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Cdk1 Is Required for the Self-Renewal of Mouse Embryonic Stem Cells

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ABSTRACT

Cyclin-dependent kinase 1 (Cdk1) is indispensible for the early development of the embryo. However, its role in maintaining the undifferentiated state of the embryonic stem (ES) cells remains unknown. In this study, we dissected the function of Cdk1 in mouse ES cells by RNA-interference and gene expression analyses. *Cdk1* expression is tightly correlated with the undifferentiated state of the ES cells. Upon differentiation, Cdk1 expression reduced drastically. *Cdk1* knock-down by RNA interference resulted in the loss of proliferation and colony formation potential of the ES cells. Consequentially, expression of self-renewal genes was reduced while differentiation markers such as *Cdx2* were induced. Our results suggest a role for Cdk1 in maintaining the unique undifferentiated and self-renewing state of the mouse ES cells. J. Cell. Biochem. 112: 942–948, 2011. © 2010 Wiley-Liss, Inc.

KEY WORDS: EMBRYONIC STEM CELLS; GENE EXPRESSION; SELF-RENEWAL

Pluripotent mouse ES cells derived from the inner cell mass (ICM) of the early pre-implantation embryo [Evans and Kaufman, 1981; Martin, 1981] are good model for the study of early embryogenesis and development. Differentiation of ES cells into multiple cell types in culture is a potential source for cell replacement therapy [Li et al., 1998; Fujikura et al., 2002; Kyba et al., 2002]. ES cells are also invaluable tools for the study of gene functions [Nichols et al., 1998; Avilion et al., 2003; Mitsui et al., 2003]. Understanding the molecular mechanisms underlying the unique properties of self-renewal and pluripotency of the ES cells is necessary to realize their clinical and scientific potentials.

Extensive studies have shown that transcription factors Oct4 and Nanog play critical roles in maintaining the undifferentiated state of mouse ES cells. Oct4 also known as Pou5f1, belongs to the POU (Pit-Oct-Unc) transcription factor family. *Oct4*-null embryo dies at embryonic day 3.5 and the blastocyst are composed mainly of trophectodermal cells without the ICM. Interestingly, Oct4 controls pluripotency of ES cells in a dose-dependent manner [Niwa et al., 2000]. A twofold induction of *Oct4* led to ES cell differentiation into primitive endoderm and mesoderm. Loss of *Oct4*, on the other hand, triggers differentiation into trophectoderm lineages. These observations indicate that the appropriate level of Oct4 is critical for the maintenance of ES cells. Nanog was first identified as "ENK" (early embryo specific NK) based on its homology with members of the *NK* gene family [Wang et al., 2003]. Two independent studies confirmed the essential roles of Nanog in ES cell maintenance and embryonic development [Chambers et al., 2003; Mitsui et al., 2003]. *Nanog*-null embryo fails in the formation of primitive ectoderm, and dies at embryonic day 4.5. Hence, Nanog is required for the ICM formation and primitive ectoderm development [Mitsui et al., 2003]. ES cells

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derived from *Nanog*−/− ICM differentiate into endoderm lineage cells [Chambers et al., 2003; Mitsui et al., 2003; Hatano et al., 2005]. Interestingly, over-expression of *Nanog* allows ES cells to bypass its dependence on the LIF and BMP signaling pathway [Chambers et al., 2003; Mitsui et al., 2003]. These results demonstrate the indispensable roles of Nanog in early embryonic development and ES cell maintenance.

Cyclin-dependent kinase 1 (Cdk1) along with cyclin B are involved in cell cycle regulation in eukaryotic cells. Deletion of *Cdk1* leads to early embryonic lethality, suggesting its critical role in early embryonic development [Santamaria et al., 2007]. Repression of Cdk1 resulted in the differentiation of trophoblast stem cells into giant cells. Furthermore, inhibition of Cdk1 caused rapid apoptosis of the ES cells [Ullah et al., 2008]. These findings suggest that Cdk1 could be involved in the maintenance of the unique undifferentiated state of early stem cells. It was recently shown that Cdk1 is a member of the Oct4 interactome in mouse ES cells [Wang et al., 2006]. Interestingly, many members of the Oct4 interactome network are necessary for the maintenance of self-renewal and pluripotency [Wang et al., 2006].

