Repression of Cyclin D1 Expression Is Necessary for the Maintenance of Cell Cycle Exit in Adult Mammalian Cardiomyocytes

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Background: How cell cycle exit is maintained in adult mammalian cardiomyocytes is largely unknown.

Results: Cyclin D1 expression causes cell cycle reentry in >40% of adult mouse cardiomyocytes.

Conclusion: Silencing the cyclin D1 expression is necessary for the maintenance of the cell cycle exit.

Significance: One of the mechanisms regulating cell cycle exit in mammalian cardiomyocytes has been uncovered.

The hearts of neonatal mice and adult zebrafish can regenerate after injury through proliferation of preexisting cardiomyocytes. However, adult mammals are not capable of cardiac regeneration because almost all cardiomyocytes exit their cell cycle. Exactly how the cell cycle exit is maintained and how many adult cardiomyocytes have the potential to reenter the cell cycle are unknown. The expression and activation levels of main cyclin-cyclin-dependent kinase (CDK) complexes are extremely low or undetectable at adult stages. The nucleic DNA content of almost all cardiomyocytes is 2C, indicating the cell cycle exit from G1-phase. Here, we induced expression of cyclin D1, which regulates the progression of G1-phase, only in differentiated cardiomyocytes of adult mice. In these cardiomyocytes, S-phase marker-positive cardiomyocytes and the expression of main cyclins and CDKs increased remarkably, although cyclin B1-CDK1 activation was inhibited in an ATM/ATR-independent manner. The phosphorylation pattern of CDK1 and expression pattern of Cdc25 subtypes suggested that a deficiency in the increase in Cdc25 (a and -b), which is required for M-phase entry, inhibited the cyclin B1-CDK1 activation. Finally, analysis of cell cycle distribution patterns showed that >40% of adult mouse cardiomyocytes reentered the cell cycle by the induction of cyclin D1. The cell cycle of these binucleated cardiomyocytes was arrested before M-phase, and many mononucleated cardiomyocytes entered endoreplication. These data indicate that silencing the cyclin D1 expression is necessary for the maintenance of the cell cycle exit and suggest a mechanism that involves inhibition of M-phase entry.

Cardiomyocytes (CMs)4 are essential for cardiac functions. If many CMs are lost and the loss is not recovered, heart failure or lethality occurs. The neonatal mouse and adult zebrafish are able to recover the loss of CMs after injury through proliferation of preexisting CM and thus regenerate their hearts (1–3). In contrast, adult mammals are not capable of substantial cardiac regeneration.

Recent studies have shown that postnatal CMs proliferate in normal mammals (4, 5). However, the percentages are very limited and insufficient to restore cardiac function after injury (at most, 0.06% per day in injured mice) (5).

These facts indicate that the cell cycle exit is strictly maintained in the majority of adult CMs. This raises two questions. How is the cell cycle exit maintained? How many adult CMs have the potential to reenter the cell cycle? Answers to these questions will contribute to regenerative medicine in the heart because reproliferation of many CMs enables substantial cardiac regeneration.

To address these questions, we have focused on cell cycle regulation in the mouse heart. The mitotic indices in CMs, and the expression and activation levels of main cyclin-CDK complexes (cyclin D-CDK4/6, cyclin E-CDK2, cyclin A-CDK1/2, and cyclin B-CDK1) in the hearts are high during early embryonic stages. These levels decrease from midgestation to birth and then show one wave in which the peak is around postnatal day 5 (P5) (6–9). The wave mainly produces binucleated cells from mononucleated cells. Eighty to ninety percent of CMs become binucleated cells (7, 9). Then all expression and activation levels of main cyclin-CDK complexes become extremely low or undetectable at adult stages. The nucleic DNA content of almost all cardiomyocytes is 2C, indicating the cell cycle exit from G1-phase. Here, we induced expression of cyclin D1, which regulates the progression of G1-phase, only in differentiated cardiomyocytes of adult mice. In these cardiomyocytes, S-phase marker-positive cardiomyocytes and the expression of main cyclins and CDKs increased remarkably, although cyclin B1-CDK1 activation was inhibited in an ATM/ATR-independent manner. The phosphorylation pattern of CDK1 and expression pattern of Cdc25 subtypes suggested that a deficiency in the increase in Cdc25 (a and -b), which is required for M-phase entry, inhibited the cyclin B1-CDK1 activation. Finally, analysis of cell cycle distribution patterns showed that >40% of adult mouse cardiomyocytes reentered the cell cycle by the induction of cyclin D1. The cell cycle of these binucleated cardiomyocytes was arrested before M-phase, and many mononucleated cardiomyocytes entered endoreplication. These data indicate that silencing the cyclin D1 expression is necessary for the maintenance of the cell cycle exit and suggest a mechanism that involves inhibition of M-phase entry.

