% donkey serum, and incubated with goat anti-mouse sarcomeric myosin heavy chain antibody (clone MF20, Developmental Hybridoma Deposit Bank) at 4 °C overnight followed by the addition of Cy-3-conjugated secondary antibody (1:500 dilution). Mounting media supplemented with DAPI were added, and EBs were observed under a fluorescence microscope. Photoshop software was used to quantify MF20 positive pixels as described elsewhere (39).

Immunostaining of ESC-induced neuronal cells was performed as described previously (36). β-III Tubulin antibody (Covance) and Alexa-488-conjugated antibody (Molecular Probes) were used as primary and secondary antibodies, respectively.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assay was performed by standard methods. Briefly, $1 \times 10^7$ ESC or EBs at days 3–6 and 8 were fixed with 1% formaldehyde and quenched by 0.125 m glycine. Genomic DNA was sonicated to 300–500 bp via BioRuptor (Diagenode). Sheared DNA was incubated with IgG, anti-CITED2, or anti-FLAG-M2, anti-trimethyl histone H3 (Lys-4) or anti-acetylated histone H3 antibodies, and protein-DNA complexes were collected by protein G magnetic beads (Invitrogen). DNA was eluted and subjected to quantitative PCR using primers designed for the Oct4 gene promoter region (40). For ChIP-quantitative PCR, differences greater than 2-fold between the control and experiment group were considered as enrichment (41).

Statistical Analysis—Student’s t test was performed to compare differences between WT and Cited2 KO groups. p value less than 0.05 was considered as statistically significant.

RESULTS

Dynamic Cited2 mRNA Expression during ESC Differentiation—To explore the role of Cited2 in the maintenance of ESC pluripotency and differentiation, we employed a sequential targeting strategy in a Cited2Flox/+ ESCs clone. First, we determined the pluripotency of this ESC clone by examining distinctive ESC characteristics. In the presence of LIF, Cited2Flox/+ ESCs formed typical compact rounded colonies, stained positive for alkaline phosphatase (AP), and expressed pluripotency-associated genes, including Oct4, Nanog, Sox2, Klf4, and c-Myc (Fig. 1A and supplemental Fig. S1). Cited2Flox/+ ESCs transfected with Oct4-GFP reporter plasmid rendered GFP fluorescence in individual ESC colonies (supplemental Fig. S1), although further quantification by flow cytometry revealed that 95% of cells in these cultures activated the Oct4 promoter (Fig. 1B).

To further determine the pluripotency of Cited2Flox/+ ESCs, we induced their differentiation via suspension EB cultures, which recapitulate early embryogenesis, including the generation of three germ layers and induction of cardiomycocytes. Oct4 and Nanog genes were silenced by day 4 EBs, although Cited2 mRNA was consistently highly expressed (Fig. 1C). This result is in agreement with a previous study demonstrating that Cited2 mRNA is highly expressed following the removal of LIF for 5 days in monolayer cultures (11). Although these data confirm that our Cited2Flox/+ ESC clone is suitable for sequential targeting to generate Cited2-null ESCs, the distinct expression of Cited2 during EB differentiation cultures also supports its critical role during the maintenance and differentiation of ESCs.

Generation of Cited2 Total Knock-out ESCs—A sequential targeting strategy to generate Cited2-null ESCs is illustrated in Fig. 2A. Cag-Cre mediated excision of lox-P flanked Cited2 (exon 2) and Neo fragments on Cited2Flox/+ ESCs was performed, and the efficiency was determined by genomic PCR using two sets of primers as illustrated in Fig. 2B. Two independent clones, designated as Cited2Flox/Neo− ESCs, were used for subsequent gene targeting on the wild type Cited2 allele by electroporation of a targeting vector we previously used for generating Cited2 total knock-out mice (19). Targeted ESC clones were screened based on genotyping and RT-PCR (Fig. 2, C and D). Total knock-out of the Cited2 gene was further confirmed via Western blot analysis using antibodies against CITED2 (Fig. 2E). Using this strategy, we efficiently generated four independent Cited2 knock-out ESC clones.

Cited2 Knock-out ESCs Maintain Undifferentiated State in the Presence of LIF—To explore the role of Cited2 in the maintenance of ESC pluripotency, Cited2Flox/+ (wild type, WT), Cited2Δ−/Δ− (Het), and Cited2Δ−/Δ− (Knock-out, KO) ESCs were grown on mitomycin-treated primary MEFs or immortalized fibroblast feeder layer (SNL cells). The undifferentiated status of Cited2Δ−/Δ− ESCs on feeder cells was reflected by a typical compact ESC morphology and positive AP staining (Fig. 3A, upper panel).