Here we employed several strategies to dissect the functional role of Cdk1 in ES cells. Upon differentiation of the ES cells by LIF withdrawal or retinoic acid induction, *Cdk1* expression was dramatically suppressed. Moreover, reduction of Oct4 or Nanog reduced the expression of *Cdk1*. Using RNAi-mediated knockdown, *Cdk1*-depleted ES cells resulted in cell death. Moreover, mRNA levels of self-renewal genes such as *Nanog*, *Tcl1*, and *Esrrb* were greatly reduced. On the other hand, trophectodermal genes such as *Cdx2*, *Hand1*, and *Mash2* increased significantly. This suggests a role of Cdk1 in regulating the self-renewal state of the ES cells.

MATERIALS AND METHODS

CELL CULTURE

Mouse E14 ES cells (ATCC) were cultured under a feeder-free condition at 37°C with 5% CO_2. The cells were maintained on gelatin-coated dishes in Dulbecco's modified Eagle medium (DMEM; GIBCO), supplemented with 15% heat-inactivated fetal bovine serum (FBS; GIBCO), 0.1 mM β-mercaptoethanol (GIBCO), 2 mM L-glutamine, 0.1 mM MEM nonessential amino acid, 5,000 units/ml penicillin/streptomycin and 1,000 units/ml of LIF (Chemicon).

KNOCKDOWN PLASMIDS AND CELL TRANSFECTION

Oct4 and *Nanog* shRNA plasmids were constructed according to the previous reports [Chew et al., 2005; Loh et al., 2006]. For *Cdk1* shRNAs, 19 base pair (bp) gene-specific oligonucleotides for RNA interference (RNAi) were designed and cloned into pSuperpuro (Oligoengine) which carries a puromycin resistant. To ensure the specificity of the oligonucleotides for RNAi, all sequences were analyzed by BLAST search to remove any cross effect with other genes. The sequences for shRNAs of different genes are listed here.

Cdk1 shRNA1	Sense strand: 5′-GATCCCCGTATAAGGGTAGACAC AGATTCAAGAGAtctgtgtctacccttatacTTTTTA-3′ Antisense strand:5′-AGCTTAAAAAGTATAAGGGTAGA CACAGATCTCTTGAAtctgtgtctacccttatacGGG-3′
Cdk1 shRNA2	Sense strand: 5′-GATCCCCGTACTTACGGTGTGGTGTATT CAAGAGAtacaccacaccgtaagtacTTTTTA-3′ Antisense strand:5′-AGCTTAAAAAGTACTTAC GGTGTGGTGTATCTCTTGAAtacaccacaccgtaagtacGGG-3′
Cdk1 shRNA3	Sense strand: 5′-GATCCCCGGACCATATTTGCAGA ACTTTCAAGAGAagttctgcaaatatggtccTTTTTA-3′ Antisense strand: 5′-AGCTTAAAAAGGACCATA TTTGCAGAACTTCTCTTGAAagttctgcaaatatggtccGGG-3′
Scrambled Cdk1 shRNA1	Sense strand: 5′-GATCCCCGAAGTGGAAACTCA GTAGATTCAAGAGAtctactgagtttccacttcTTTTTA-3′ Antisense strand:5′-AGCTTAAAAAGAAGTGGAAA CTCAGTAGATCTCTTGAAtctactgagtttccacttcGGG-3′
Scrambled Cdk1 shRNA2	Sense strand: 5′-GATCCCCGTGGTCTTATCGTAGGA GTTTCAAGAGAactcctacgataagaccacTTTTTA-3′ Antisense strand:5′-AGCTTAAAAAGTGGTCTT ATCGTAGGAGTTCTCTTGAAactcctacgataagaccacGGG-3′
Scrambled Cdk1 shRNA3	Sense strand: 5′-GATCCCCGCAAACATTAGCGAGTT CTTTCAAGAGAagaactcgctaatgtttgcTTTTTA-3′ Antisense strand:5′-AGCTTAAAAAGCAAACATTAGC GAGTTCTTCTCTTGAAagaactcgctaatgtttgcGGG-3′

Transfection of shRNA plasmids was performed using Lipofectamine 2000 (Invitrogen). For knockdown, 2 μg of shRNA plasmids were transfected into ES cells on 35 mm plates. Puromycin (Sigma) selection at 1.0 μg/ml was introduced 24 h after transfection, and maintained for 2–6 days prior to RNA harvesting or Alkaline phosphatase staining (Sigma).