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4 The abbreviations used are: CM, cardiomyocyte; CDK, cyclin-dependent kinase; ATM, ataxia telangiectasia mutated; ATR, AT- and RAD3-related; P, postnatal day; NLS, nuclear localizing signal; CCD, CAG/loxP-CAT-polyA-loxP-NLS-cyclinD1-IRES-EGFP; MER, Myh6::MERCreMER; Myh6, myosin heavy chain 6 gene; Tam, tamoxifen; PCNA, proliferating cell nuclear antigen; pH3-S10, phospho-histone H3-Ser-10; EdU, 5-ethyl-2’-deoxyuridine; d.p.i., days post-injection; Rb, retinoblastoma protein; H2AX, histone H2A variant X; EGFP, enhanced GFP.
low or undetectable after P14, and the levels are maintained for life. Simultaneously, almost all CMs exit the cell cycle, and the state is strictly maintained. The nuclear DNA content of almost all CMs is 2C (6, 9), showing the cell cycle exit from G1-phase.

Generally, the G1 cell cycle progression is regulated by D-type cyclins-CDK4/6 complexes (10). D-type cyclins are regarded as sensors of the extracellular environment that link the mitogenic pathways to the core cell cycle machinery (11). One of these cyclin, cyclin D1, is required for repression of CM proliferation at midgestation (8). Expression of D-type cyclins as well as other main cyclins in the mouse hearts is very low after P14 (7–9). In the present study we induced expression of cyclin D1 only in differentiated CMs of adult mice to reactivate the cyclin D-CDK4/6 complex and examined the effects on cell cycle progression. We found that >40% of adult CMs reentered the cell cycle due to the induction, indicating that silencing the cyclin D1 expression is necessary for the maintenance of the cell cycle exit.

EXPERIMENTAL PROCEDURES

Vector Construction—The CCD vector was produced by insertion of the nucleotide sequence for HA tag and nuclear localization signal (NLS)-cyclin D1-internal ribosome entry site (IRES)-EGFP into the cloning site of a plasmid containing CAG promoter-loxP-CAT-loxP-cloning site-poly(A) (pBSKSec1 CAG-CAT-Poly(A), a kind gift from Dr. Saga, National Institute of Genetics, Japan). The nucleotide sequence for NLS was tagged to the cyclin D1 transgene, because nuclear import of cyclin D1 is inhibited in rat neonatal and most likely adult CMs (12). That of HA was also tagged to the cyclin D1 transgene.

Mice—Mice with C3H/HeJ Jcl genetic backgrounds were used in all experiments other than experiments using p21Cip1 KO mice. Original MER (Myk6: MERCrmER) (13) mice were backcrossed with C3H/HeJ mice. MER mice have been frequently used in CM-specific gene expression or knock-out studies and also in labeling differentiated CMs (14). The CCD transgenic mouse lines (CAG:loxP-CAT-Poly(A)-loxP-NLS-cyclin D1-ires-EGFP) were produced using the CCD vector, as described previously (15). The CCD (+)/MER (+) double-hemizygote mice were generated by intercrossing between CCD (+) and MER (+) mice. CCD (+)/MER (+); p21Cip1−/− mice were obtained by multiple crossing of CCD (+), MER (+), and p21Cip1−/− (a kind gift from Dr. Leder, Harvard University; C57BL/6 J) mice. Adult mice >6 weeks old were used in this study. For CM-specific induction of NLS-cyclin D1 and EGFP, tamoxifen (Tam, Sigma) was dissolved at 10 mg/ml in corn oil (Sigma), and adult CCD (+)/MER (+) mice were administered 0.1 ml of dissolved Tam into the peritoneal cavity. The expression of transgenes by Cre recombination was confirmed by EGFP expression. The presence of a vaginal plug was regarded as E0.5. All mice were genotyped by PCR. All animals were handled and maintained in accordance with institutional guidelines (Animal Care and Use Committee, Tottori University) and the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan).

Histology and Immunostaining—The immunostaining was performed as described in previous reports (16–18). Frozen sections were used with the antibodies shown in Table 1. EGFP fluorescence was not detected in sections derived from CCD (+)/MER (+) mice with administration of Tam, most likely because of the fixation procedure. For analyses of cyclin D1−/−, BrdU−, Ki67−, and PCNA-positive CMs, frozen sections of the hearts were stained with each antibody (Table 1) using an immunofluorescence method. After these images were photographed, the same sections were stained with an antibody to Nkx2.5 (nuclear marker of CM; Table 1) using a peroxidase-labeled antibody method, because signals for Nkx2.5 are very weak at adult stages. The photographed images were merged, and positive CM (%) was determined from the number of double-positive cells/number of Nkx2.5 positive cells × 100. >100 Nkx2.5 positive cells per mouse were counted. CMs were also identified by staining with an antibody to sarcomeric actin (Table 1). BrdU was injected 24 h before sampling. For analyses of phosphohistone H3-Ser-10 (pH3-S10), cardiac sections were coimmunostained with antibodies against cyclin D1 and pH3-S10. Because signals for Nkx2.5 in pH3-S10 positive nuclei are very weak, cyclin D1 was used as a marker for cardiomyocytes. Images were acquired with microscopes (Axiolmager M1, Carl Zeiss) equipped with imaging software (Axiowision 4.8, Carl Zeiss) at room temperature. A microscopy camera (AxioCam MRC5, Carl Zeiss) was used for immunofluorescence staining and immunohistochemistry.