To facilitate further biochemical and phenotypic analysis of Cited2 KO ESCs, we generated gelatin-adapted ESCs by serially weaning from feeder cells. Cited2 deletion did not affect undifferentiated status of gelatin-adapted Cited2 KO ESCs at early passages (less than 4 passages) as shown by compact ESC morphology and strong AP staining pattern (Fig. 3A, lower panel). Levels of Oct4 and Nanog transcripts in early passages of gelatin-adapted WT and Cited2 KO ESCs were comparable (Fig. 3B). Annexin V staining demonstrated that there was no difference in the basal level of cell apoptosis between gelatin-adapted WT and Cited2 KO ESCs (Fig. 3C). Early passage gelatin-adapted WT and Cited2 KO ESCs displayed similar cell cycle distribution, suggesting knock-out of Cited2 does not influence...
ESC cell proliferation (Fig. 3D and supplemental Fig. S2). These results support that loss of Cited2 does not alter the undifferentiated state of ESCs in the presence of LIF.

**Knock-out of Cited2 Delays ESC Differentiation by Deregulation of Oct4**—To investigate whether Cited2 deletion affects ESC differentiation, we cultured WT, Het, and Cited2 KO ESCs on feeder cells in the absence of LIF (supplemental Fig. S3A). At day 6, WT and Het ESC cultures differentiated as evidenced by flattened morphology and loss of AP staining. Interestingly, some of Cited2 KO ESC colonies maintained compact morphology and were stained positive for AP, indicating that differentiation of Cited2 KO ESCs may be inhibited or delayed. To exclude feeder cell-derived factors hampering Cited2 KO ESC differentiation, we seeded WT and Cited2 KO ESCs at lower densities on gelatin-coated plates in the absence of LIF. On day 4 of differentiation, WT ESCs displayed flattened morphology and loss of AP staining, but Cited2 KO ESC cultures continued to stain positive for AP (supplemental Fig. S3B), suggesting that loss of Cited2 influences the differentiation of ESCs normally induced by LIF withdrawal.

To closely monitor ESC differentiation, WT and Cited2 KO ESCs were stably transfected with Oct4 promoter-driven GFP reporter plasmid. On day 0, a similar percentage of Oct4-GFP+ cell numbers was observed in WT and Cited2 KO ESC cultures (Fig. 4A), supporting that Cited2 KO ESC can maintain an undifferentiated state in the presence of LIF. Following LIF removal, Oct4-GFP+ cells in both WT and Cited2 KO EBs decreased to ∼30% at day 2 post-differentiation. In WT cultures, Oct4-GFP+ cell numbers further decreased to 10 and 5% on days 4 and 5 of EB differentiation, respectively. In contrast, Cited2 KO EBs contained significantly higher percentages of Oct4-GFP+ cell numbers (30 to 20%). Under fluorescence microscopy, GFP+ cells were undetectable in day 5 WT EBs, although a patchy distribution of GFP intensity was detected within Cited2 KO EBs (Fig. 4B). Oct4-GFP expression in Cited2 KO EBs correlated with significantly higher stem-related transcript levels in day 4 (Oct4 and Sox2) and day 6 (Oct4, Sox2, c-Myc, and Klf4) EBs, further suggesting a significant delay or block in the differentiation of Cited2 KO ESCs (Fig. 4C and supplemental Fig. S4).