RNA ISOLATION, REVERSE TRANSCRIPTION AND REAL-TIME PCR ANALYSIS

Total RNA was extracted using Trizol reagent (Invitrogen). cDNA synthesis was performed with 500ng of total RNA using RevertAid™ First Strand cDNA Synthesis kit (Fermentas) according to the manufacturer's instructions. Endogenous mRNA levels were measured by real-time PCR analysis based on SYBR Green detection (Fermentas) with the BioRad real-time PCR machine. Results were normalized with β-actin. All the primers used in the study gave rise to single product of the right size in agarose gel analysis.

PROTEIN EXTRACTION AND WESTERN BLOTTING

Total protein was extracted by lysing cells with the whole cell extraction buffer (Tris, 50 mM; Nacl, 150 mM; NP40, 1%; Glycerol, 10%; EDTA, 1 mM; PMSF, 1 mM). Thirty micrograms of the total protein were separated by SDS–PAGE and transferred to PVDF 45 membrane. The membrane was blocked with 5% milk and probed with specific primary antibodies and secondary antibodies. The blots were developed with ECL Advance Western Blotting Detection Kit (Amersham). Anti-Cdk1 antibodies (Bioworld, BS1820; Cell Signaling, Y15; Abcam, E161) and mouse anti-β actin antibody (Boster, BM0627) were used.

MICROARRAY

For the *Cdk1*-depleted ES cells, NimbleGene microarray platform (NimbleGene mouse Gene Expression 12 × 135K Array) was used. The mRNAs derived from *Cdk1* shRNA 2 and vector-treated ES cells were reverse-transcribed, labeled and analyzed. Arrays were processed following the manufacturer's instructions. Three biological replicates of the profiles were performed for both *Cdk1*-depleted ES cells and vector control. RMA normalization was use to normalize the microarrays. Significance analysis of microarrays

(SAM) was used to select differentially expressed genes (Fold change (FC) >1.5 for up-regulated, FC <0.6 for down-regulated; FDR <5%).

The differentially expressed genes were mapped to Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways through the Molecule Annotation System (MAS) platform (http://bioinfo.capitalbio.com/mas). The GO terms and KEGG pathways with q values <0.01 were considered statistically significant.

FLOW CYTOMETRY ASSAY

ES cells were plated at 3×10^6 cells/well density in 60 mm plates and transfected with control and *Cdk1* shRNA vectors. After 3 days of 1 μg/ml puromycin selection, cells were collected for Flow cytometry analysis. Briefly, cells were washed twice with PBS and re-suspended in 75% ice-cold ethanol at 4°C overnight. Cells were then stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) at a concentration of 1 μg/ml at room temperature for 5 min in dark. Cell cycle was analyzed using the FACSort flow cytometer (Becton Dickinson, USA). For some analysis, ES cells were stained with Annexin V FITC kit (Biosea).

RESULTS

EXPRESSION OF CDK1 CORRELATES WITH THE UNDIFFERENTIATED STATE OF ES CELLS

We were interested to know whether the expression level of *Cdk1* correlates with the undifferentiated state of ES cells. To achieve this, we induced ES cells differentiation by the addition of retinoic acid (RA) or the removal of LIF from the culture medium. Both the RA induction and LIF withdrawal led to the reduction in the expression levels of pluripotency genes *Oct4*, *Nanog*, and *Sox2* (Fig. 1A). Interestingly, differentiation of ES cells induced the suppression of *Cdk1* mRNA level. Next, we performed knockdown of *Oct4* or *Nanog* using RNA interference. Both the *Nanog* and *Oct4* shRNA constructs were effective in reducing the mRNA level of *Nanog* and *Oct4* mRNA respectively (Fig. 1B,C). Strikingly, either *Oct4* or *Nanog* depletion resulted in the dramatic down-regulation of *Cdk1* level (Fig. 1D). These findings suggested that the expression of *Cdk1* is highly correlated with the pluripotent ES cell state, and key regulators such as Oct4 and Nanog may regulate the high level of Cdk1 expression in undifferentiated ES cells (Fig. 1A).