Enzymatic Dissociation of CMs into Single Cells and Measurement of DNA Content on Slide Glasses—Enzymatic dissociation of CMs of the cardiac ventricles into single cells was performed as described previously (9, 19). 1 mg/20 g body

| Table 1 | Antibodies used in this study |
|---------|-------------------------------|
| WB      | Western blotting; IP; immunoprecipitation; IF; Immunofluorescence; IHC, Immunohistochemistry. |
| Antigens | Antibodies | Assays |
| α-Sarcomeric actin |  |  |
| BrdU | ab6326; Abcam | IF |
| Cdc25a | sc7389; Santa Cruz Biotechnology | WB |
| Cdc25b | sc327; Santa Cruz Biotechnology | WB |
| CDK1 | sc-54; Santa Cruz Biotechnology | WB |
| CDK2 | sc-163; Santa Cruz Biotechnology | WB, IP |
| CDK4 | sc-260; Santa Cruz Biotechnology | WB, IP |
| Cdt1 | A gift from Dr. Hideo Nishitani | WB |
| Chk1 | sc-8408; Santa Cruz Biotechnology | WB |
| Cyclin A | sc-751; Santa Cruz Biotechnology | IP |
| Cyclin A | CY-A1; Sigma | WB |
| Cyclin B1 | GNS1; Thermo | IP |
| Cyclin B1 | V152; Cell Signaling Technology | WB |
| Cyclin D1 | SP4; Lab Vision | WB, IF |
| Cyclin E | 07–683; Millipore | WB, IP |
| γ-H2AX | 2577; Cell Signaling Technology | WB |
| GAPDH | sc-32233; Santa Cruz Biotechnology | WB |
| Geminin | A gift from Dr. Keiko Nakayama | WB |
| H2A.X | 07–627; Millipore | WB |
| Ki-67 | ab15580; Abcam | IF |
| Mcm3 | ab4460; Abcam | WB |
| Mcm4 | A gift from Dr. Hideo Nishitani | WB |
| Nkx2.5 | sc-8657; Santa Cruz Biotechnology | IHC |
| p21Cip1 | SX118; BD Pharmingen | WB |
| p53 | G173–524; BD Pharmingen | WB |
| p53 | P0357; Sigma | WB |
| p53 | sc-6243; Santa Cruz Biotechnology | WB |
| pCDK1-Y15 | 9111; Cell Signaling Technology | WB |
| pCDK2-T160 | 2561; Cell Signaling Technology | WB |
| pChk1-S345 | 2341; Cell Signaling Technology | WB |
| PCNA | sc-56; Santa Cruz Biotechnology | IF |
| pH3-S10 | 06–570; Millipore | IF |
TABLE 2

| Gene         | Forward primer         | Reverse primer         |
|--------------|------------------------|------------------------|
| Cdc25a       | AAAGACCACTTATGGGCTTCTAC    | CATACAGTCGAGGGTGATGG |
| Cdc25b       | TCCACTTGGAAGTCTGCTCT    | ATGTCTTGGAAGAAGCTCC  |
| Cdc25c       | AGTCAAGACGGAGACCTCCAG   | CAGGAGACCCGATTCGAG |
| CDK1         | CAGGAGATGCCAGAAGCTTT    | GAAAGGTTGCTTCTGAGTCC |
| CDK2         | CAGGAGATGGACTCTTATGCCC | ATAGTGACGGATTTCGGAA |
| cyclin A2    | CTTCCCTTCTAGCTATCTGCTG  | GGTGSSCTTGTGAGATGTT |
| cyclin B1    | TTATCTTCTAGTGAAGACTCTC | TCTTCTAGTCTGATAC    |
| cyclin B2    | GGATGGAAGCTCTTGGAAGA   | GTCCTGAGCTCAGGTAGG |
| cyclin E1    | GTCTCAATTGAGCTGATGTC   | GAAAGAACAATTTCTTCTTC |
| cyclin E2    | TGACTCTGACAGCTGACAG   | GCACCACTGAGCTACAC  |
| GAPDH        | GGTGAGGCCAAGAAAGGT3C    | GCCAOAGGACCTCGCTTACG |

weight of EdU was injected into Tam-treated CCD (+)/MER (+) mice at 5 days post-injection (d.p.i.) for analysis at 7 d.p.i. or at both 5 and 7 d.p.i. for analysis at 14, 28, or 91 d.p.i. Dissociated CMs were fixed with 4% paraformaldehyde at 4 °C for 24 h. The CMs were washed once with distilled water and then smeared on slide glasses. EdU incorporation was detected as described previously (20). CMs were identified by staining with an antibody to sarcomeric actin (Table 1), as described in the histological methods (18). DNA was stained with 1 μg/ml DAPI for 30 min. After staining, fluorescence images were acquired with a microscope (BZ-9000, Keyence) equipped with imaging software (Viewer BZ-II, Keyence) at room temperature. A microscopy camera in BZ-9000 was used. Then DNA content per nucleus of CMs was measured with a Cell Cycle Application Module of MetaMorph software (Molecular Devices). The cell cycle distribution patterns of mono- and binucleated CMs was analyzed independently. We analyzed 1500–2000 nuclei of total and binucleated CMs and 100–200 nuclei of mononucleated CMs.