We next investigated if cells in differentiating EB cultures remained pluripotent by replating single cell suspension from day 4 and 5 EBs and seeding back to gelatin-coated plates with complete ESC maintenance media containing LIF. Day 4 WT EBs maintained some extent of stemness reflected by the number of AP-positive colonies (92 AP colonies), which was greatly reduced in day 5 WT EBs (534 and 66 AP colonies) (Fig. 4D). In contrast, numbers of AP-positive colonies were significantly higher in day 4 and 5 Cited2 KO EBs (534 and 66 AP colonies, respectively) than WT controls, correlating with higher percentages of Oct4-GFP+ cell numbers (Fig. 4A), increased Oct4-GFP fluorescence intensity (Fig. 4B), and significantly elevated Oct4 transcript levels (Fig. 4C) at the indicated time points. Collectively, these results demonstrate that loss of Cited2 impairs the normal differentiation of ESCs.
Defective Hematopoietic and Neuronal Differentiation in Cited2 Knock-out ESCs—The Oct4 transcription factor not only is a master regulator for the pluripotency of ESCs but also controls their differentiation (9). It was demonstrated that 2–3-fold overexpression of Oct4 inhibits hematopoietic differentiation of ESCs (8). Because ineffective down-regulation of Oct4 expression was observed in Cited2 KO EBs and Cited2 deletion affects fetal and adult hematopoiesis (23, 26, 27), we examined whether Cited2 depletion in ESCs affects hematopoietic differentiation. Cells from primary day 9 EBs were re-plated in methylcellulose media containing cytokines supporting hematopoietic colony formation and scored for CFU-E, CFU-GM, and CFU-Mix by day 7. Compared with WT, cells from Cited2 KO EBs displayed significantly reduced numbers of hematopoietic colonies (Fig. 5A). Reduced expression of Brachyury (T gene) mRNA in early EBs (day 3) supports the importance of Cited2 in promoting mesoderm differentiation (Fig. 5B). We examined the expression of two genes associated with hemoglobin switching, Hbb-bh1 and Hbb-b1, considered to be expressed in primitive versus definitive erythroid cells, respectively (42). Embryonic Hbb-bh1 mRNA expression peaked by day 6 and was then followed by induction of adult Hbb-b1 transcript levels in day 8 WT EBs (Fig. 5B). In contrast, although transcript levels of either globin gene were significantly reduced throughout the differentiation of Cited2KO EBs, Hbb-b1 levels became indistinguishable exclusively in day 8 cultures (Fig. 5B).

Because improper silence of Oct4 in Cited2 KO ESCs affects mesoderm derived hematopoietic cell differentiation, we next investigated whether deletion of Cited2 also affected ectoderm development. Using culture conditions that promote neuronal
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**FIGURE 3. Cited2 knock-out ESCs maintain undifferentiation in the presence of LIF.** A, AP staining of WT and Cited2 KO ESCs grown on feeder cells (upper panel) and gelatin-coated plates (lower panel). B, expression of Oct4 and Nanog mRNA in WT and Cited2 KO ESCs grown on gelatin-coated plates. Relative gene expression was collected from three independent experiments on three independent Cited2 KO clones, and the data are presented as mean ± S.D. C, apoptosis of WT and Cited2 KO ESCs grown on gelatin. Apoptotic cells were calculated by percentage of annexin-V-positive cells via flow cytometry. Three independent experiments on WT and Cited2 KO ESCs were performed, and data shown are mean ± S.D. D, cell cycle distribution of G1, G2/M, and S phase cells in WT and Cited2 KO ESCs was assayed by propidium iodide staining and subsequent flow cytometry. Results of repeated experiments on two independent Cited2 KO clones (KO#1 and KO#2) are presented. Data shown are means ± S.D. (n = 3).

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differentiation, WT ESCs exhibited robust numbers of neurons at 8 days of ESC differentiation as evidenced by the enrichment of β-III Tublin-positive cells as shown in Fig. 5C. In contrast, the number of neurons was greatly diminished in Cited2 KO ESC differentiation cultures. Defective neuronal differentiation in Cited2 KO ESCs was further supported by significantly reduced expression of neuronal associated genes (Pax6, Nestin, and β-III tublin) in day 6 and 8 EBs (Fig. 5D).

**Knock-out of Cited2 in ESCs Impairs Cardiomyocyte Differentiation**—Mutations of the human CITED2 gene have been demonstrated to associate with congenital heart diseases (43). Although a prominent phenotype of Cited2 knock-out embryos is cardiac anomalies, the molecular regulation by Cited2 during differentiation and maintenance of cardiomyocytes has never been investigated (17, 19). Thus, we differentiated WT and Cited2 KO ESCs was further supported by significantly reduced expression of neuronal associated genes (Pax6, Nestin, and β-III tublin) in day 6 and 8 EBs (Fig. 5D).