KNOCKDOWN OF CDK1 RESULTED IN THE REDUCED EXPRESSION OF SELF-RENEWAL GENES AND THE INDUCTION OF DIFFERENTIATION MARKERS

To investigate the role of Cdk1 in ES cell maintenance, we depleted its expression using two shRNA constructs that targeted different sites of the *Cdk1* mRNA. After 2 days of puromycin selection, both constructs efficiently reduced endogenous *Cdk1* mRNA by 80% and 70%, respectively (Fig. 2). By western blotting analysis, we confirmed that both *Cdk1* shRNAs resulted in dramatic decrease of Cdk1 protein (Supplementary Fig. S2). To further characterize the role of Cdk1, we measured the expression levels of marker genes

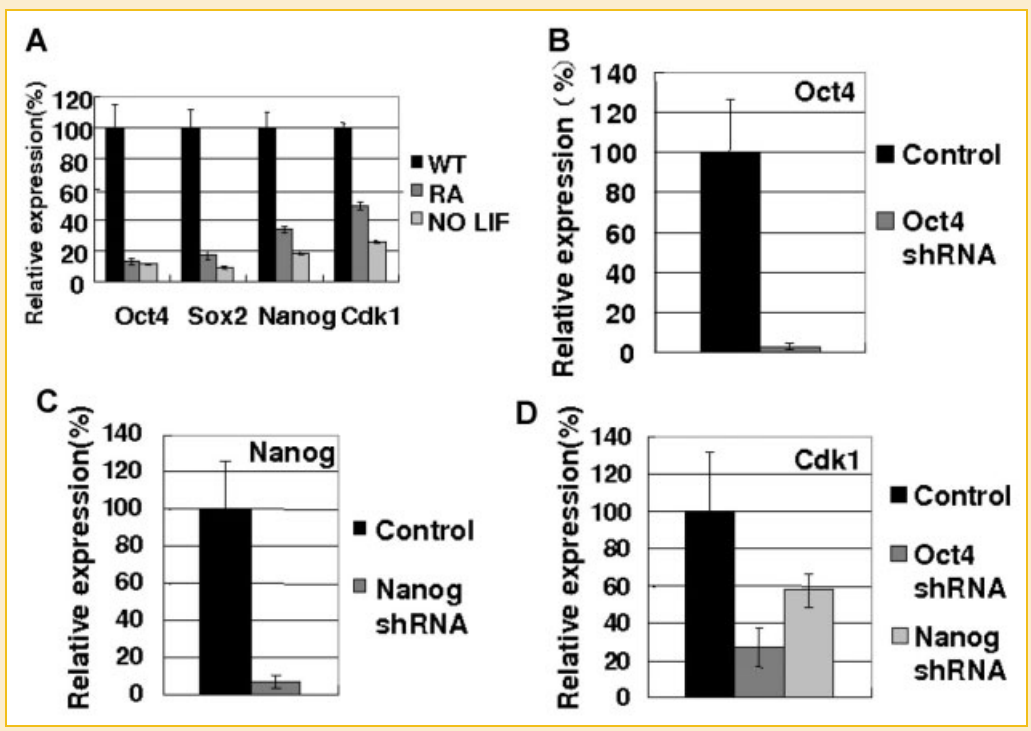

Fig. 1. Undifferentiated mouse ES cells express high level of Cdk1. A: Reduction of *Cdk1* expression in ES cells cultured in differentiation-inducing conditions. ES cells were cultured in medium with RA for 3 days or withdrawn of LIF for 5 days. The expression of *Cdk1* was measured by quantitative real-time PCR analysis. Data are presented as the mean ± SEM. B,C: *Oct4* and *Nanog* mRNA were depleted by their respective shRNA. The mRNA levels of *Oct4* and *Nanog* were determined by real-time PCR after 4 days of puromycin selection. Data are presented as the mean ± SEM. D: The expression of *Cdk1* was measured by real-time PCR in both *Oct4*- and *Nanog*-depleted ES cells. Data are presented as the mean ± SEM.