Western Blot Analysis, Immunoprecipitation, and in Vitro Kinase Assay for CDKs—The cardiac ventricles were lysed, and then immunoprecipitation, Western blot analysis, and in vitro kinase assays for CDKs were performed as described previously (9, 18, 21, 22). Antibodies used for immunoprecipitation and Western blot analysis are shown in Table 1. The intensity of the bands in Western blot analysis or in vitro kinase assay was quantified using Image J.

Real Time PCR—Real-time RT-PCR was performed as described previously (8). Primers are shown in Table 2. To standardize the amount of sample cDNA, GAPDH was used as an endogenous control.

Statistical Analysis—The experimental data were analyzed using Student’s t test. Tukey’s multiple comparison test was used after obtaining a significant difference with one-way analysis of variance for multiple comparison tests.

RESULTS

Induction of Cyclin D1 Only in Adult CMs—To investigate whether the reactivation of the cyclin D-CDK4/6 complex promoted the cell cycle reentry of adult mouse CMs in vivo, we induced expression of cyclin D1 only in differentiated adult CMs using an inducible Cre-loxP system (Fig. 1A). Expression of cyclin D1 and EGFP was induced in the hearts of double transgenic mice (CCD (+)/MER (+)) by a single administration of Tam (Fig. 1, B–E). Induction of cyclin D1 was observed in almost all CCD (+)/MER (+) CMs, as >95% of the CM marker, Nkx2.5-positive nuclei, were also positive for exogenous cyclin D1 at 7 d.p.i. (Fig. 1C). These cells were also positive for sarcomeric actin (data not shown). Conversely, all exogenous cyclin D1-positive cells were also positive for Nkxx2.5 and sarcomeric actin. These data indicated that cyclin D1 induction was limited to CMs. In addition, the cyclin D-CDK4 activity increased in the hearts of CCD (+)/MER (+) mice after administration of Tam and was maintained constantly from at least 5 d.p.i. (Fig. 1F).

Increase in Proliferation Marker-positive CMs by Cyclin D1 Induction—We examined the effects of cyclin D1 induction on the cell cycle progression in adult CMs with immunostaining using antibodies against three cell proliferation markers (BrdU, Ki-67, and PCNA) in sections of hearts. The percentages of positive CMs with these markers increased significantly at 7 d.p.i. in CCD (+)/MER (+) mice when compared with control mice (Fig. 2A). Especially, >40% of CMs were positive with PCNA (Fig. 2, A and B). These proliferation marker-positive CMs were uniformly distributed in whole ventricles.

We next examined CMs in M-phase. An extremely low number of CMs (4–8 nuclei/section) were positive with a late G2-M phase marker, pH3-S10, at 7 d.p.i., and judging from the morphology of their nuclei, these cells appeared to be at late G2-phase (Fig. 2C). These data suggested that many CMs reentered the cell cycle, but the cycle was arrested before M-phase.

Expression and Activities of Cyclin-CDK Complexes after Cyclin D1 Induction—Induction of cyclin D1 suggested cell cycle reentry in many CMs. Next, we examined the expression and activities of main cyclin-CDK complexes.

Western blot analysis showed that expression of exogenous cyclin D1 was detected from 3 d.p.i., after which the protein level remained constant thereafter (Fig. 3A). The expression levels of other main cyclins and CDKs were very low or at the background levels in control mice (see 0 d.p.i. in Fig. 3A) (9). However, these protein levels increased markedly by induction of cyclin D1 and showed a peak at 5 or 7 d.p.i. In addition, these patterns were similar to those of the mRNAs (Fig. 3B). These results showed that the expression of main cyclins and CDKs was induced by cyclin D1 induction; however, the levels decreased after 7 d.p.i.

We also examined the expression of proteins related to DNA replication (Cdt1, Geminin, Mcm3, and Mcm4; Fig. 3, C and D). These protein levels were increased by administration of Tam and showed a peak at 5 or 7 d.p.i., although the increase in Cdt1 was only slight. Expression of Cip/Kip family proteins was also examined (Fig. 3E). p21Cip1 levels increased by induction of
cyclin D1, although those of p27Kip1 did not show apparent changes. p57Kip2 was not detected in all adult heart samples. The result was consistent with that of a previous study (23).