**FIGURE 3. Cited2 knock-out ESCs maintain undifferentiation in the presence of LIF.** A, AP staining of WT and Cited2 KO ESCs grown on feeder cells (upper panel) and gelatin-coated plates (lower panel). B, expression of Oct4 and Nanog mRNA in WT and Cited2 KO ESCs grown on gelatin-coated plates. Relative gene expression was collected from three independent experiments on three independent Cited2 KO clones, and the data are presented as mean ± S.D. C, apoptosis of WT and Cited2 KO ESCs grown on gelatin. Apoptotic cells were calculated by percentage of annexin-V-positive cells via flow cytometry. Three independent experiments on WT and Cited2 KO ESCs were performed, and data shown are mean ± S.D. D, cell cycle distribution of G1, G2/M, and S phase cells in WT and Cited2 KO ESCs was assayed by propidium iodide staining and subsequent flow cytometry. Results of repeated experiments on two independent Cited2 KO clones (KO#1 and KO#2) are presented. Data shown are means ± S.D. (n = 3).

We further investigated cardiomyocyte differentiation by performing whole-mount immunostaining using an antibody against muscle marker sarcomeric myosin heavy chain (clone MF20) that labels cardiomyocytes in these same EB cultures. As shown in Fig. 6B and quantified in supplemental Fig. S5D, on day 8 of ESC differentiation, MF20-positive cells corresponded to the beating foci observed under a microscope. In contrast, MF20 staining signal was not detected in Cited2 KO EBs. Western blot analysis was also performed at a later time point using the cardiac-specific structural protein, cardiac troponin T, which was only detected at day 12 of WT but not Cited2 KO EBs (supplemental Fig. S5E). Consistent with cardiomyocyte defects in Cited2 KO EBs with delayed differentiation, Oct4 mRNA was higher in Cited2 KO EBs at day 4 in these culture conditions (supplemental Fig. S5C). These data demonstrate that Cited2 knock-out ESCs display reduced cardiomyocyte differentiation and further confirm the delayed silencing of the Oct4 gene.

**Defective Cardiomyocyte Differentiation in Cited2 Knock-out ESCs Associates with Delayed NFAT3 Induction**—Calcineurin/NFAT signaling is an essential regulator of ESC stemness and differentiation (44). Inhibition of NFATs by cyclosporin A
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To explore whether reduced NFAT3 expression in Cited2 KO ESCs affects the induction of ESC differentiation markers, we examined protein expression of E-cadherin, N-cadherin, and SM-actin in WT and Cited2 KO ESCs/EBs. As shown in Fig. 6C, E-cadherin protein expression was similar in undifferentiated WT and Cited2 KO ESCs, but it was modestly decreased in Cited2 KO EBs at day 8 and 10 of EB differentiation relative to WT controls. Significant induction of N-cadherin was observed in day 8 WT EBs, although only a partial induction of N-cadherin was observed in Cited2 KO day 10 EBs. SM-actin induction in WT ESCs occurred at EB day 6, although comparable expression level of SM-actin was not detected until day 10 in Cited2 KO EBs (Fig. 6C). The dysregulated expression of these proteins further confirms that cardiomyogenic differentiation is impaired in Cited2 KO ESCs.

To further link reduced NFAT3 activity with defective cardiomyocyte differentiation in Cited2 KO ESCs, we examined the expression of cardiomyocyte associated NFAT gene targets, Nkx2.5 and β-MHC (45, 46). As shown in Fig. 6D, in three independent Cited2 ESC clones with reduced cardiomyocyte differentiation, the expression of Nkx2.5 and β-MHC was significantly decreased in day 8 EBs.

Dynamic Regulation of Oct4 in Pluripotent and Differentiating ESCs—Cited2 was recently characterized as one of the direct target genes for FoxP1 in undifferentiated and differen-

maintains ESCs in an undifferentiated status in the absence of LIF and reduces epitheliun-to-mesenchymal transition by deregulating Src (44). In contrast, ectopic expression of constitutively activated forms of NFAT3 or NFAT4 induces ESC differentiation. NFAT3 also plays important roles in ESC-derived cardiomyocyte differentiation by regulating the expression of Nkx2.5 in early cardiac progenitor cells and controlling β-MHC expression in adult cardiomyocytes (45,46). Importantly, inhibition of NFAT by FK-506 at early time points (day 0–2) of ESC differentiation potentely inhibited cardiomyocyte differentiation (46). Because we observed impaired cardiomyocyte differentiation in Cited2 KO ESCs, we sought to examine whether defects in Cited2 knock-out ESCs correlate with deregulated NFAT signaling. It has been demonstrated that NFAT3 and NFAT4 are two NFAT members highly induced in differentiating ESCs (47). Consistent with previous studies, we found that NFAT3 mRNA expression was induced in differentiating WT EBs (days 3–6) relative to undifferentiated ESC (day 0) (supplemental Fig. S6, A and B). In contrast, NFAT3 mRNA levels were significantly reduced in undifferntiated Cited2 KO ESC as well as throughout EB differentiation (supplemental Fig. S6, A and B). By day 10, NFAT3 mRNA expression in WT cultures tapered to similar levels quantified from Cited2 KO EBs (supplemental Fig. S6B). Similar expression patterns were observed for NFAT3 protein levels (Fig. 6C).