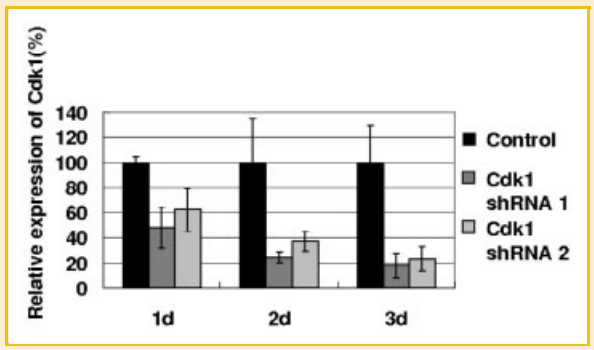

Fig. 2. shRNA knockdown of *Cdk1* in ES cells. Mouse ES cells were transfected with *Cdk1* shRNA constructs. Levels of knockdown were determined by real-time PCR quantification of mRNA harvested after 1, 2, or 4 days of puromycin selections. Data are presented as the mean ± SEM.

important for self-renewal and differentiation. The expressions of *Sox2*, *Esrrb*, and *Tcl1* were significantly reduced to 30–40%, while the expressions of *Nanog* and *Tdgf1* were reduced to 50–60% (Fig. 3A). *Oct4* expression, however, did not change with the *Cdk1* depletion. Conversely, the expressions of trophectoderm genes, such as *Cdx2*, *Hand1*, and *Mash2*, were induced significantly upon the knockdown of *Cdk1*. Changes in the expression of other lineage specific genes such as *Fgf5* and *Msx1* were also observed (Fig. 3B). A third *Cdk1* shRNA construct targeting different site of *Cdk1* gene also show similar maker gene expression profiles (Supplementary Fig. S1).

To further confirm the specificity of the knockdown, three shRNA constructs with scrambled sequences were used. As expected, the scrambled shRNA constructs did not reduce *Cdk1* level (Supplementary Fig. S3A), nor result in perturbation of the marker gene expressions as seen in the *Cdk1* knockdown (Supplementary Fig. S3B). Taken together, the *Cdk1* shRNA constructs were specific and the cellular effects induced by the knockdown were not due to aberrant off-targeting effects.

Next, we performed global gene expression profiling of the *Cdk1*-knockdown ES cells using the NimbleGene microarray. We found about 2241 genes to be differentially expressed upon *Cdk1* depletion (q value ≤ 0.05) (Supplementary Table I). To further examine the functional roles of Cdk1 in ES cell biology, we performed gene ontology (GO) analysis. Notably, we found significant representation of various cellular processes, such as transcription regulation and developmental processes (Supplementary Table II). Pathway mapping of the genes regulated by Cdk1 identified their involvement in various signaling pathways including the MAPK pathway and Wnt signaling pathway (Supplementary Table III).

CDK1 DEPLETION REPRESSED ES CELL PROLIFERATION AND RESULTED IN INCREASED APOPTOSIS

Next, we performed alkaline phosphatase (AP) staining of the *Cdk1*-depleted ES cells. *Cdk1* knockdown cells maintained positive signals for the AP staining (Fig. 4A). However the cells grew in smaller colonies as compared with the mock RNAi control. In cell proliferation assay, *Cdk1* knockdown significantly repressed the