We next examined the activities of cyclin-CDK complexes with in vitro kinase assay after immunoprecipitation using antibodies against cyclin E, A, or B1 (Fig. 4, A and B). The patterns

**FIGURE 1. CM-specific induction of cyclin D1 expression in adult CCD(+/+)/MER (+) mouse by Tam administration.** A, the constructs of transgenes are shown. CCD(+/+)/MER(+) mice were generated by intercrossing between CCD(+) and MER(+) mice. MER-Cre-MER localizes from cytoplasm to nuclei in CMs by administration of Tam and excludes the sequences containing the CAT gene with poly(A) signal between loxP sites only in differentiated CMs, resulting in CM-specific induction of cyclin D1 and EGFP expression. B, the bright field (upper panels) and EGFP fluorescence (lower panels) images of the hearts of cyclin D1-induced (CCD(+/+)/MER(+), Tam(+)) and control (CCD(+/+)/MER(-), Tam(-)), and CCD(+/+)/MER(+), Tam(-)) mice at 7 d.p.i. are shown. Tam(+) and Tam(−) indicate mice administered Tam and only vehicle, respectively. These images were photographed immediately after thoracotomy. Scale bar, 5 mm. C, cardiac sections of mice at 7 d.p.i. of Tam were immunostained with an antibody against a CM marker, Nkx2.5, using a peroxidase-labeled antibody method (DAB, upper panels), and then the same sections were stained with an antibody against cyclin D1 (red) using an immunofluorescence method (middle panels). These images were merged (lower panels), and double-positive CM nuclei with Nkx2.5 (pseudo-color, green) and exogenous cyclin D1 (red) are visualized in yellow. These double-positive cells were also positive for sarcomeric actin. Scale bar, 100 μm. D, tissue specificity of exogenous cyclin D1 expression. A CCD(+/+)/MER(+) adult mouse was administered Tam, and equal amounts of extracts from the indicated tissues at 7 d.p.i. were analyzed by Western blot analysis. Whole embryos at E14.5 were used as a positive control. Arrowheads show the positions of positive signals. E, genotype and Tam specificity of exogenous cyclin D1 induction. Equal amounts of extracts from the cardiac ventricles of the indicated mice at 28 d.p.i. were analyzed by Western blot analysis. Whole embryos at E14.5 were used as a positive control. GAPDH was used as an endogenous control. F, CDK4 activation by cyclin D1 induction. Equal amounts of extracts from the cardiac ventricles of CCD(+/+)/MER(+) adult mice administered Tam were immunoprecipitated with an antibody against CDK4 at indicated d.p.i. The immunoprecipitates (IP) were analyzed with an in vitro kinase assay using Rb as a substrate. 32P-Labeled Rb (upper panel), autoradiograph for phosphorylated Rb. Rb (lower panel), Coomassie Brilliant Blue staining images of Rb. Arrowheads show the positions of positive signals.
of the immunoprecipitated cyclins and CDKs were similar to those in Fig. 3A (Fig. 4A). However, the kinase activity of cyclin A-CDK was low, and that of cyclin B1-CDK was extremely low at 5 and 7 d.p.i., although that of cyclin E-CDK was high (Fig. 4, A and B).

The cyclin-CDK1 complex is the master regulator of mitosis, and inhibition of its activation induced G2 arrest or endoreplication in various species and cells (24, 25). Because the dephosphorylation of both Thr-14 and Tyr-15 is required for the activation of CDK1 and entry into M-phase (26), we examined the phosphorylation pattern in the hearts of CCD (+)/MER (+) mice. Mammalian CDK1 that co-immunoprecipitated with cyclin B1 was detected as three different bands on SDS-PAGE. The upper, intermediate, and lower bands represent CDK1 of cyclin B1 was detected as three different bands on SDS-PAGE. The upper, intermediate, and lower bands represent CDK1 of cyclin B1. Mammalian CDK1 that co-immunoprecipitated with cyclin B1 was detected as the major band at 5 and 7 d.p.i. of Tam were co-immunostained with antibodies against PCNA (red) and Nkx2.5. The merged images in the left ventricle are shown. The same methods as described in Fig. 1C were used. Double-positive CM nuclei with Nkx2.5 (pseudo-color, green) and PCNA (red) are visualized in yellow. Scale bar, 100 μm. C, cardiac sections of CCD (+)/MER (+) mice at 7 d.p.i. of Tam were co-immunostained with antibodies against cyclin D1 and pH3-S10. Arrowheads show very rare double-positive cardiomyocyte nuclei with cyclin D1 (green, exogenous expression) and pH3-S10 (red) in three independent images in the left ventricle. Insets show the high power views of the pH3-S10-positive nuclei stained with DAPI. These nuclei appear to be at late G2-phase because nuclear membrane and nucleoli, but not any chromosome condensation, can be seen. Because signals for Nkx2.5 in phospho-H3-S10 positive nuclei are very weak, cyclin D1 was used as a marker for cardiomyocytes. Scale bar, 50 μm.