FIGURE 4. Cited2 knock-out ESCs exhibit delayed differentiation. A, WT and Cited2 KO ESCs were transfected with Oct4-GFP-Puro reporter plasmid and induced to differentiate by depleting LIF via a hanging-drop method. Percentage of Oct4-GFP-positive cells at indicated time points was recorded by flow cytometry (n = 3). The experiments were performed on two independent Cited2 KO clones (KO#2 and KO#3), and similar results were obtained. Data from WT and Cited2 KO#3 are presented. B, morphology and GFP expression of WT and Cited2 KO EBs 5 days post-differentiation. C, Oct4 mRNA expression during differentiation in WT and Cited2 KO ESCs assessed by RT-PCR. The experiments were repeated twice independently on WT and Cited2 KO#3. Representative data are shown as mean ± S.D. Error bars stand for S.D. of three replicates. D, repopulating capacity of WT and Cited2 KO EBs. Day 4 and day 5 EBs were trypsinized to single cell suspension, and 1 × 10⁶ cells were seeded in complete ESC culture media for 5 days. AP staining was used to evaluate EB repopulating ability. Upper panel, representative field of AP-positive clones derived from day 4 WT and Cited2 KO#3 EBs. Lower panel, quantitative analysis of AP (+) colonies originated from EBs at day 4 and 5. Data shown are mean ± S.D. (n = 3), *, compared with WT controls; p < 0.05.
tiated ESCs. The Cited2 promoter is occupied by both the ES form (FoxP1-ES, essential for ESC pluripotency) and the constitutive form of FoxP1 (FoxP1, critical for ESC differentiation) (14). Interestingly, although Oct4 is a FoxP1 target gene, its promoter is also occupied by both FoxP1-ES and FoxP1. To dissect potential downstream mechanisms mediated by FoxP1-Cited2 pathway in undifferentiated and differentiating ESCs, we performed ChIP assays to examine the recruitment of CITED2 to the regulatory machinery of the Oct4 gene, which is critical for ESC pluripotency and differentiation.

A 3-fold enrichment of CITED2 on the Oct4 R10 promoter region was detected in undifferentiated ESCs by the ChIP assay using anti-CITED2 antibody (Fig. 7A). Binding specificity of CITED2 to the Oct4 R10 region was confirmed by transfecting FLAG-tagged CITED2 into Cited2 KO ESCs (supplemental Fig. S7A) and using anti-FLAG antibody for the ChIP assay (supplemental Fig. S7B). As Cited2 is consistently highly expressed in WT ESCs during cardiomyocyte differentiation (Fig. 1C), we further monitored recruitment of CITED2 to the Oct4 promoter during this specific differentiation process. We found the enrichment of CITED2 to the Oct4 R10 region is maintained in differentiating WT EBs at days 3 and 4 (Fig. 7A) but not in EB days 5, 6, and 8 (data not shown).

Furthermore, accumulated evidence has demonstrated that histone code is critical for ESC differentiation (1). An epigenetic hallmark of undifferentiated ESCs is the presence of permissive and repressive histone marks on key developmental genes such as Oct4. Dynamic gain and loss of permissive and repressive histone marks also dictate proper ESC differentiation. CITED2 is a cofactor for CBP/p300, which are bona fide histone acetyltransferases (48, 49). We investigated dynamic modifications of two activating marks (acetylated histone H3, AcH3, and trimethylated histone H3 at lysine 4, Me3H3K4) on the Oct4 promoter during cardiomyocyte differentiation. As CITED2 is recruited to the Oct4 promoter R10 region in undifferentiated ESCs and early differentiating EBs (Fig. 7A), epigenetic regulation of this R10 region and several other amplicons in the Oct4 gene was also monitored. As compared with WT groups, Cited2...
KO EBs at days 3–5 were featured by significantly increased occupancy of ACh3 and Me3H3K4 marks in the Oct4 gene (Fig. 7, B and C, and supplemental Fig. S7C), which correlates with significantly elevated Oct4 transcript levels at EB days corresponding time points of cardiomyocyte differentiation (supplemental Fig. S6C). We observed no difference of ACh3 and Me3H3K4 occupancy on the Oct4 promoter regions at EB days 6 and 8 of cardiomyocyte differentiation, at the time when Oct4 mRNA was silenced in both WT and Cited2 KO EBs (supplemental Fig. S5C). Collectively, our data suggest that Oct4 gene expression during ESC early differentiation is coordinated by the recruitment of CITED2 and histone active marks to the Oct4 promoter.