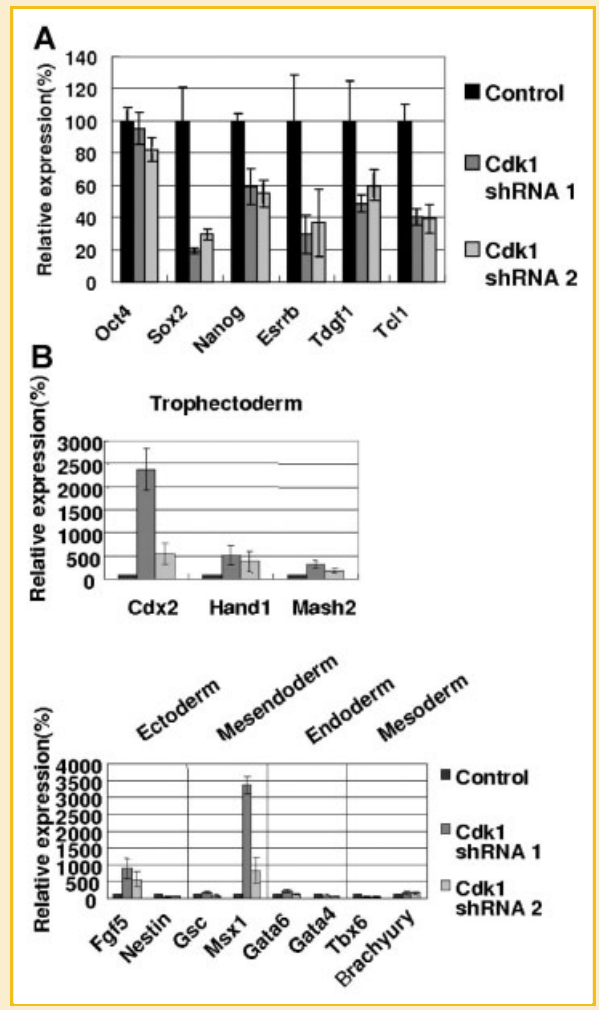

Fig. 3. The expressions of marker genes in *Cdk1*-depleted ES cells. A: Real-time PCR analysis of pluripotency associated genes in the *Cdk1*-depleted ES cells. Cells were harvested after 4 days of puromycin selection. The levels of the transcripts were normalized against the control vector-transfected cells. Data are presented as the mean ± SEM. B: Real-time PCR analysis of lineage-specific marker gene expressions in the *Cdk1*-depleted ES cells. Cells were harvested after 4 days of puromycin selection. The levels of the transcripts were normalized against the control pSuper-transfected cells. Data are presented as the mean ± SEM.

ES cell growth and proliferation (Fig. 4B). Moreover, in the colony re-plating assay, *Cdk1* depleted ES cells did not form any colony (Fig. 4C).

Furthermore, we analyzed the cell cycle profile of the *Cdk1*-depleted ES cells. Using flow cytometry, we found that 4 days after *Cdk1* knockdown, ES cells were arrested at G2 phase (Fig. 5). Together with the earlier findings demonstrating the reduction in cell proliferation (Fig. 4), we speculated that the *Cdk1* depletion in ES cells may result in apoptosis. To demonstrate the possibility, we performed Annexin V staining followed by flow cytometry. Compared to the control cells, *Cdk1* shRNA treated cells had higher rate of apoptosis. This indicates that Cdk1 may be involved in the inhibition of ES cell apoptosis (Fig. 6). For the cell cycle profiling and Annexin V staining experiments, *Cdk1* shRNA 3 showed similar