The lower band of CDK1 in whole embryos at E10.5 was apparent, which is consistent with high CDK1 activity (Fig. 4A). The intermediate and lower bands were not detected at 5 and 7 d.p.i., although those in Fig. 3A were similar to our results (Fig. 4). In addition, conditional knock-out (KO) experiments of single, double, and triple Cdc25 subtypes revealed that only triple and double (Cdc25a and -b) KO mice show robust phenotypes in intestinal epithelial cells, such as the inhibition of mitosis (30, 31). These reports indi-
cated that both Cdc25a and -b are necessary for M-phase entry through dephosphorylation of CDK1. Thus, a deficiency of increases of Cdc25a and -b expression can explain the mechanism that inhibits activation of CDK1.

It is known that the activation of ATM/ATR pathways is involved in the inhibition of CDK1 activation in the G2 checkpoint (32, 33). Phosphorylation of ATM/ATR targets (histone H2AX, Chk1, and p53) are tightly associated with the activation of ATM/ATR pathways (34–37). Therefore, we investigated the expression and phosphorylation of these ATM/ATR targets after induction of cyclin D1. Although H2AX and Chk1 were detected, the phosphorylated forms (γ-H2AX and pChk1-S345) were not observed in the cardiac ventricles of CCD (+)/MER (+) adult mice at indicated d.p.i. The relative mRNA levels are presented as the mean ± S.E.*p < 0.05; **p < 0.01; ***p < 0.005 versus 0 d.p.i. (Tukey’s multiple comparison test after obtaining a significant difference with one-way analysis of variance). The vertical axis is shown as a logarithmic scale.

Reentry by >40% of CMs to the Cell Cycle by Cyclin D1 Induction—Remarkable increases in proliferation markers (Fig. 2A) and in the expression and activities of cyclin-CDK complexes (Fig. 3) strongly suggested that many adult CMs reentered the cell cycle by induction of cyclin D1. To study how many adult CMs reentered the cell cycle and to determine to what phase the cell cycle of these CMs proceeded, we next analyzed the cell cycle distribution patterns in CCD (+)/MER (+) CMs after Tam administration. We measured the DNA content per nucleus of a single CM dissociated from the ventricles on slide glasses, because FACS analysis can neither measure the DNA content in each nucleus in binucleated CMs nor distinguish between mono- and binucleated CMs. The method for mouse cardiomyocytes has been established (9). Nuclei with 2C, 2C-4C, and 4C were regarded as those in G1-, S-, and G2/M-phase, respectively. In addition, to monitor the cell cycle progression of adult CMs after induction of cyclin D1, we simultaneously performed EdU pulse-chase experiments.
The induction of cyclin D1 caused prominent cell cycle entry in adult CMs. DNA content of the majority of nuclei (91.5%) was 2C in total CMs of control mice, whereas 4C nuclei were rare (3.9%) (Fig. 6, A and B). After induction of cyclin D1, 2C nuclei gradually decreased, whereas 4C nuclei increased (Fig. 6, A–C). The final percentages of 2C and 4C were 44.2 and 30.8%, respectively (Fig. 6B), showing that >40% of total CM nuclei entered the cell cycle (91.5 – 44.2 = 46.3%). These percentages of binucleated cells were almost the same as those of total CMs (Fig. 6, A and B). The cell cycle progression of mononucleated CMs was more prominent. The percentages of 2C and 4C in mononucleated CMs of control were 72.8 and 18%, respectively.
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Subsequently, 8C CMs were detected at later time points (Fig. 6B). These results were consistent with the findings that mitotic CMs were not observed (Fig. 2C) and that the kinase activity of cyclin B1-CDK was extremely low (Fig. 4). These data showed that >40% of CMs reentered the cell cycle. The cell cycle of almost all of these binucleated CMs was arrested before M-phase, and many mononucleated CMs entered endoreplication, suggesting inhibition of M-phase entry in both CMs.

Increase in p21^{Cip1} Is Not Essential for Inhibition of the M-phase Entry—Finally, we examined whether the increase in p21^{Cip1} level (Fig. 3E) inhibited M-phase entry. We produced double transgenic and p21^{Cip1} knock-out mice (CD(CD) (+) /MER (+); p21^{Cip1}+/−) by crossing, and then Tam was administered. The cell cycle distribution patterns of these mice and control KO mice (CD (−) /MER (−); p21^{Cip1}+/−) were examined. The patterns of the control mice showed abnormalities (increases in nuclei in 4C in both mono- and binucleated CMs and 8C in mononucleated CMs) (Fig. 7A, left) as compared with wild type control mice (Fig. 6Aa). However, the abnormalities would result from those that occurred during postnatal stages, because CMs in p21^{Cip1}+/− mice already showed the same phenotypes at P14 (23) and no EdU-positive CMs were detected in the adult CD (−) /MER (−); p21^{Cip1}+/− mouse (Fig. 7A, left). Effects of cyclin D1 induction were observed in the CD (CD) (+) /MER (+); p21^{Cip1}+/− mouse, as 2C nuclei decreased and those of 4C (both mono- and binucleated CMs) and 8C (mononucleated CMs) increased compared with the CD (−) /MER (−); p21^{Cip1}+/− mouse (Fig. 7, A and B). These features were similar to those of CD (CD) (+) /MER (+); p21^{Cip1}+/− mice (Fig. 6D). If CMs enter the M-phase, increases in EdU-positive 2C nuclei and/or tri- or tetr triturated CMs are expected. However, most of the EdU-positive nuclei were not in 2C in both mono- and binucleated CMs. Only two 2C nuclei were observed in a binucleated CM. In addition, the percentages of tri- and tetranucleated CMs increased slightly, a similar increase was observed in the CD (CD) (+) /MER (+); p21^{Cip1}+/− mice (Fig. 6Bb). Therefore, we concluded that an increase in p21^{Cip1} is not essential for the inhibition of M-phase entry.