**DISCUSSION**

In this study, we generated Cited2 knock-out mESCs to characterize its role in ESC pluripotency and differentiation. We showed that loss of Cited2 impairs ESC differentiation in the neuronal, hematopoietic, and cardiomyocyte lineages whereby Cited2 KO EBs displayed temporally inappropriate expression and delayed silencing of the Oct4. We further demonstrated the recruitment of CITED2 to the Oct4 gene. Cited2 therefore plays a regulatory role in ESC cell fate decision in which Cited2 directly regulates the expression of Oct4.

Cited2 was previously identified as one of the genes that is involved in the maintenance of mESC identity in the absence of LIF (11). Although the overexpression study proposes a requirement for Cited2 in “rescuing” the mESC stemness, the essential role of Cited2 in mESC maintenance was not addressed. Our results suggest that Cited2 is not essential for the maintenance of undifferentiated ESCs but rather in promoting the conventional differentiation induced by LIF removal. We argue that similarities or differences between our results and those of others could be due to the origin of ES clones (primary versus polypoma large T immortalized lines), the expression strategies utilized (overexpression versus knock-out genetic studies), or the culture conditions (passage number or differentiation assays).

It is well established that Oct4 functions as a pluripotency determinant in ESCs, and proper levels of Oct4 critically dictate ESC identity. Emerging evidence points to crucial roles of Oct4 during ESC germ layer differentiation as levels of Oct4 expression have been found to influence hematopoietic development (50). The finding that Cited2 mRNA is significantly increased (~80-fold difference) in Oct4-GFP<sup>+</sup> population during EB differentiation (13) further indicates the potential regulation of Oct4 expression by Cited2 during EB differentiation. In this study, the sustained expression of Oct4 in day 4–6 EBs further confirms the importance of regulating Oct4 expression that otherwise impairs proper development, as we noted for cardiomyocyte, hematopoietic, and neuronal differentiation in

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**FIGURE 6. Impaired cardiomyocyte differentiation in Cited2 knock-out ESCs associates with retarded NFAT3 induction.** A, percentage of beating foci in WT and Cited2 KO EBs at different time points. Single EBs were seeded in gelatin-coated 96-well plates. At least 80 attached EBs were enumerated for the occurrence of beating EBs for each genotype. Experiments were independently repeated at least four times on WT and three independent Cited2 KO clones. Data are shown as mean ± S.D. N.D., no beating EBs detected. *, compared with WT controls, p < 0.05. B, whole-mount EB immunostaining of sarcomeric myosin heavy chain (MF20) in WT and Cited2 KO EBs differentiated for 8 days. DAPI served as a positive control for nuclear staining. C, NFAT3, E-cadherin, N-cadherin, and SM-actin protein expression in WT and Cited2 KO ESCs during differentiation. These experiments were repeated three times on WT and three independent Cited2 KO clones. Representative data from one experiment on WT and Cited2 KO#2 are presented. Numbers shown are relative protein expression levels measured by densitometry and normalized by an internal control, β-actin. D, reduced NFAT3 activity in Cited2 KO ESCs is reflected by the down-regulation of NFAT3 target genes (Nkx2.5 and β-MHC) in cardiomyocytes. Triplicate experiments were performed on three independent Cited2 KO ESC clones at EB day 8 and data are presented as mean ± S.D. *, compared with WT controls, p < 0.05.
Cited2 KO ESCs. Delayed silencing of the Oct4 gene during ESC differentiation is also observed in SIRT1 KO ESCs and is associated with defective hematopoietic differentiation (51), providing additional cross-talk between Cited2 and SIRT1-FoxO1/-FoxO3-mediated signaling in ESC cell fate decision.