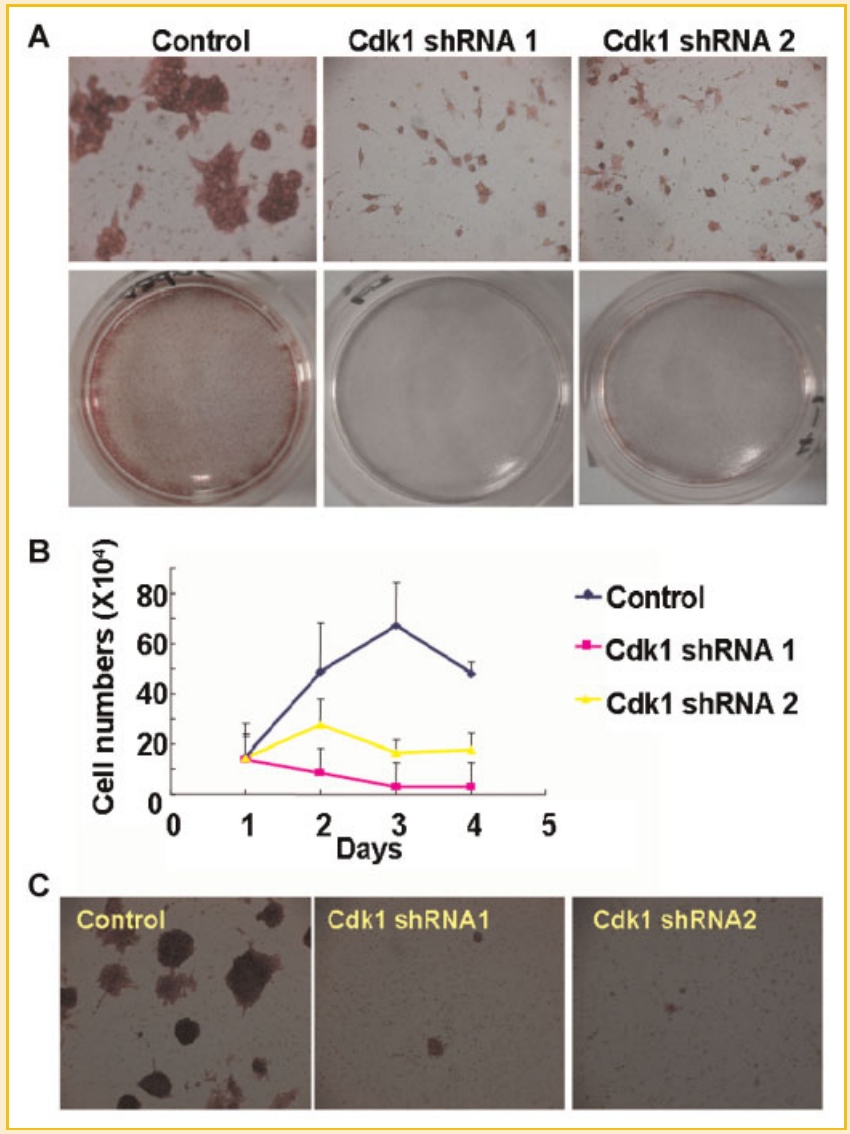

Fig. 4. *Cdk1* depletion inhibits self-renewal of ES cells. A: Alkaline phosphatase staining assay was used to define undifferentiated cells. The cells were stained after 4 days of puromycin selection. Pictures were taken of the bright-field magnification (upper panel) and the whole culture plate (lower panel) after AP staining. B: ES cells were seeded at 10^4 cells/well in a 24-well culture plate and transfected with vector control, *Cdk1* shRNA1 and *Cdk1* shRNA2, respectively. The cell growth was monitored for 4 days after transfection. C: Transfected ES cells from B were dissociated after 4 days of puromycin selection and re-seeded in a 6-well plate at 3×10^5 cells per well. The cells were cultured in the medium with 1 μg/ml puromycin and stained on day 7 after seeding.

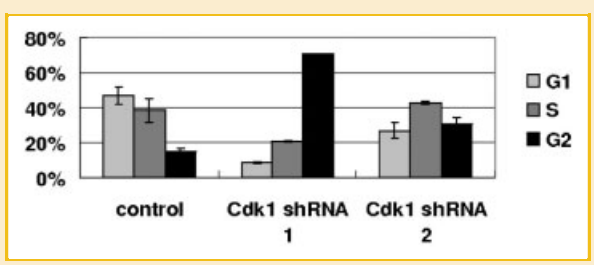

Fig. 5. *Cdk1*-depleted ES cells were arrested at G2 phase. ES cells were seeded at a density of 60×10^4 cells in 60 mm culture plate and transfected with vector control, *Cdk1* shRNA1 or *Cdk1* shRNA2. The cells were fixed with 75% ethanol after 4 days of puromycin selection and analyzed by flow cytometry.

results with the shRNA 1 and 2 (Supplementary Figs. S4 and S5). Together, our results indicate a role for *Cdk1* in the regulation of proliferation and the self-renewal of ES cells.