DISCUSSION

The expression of cyclin D1 as well as other D-type cyclins and main cyclins decreases to very low levels after P14 when binucleation is almost completed. The silent state of cyclin D1 maintained for life (7, 9). In the present experiments we showed that induction of cyclin D1 caused an increase in expression of main cyclins and CDKs and cell cycle reentry in >40% of CMs (Figs. 2, 3, 6, and 7). These results show that the cyclin D1 expression is necessary for the maintenance of the cell cycle exit in many CMs. D-type cyclins (D1, D2, and D3) have critical and redundant functions in cardiomyocyte proliferation because triple, but not single or double knock-out, mice showed phenotypes in the cardiomyocyte

FIGURE 5. Phosphorylated forms of histone H2AX and Chk1 and expression of p53 are not detected after induction of cyclin D1. Phosphorylated histone H2AX (γ-H2AX), histone H2AX (H2AX) (A), phosphorylated Chk1 (pChk1-S345), Chk1 (B), and p53 (C) were analyzed by Western blot analysis using extracts from the cardiac ventricles of CD(CD) (+) /MER (+) adult mice or other genotyped mice at indicated d.p.i. of Tam. NIH3T3 cells were used as positive (+IR and +Thy) and negative controls (−IR and −Thy) for phosphorylation. +IR, irradiated at 10-gray dose; −IR, no irradiation; +Thy, treated with 2 μM thymidine for 20 h; −Thy, no treatment with thymidine. Whole embryos from 13.5 d.p.c and later (CCD MER(+) and 8C CMs of multiple mice at 14 d.p.i., and the changes in 2C and 4C in both bi- and mononucleated CMs and in >4C CMs of mononucleated CMs were significant (Fig. 6D). These data also showed that ~40 and 60% of bi- and mononucleated CMs entered the cell cycle at the stage, respectively (control 2C (%) − CD (−) /MER (−); 2C (%): binucleated CMs, 90.1–51.6 = 38.5%; mononucleated CMs, 72.6–12.9 = 59.7%).

EdU was injected at 5 d.p.i. or at both 5 and 7 d.p.i. EdU-positive nuclei of the total and binucleated CMs were mainly detected in the 4C population (Fig. 6Aa). EdU-positive mononucleated CMs were found first in the 4C population at 7 d.p.i. Subsequently, 8C CMs were detected at later time points (Fig. 6Aa), indicating that these CMs entered endoreplication by skipping M-phase. No EdU-positive 2C nuclei was observed in either mono- or binucleated CMs, indicating that these cells could not complete karyokinesis or cytokinesis (Fig. 6Aa). These results were consistent with the findings that mitotic CMs were not observed (Fig. 2C) and that the kinase activity of cyclin B1-CDK was extremely low (Fig. 4). These data showed that >40% of CMs reentered the cell cycle. The cell cycle of almost all of these binucleated CMs was arrested before M-phase, and many mononucleated CMs entered endoreplication, suggesting inhibition of M-phase entry in both CMs.

Increase in p21^{Cip1} Is Not Essential for Inhibition of the M-phase Entry—Finally, we examined whether the increase in p21^{Cip1} level (Fig. 3E) inhibited M-phase entry. We produced double transgenic and p21^{Cip1} knock-out mice (CD(CD) (+) /MER (+); p21^{Cip1}+/−) by crossing, and then Tam was administered. The cell cycle distribution patterns of these mice and control KO mice (CD (−) /MER (−); p21^{Cip1}+/−) were examined. The patterns of the control mice showed abnormalities (increases in nuclei in 4C in both mono- and binucleated CMs and 8C in mononucleated CMs) (Fig. 7A, left) as compared with wild type control mice (Fig. 6Aa). However, the abnormalities would result from those that occurred during postnatal stages, because CMs in p21^{Cip1}+/− mice already showed the same phenotypes at P14 (23) and no EdU positive CMs were detected in the adult CD (−) /MER (−); p21^{Cip1}+/− mouse (Fig. 7A, left). Effects of cyclin D1 induction were observed in the CD (CD) (+) /MER (+); p21^{Cip1}+/− mouse, as 2C nuclei decreased and those of 4C (both mono- and binucleated CMs) and 8C (mononucleated CMs) increased compared with the CD (−) /MER (−); p21^{Cip1}+/− mouse (Fig. 7, A and B). These features were similar to those of CD (CD) (+) /MER (+); p21^{Cip1}+/− mice (Fig. 6D). If CMs enter the M-phase, increases in EdU-positive 2C nuclei and/or tri- or tetr triturated CMs are expected. However, most of the EdU-positive nuclei were not in 2C in both mono- and binucleated CMs. Only two 2C nuclei were observed in a binucleated CM. In addition, the percentages of tri- and tetr triturated CMs did not increase in the CD (CD) (+) /MER (+); p21^{Cip1}+/− mouse at 14 d.p.i. of Tam (Fig. 7C). Although the percentage of binucleated CMs increased slightly, a similar increase was observed in the CD (CD) (+) /MER (+); p21^{Cip1}+/− mice (Fig. 6Bb). Therefore, we concluded that an increase in p21^{Cip1} is not essential for the inhibition of M-phase entry.