Our results are consistent with a recent study of FoxP1 implicating the dual role of Cited2 in ESC pluripotency and differentiation (14). Both the FoxP1-ES isoform, encoded by an alternatively spliced FoxP1 RNA, and the constitutive form of FoxP1 occupy the Cited2 promoter region, controlling ESC pluripotency and differentiation, respectively. Interestingly, the Oct4 promoter region is also bound by both FoxP1 isoforms suggesting that proper regulation of Oct4 expression is rendered by FoxP1-isoform/Cited2 pathway in ESCs. Interestingly, Oct4 is also a direct target gene for FoxO1/FoxO3-mediated ESC identity, and Cited2 is a well established FoxO1/FoxO3 target gene in hematopoietic cells and cardiomyocytes exposed to hypoxia and oxidative stress (52–54). Because deacetylation of FoxO1/FoxO3 is in part mediated by SIRT1 and Sirt1 KO and ESCs display resistance to oxidative stress, it will be important to determine whether Cited2 also plays an important role in ESCs in response to stress conditions (55).

Our present findings further support the critical role of Cited2 in cardiomyocyte differentiation by affecting the expression of NFAT3 leading to down-regulation of Nkx2.5 and β-MHC cardiomyocyte genes. Calcineurin-NFAT pathway is activated in T cells, cardiomyocytes, neural cells, and cancer cells and is emerging as a potent regulator of ESC cell fate decision (44, 56). Although inhibiting of NFAT by cyclosporin A maintains ESCs, constitutive activation of NFAT in ESCs leads to differentiation as evidenced by reduced Oct4 expression and induction of N-cadherin and SM-actin through activation of Src and increased expression of epithelial-mesenchymal transition (EMT) markers (44). Although the critical role of calcineurin-NFAT signaling in regulating early lineage specification in ESCs was established, the actual regulation of NFAT expression in ESCs remains elusive. Interestingly, a modulatory role of Cited2 in the expression of EMT markers has been demonstrated in
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MCF10A cells.4 Because EMT is a process required for lineage specification in ESCs in response to different stimuli, future studies are warranted in establishing the role of Cited2 in lineage specification and EMT.

CBP/p300, interacting proteins of CITED2, exert epigenetic modifications through histone acetylation of multiple genes, including some involved in the differentiation of ESC imparting further evidence that transcriptional complexes containing Cited2 are critical in modulating important developmental cellular functions (10). The potential role of Cited2 in epigenetic gene regulation is not limited to ESCs. In somatic cells, Cited2 has been shown to modulate Bmi-1, a key member of polycomb repressor complex-1, in mouse embryonic fibroblast cells (57). Induction of Cited2 expression is regulated by histone deacetylase inhibitors in gastroenterological cancer cells, and induced Cited2 expression renders cancer cells more sensitive to chemotherapy (58, 59). These studies suggest a potential role of Cited2 in the remodeling of chromatin structures, and future studies designed to elucidate the mechanism(s) are also clinically relevant.

Our finding of Cited2 as a key player in mESC differentiation via regulation of Oct4 has important implications for regenerative and clinical medicine. First, it has been demonstrated that overexpression of Cited2 does not increase the efficiency of induced pluripotent stem cells (iPS) (60). However, it is possible that reduced expression of Cited2 may enhance iPS efficiency given our finding that loss of Cited2 increases the replating capacity of EBs. Second, CITED2 mutations are associated with human congenital heart disease in a human population control study (43). Therefore, it might be informative to generate human iPS lines with CITED2 mutation and investigate if the capacity to differentiate into cardiomyocytes is affected. Inhibitors of Cited2 could also be developed based on the known structural information (61) to enhance iPS efficiency.

Acknowledgments—We thank Michael Sramkoski at Case Comprehensive Cancer Center for help with flow cytometry analysis; members in the Yang, Proweller, and Ramirez laboratories for helpful discussions; Dr. Richard Krzyzek at R&D Systems for anti-Cited2 antibody; Dr. David LePage at Case Transgenic and Targeting Facility for SNL 7C/7 feeder cells; Dr. Austin Smith (Wellcome Trust Centre for Stem Cell Research) for Oct4-GFP reporter plasmid; Dr. Paul Tasar (Dept. of Genetics, Case Western Reserve University) for advice on neuronal differentiation; Dr. Xiangzi Han for pcDNA3.1-hygromycin vector plasmid; Dr. Yiwei Wang for help on immunostaining and Drs. Zhenghe Wang and Guangbin Luo (Dept. of Genetics, Case Western Reserve University) for suggestions on gene targeting.

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