DISCUSSION

The cell cycle progression in eukaryotic somatic cells is tightly regulated. Cdks, together with cyclins, are the major components of the cell cycle machinery [Morgan, 1997]. Different Cdk-cyclin complexes are respectively involved in specific cell cycle stages. For example, the G1/S transition checkpoint is mainly regulated by the Cdk4/6-cyclin D and Cdk2-cyclin E complexes which phosphorylate Rb and release E2F [Mittnacht, 1998; Trimarchi and Lees, 2002].

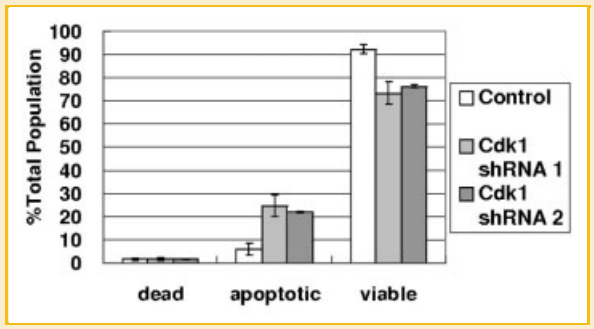

Fig. 6. Reduction of *Cdk1* expression resulted in ES cells apoptosis. ES cells were transfected with vector control, *Cdk1* shRNA1 and *Cdk1* shRNA2, respectively. After 4 days, transfected cells were double-stained with Annexin V and propidium iodide and analyzed by flow cytometry.

However, in ES cells, the cell cycle is uniquely short, primarily owing to absence of the G1/S checkpoint [Savatier et al., 1996; Becker et al., 2006]. And the Cdk4/Cdk6-associated kinase activity is not present whereas the Cdk2-cyclinA/E activity is constitutively active throughout the cell cycle in murine ES cells [Savatier et al., 1996; Stead et al., 2002]. It is of great interest to determine the relationship between the cell cycle regulation and pluripotency maintenance of ES cells. Recently, Zhang et al. showed that pluripotent gene NANOG is involved in cell cycle regulation through direct interaction with CDK6 and CDC25A in human ES cells [Zhang et al., 2009]. The kinase activity of Cdk6 and its interaction with Cyclin D can be detected in murine ES cells, and importantly, its kinase activity decreased significantly upon differentiation [Faast et al., 2004]. Genome-wide mapping of the core transcription factors in mouse ES cells had previously identified binding of Nanog (chr10:68755182–68755252) and Esrrb (chr10:68797315–68797328) at the Cdk1 gene locus, indicating that Cdk1 may be involved in the regulatory network responsible for maintaining the properties of ES cells [Chen et al., 2008].

It was reported that deletion of *Cdk1* leads to early embryonic lethality prior to day E3.5. This indicates an essential role of Cdk1 in early embryonic development [Santamaria et al., 2007; Satyanarayana et al., 2008]. In our study, we show that *Cdk1* depletion compromised the proliferation and self-renewal of the ES cells. This is consistent with a previous study where treatment of mouse ES cells with Cdk1 inhibitor resulted in cell death and increased apoptosis [Ullah et al., 2008]. In *Cdk1* knockdown cells, we found reduction in the level of *Sox2*, *Esrrb*, and *Tcl1*. Interestingly, we did not detect a reduction in the Oct4 mRNA level during *Cdk1* knockdown. The regulation of Cdk1 on self-renewal genes could be direct or indirect. Of note, Cdk1 has been previously shown to interact with Oct4, a key transcription factor that regulates expression of many genes critical for ES cells [Wang et al., 2006]. The mechanistic role of Cdk1 and Oct4 interaction remains to be identified. Cdk1 repression also caused the up-regulation of marker genes for trophectoderm lineages. Interestingly, a previous study has indicated role for Cdk1 in regulating trophoblast stem cells differentiation [Ullah et al., 2008]. How Cdk1 regulates the repression of ES cell differentiation to the trophectoderm lineage, and whether it shares similar mechanistic role in ES cells and trophoblast stem cells will be of great interest for future studies.

In conclusion, our study demonstrated high expression of Cdk1 in undifferentiated state of ES cells. We confirmed the important role of Cdk1 in maintaining ES cell proliferation and self-renewal. Furthermore, we uncovered the role for Cdk1 in the inhibition of trophectoderm differentiation of the ES cells.

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