DISCUSSION

The expression of cyclin D1 as well as other D-type cyclins and main cyclins decrease to very low levels after P14 when binucleation is almost completed. The silent state of cyclin D1 maintained for life (7, 9). In the present experiments we showed that induction of cyclin D1 caused an increase in expression of main cyclins and CDKs and cell cycle reentry in >40% of CMs (Figs. 2, 3, 6, and 7). These results show that the cyclin D1 expression is necessary for the maintenance of the cell cycle exit in many CMs. D-type cyclins (D1, D2, and D3) have critical and redundant functions in cardiomyocyte proliferation because triple, but not single or double knock-out, mice showed phenotypes in the cardiomyocyte

(6Aa)
proliferation (39). These results suggest that silencing the expression of other D-type cyclins (D2 and D3) is also necessary for the maintenance of the cell cycle exit in CMs. After cell cycle reentry, the cell cycle of CMs was arrested in 4C (binucleated cells) and 4C or 8C (mononucleated cells) (Fig. 6). These results suggest two possibilities.
p21Cip1 is not essential for inhibition of the M-phase entry. A, the histograms show the number of nuclei with various DNA contents for bi- (upper) and mononucleated (lower) CMs in CCD/+/MER−; p21Cip1+/− and CCD/+/MER+; p21Cip1+/− mice at 14 d.p.i. of Tam. EdU was injected at both 5 and 7 d.p.i. No EdU positive CMs were detected in the CCD/−/MER−; p21Cip1+/− mouse. B, percentages of 2C, 2C-4C, 4C, and >4C populations in bi- and mononucleated CMs of mice in A. C, percentages of mono-, bi-, and tri-/tetraneucleated CMs of mice in A.

One is that the G2 checkpoint was induced by negative effects due to cyclin D1 induction, such as DNA damage, incomplete DNA replication, or prolonged expression of cyclin D1, and the checkpoint prevented CMs from entering mitosis. The G2 checkpoint is regulated by ATM/ATR pathways, including p21Cip1 and Cdc25 (40, 41). However, no signs indicating activation of ATM/ATR or DNA damage were detected in the hearts of control or cyclin D1-induced mice (before and after cell cycle reentry, Fig. 5). In addition, the DNA content of many mononucleated CMs in cyclin D1-induced mice increased to 8C from 4C (Fig. 6), suggesting completion of DNA replication.

Regarding p21Cip1, the protein level increased after cyclin D1 induction (Fig. 3E). However, the upstream protein, p53 in ATM/ATR pathways (40, 41) was not detected in any adult hearts (Fig. 5C). Moreover, CMs of p21Cip1−/− knock-out mice, in which cyclin D1 was induced, still did not exhibit robust M-phase entry (Fig. 7), indicating that an increase in p21Cip1 is not essential for inhibition of the M-phase entry. p21Cip1 has positive functions for cell cycle progression as well as negative functions (11), suggesting that the increase was related to cell cycle progression. In fact, p21Cip1 is highly expressed in mouse embryonic hearts when the proliferation activities are high (23).

Regarding Cdc25, the expression of Cdc25a and -b did not increase after cyclin D1 induction (Fig. 4, C and D). In ATM/ATR pathways, Cdc25c among Cdc25 subtypes is repressed by p53 (40, 41). These facts cannot explain the deficiency in increases of Cdc25a and -b. These results suggest that an increase in p21Cip1 and deficiency in increases of Cdc25a and -b are not related to ATM/ATR pathways.

Another possibility is that CMs have a natural system independent of the negative effects due to cyclin D1 induction, and the system also prevents CMs from entering mitosis after cell cycle reentry. To prove the possibility, we need to demonstrate the same phenotypes in other mouse systems that are independent of the negative effects of cyclin D1 induction and show cell cycle reentry. We have observed similar phenotypes to cyclin D1-induced mice; increases in 4C (both mono- and binucleated CMs) and 8C (mononucleated CMs) populations, in p21Cip1−/− and p21Cip1−/− knockout mice (23).

From the discussion above, the second possibility, which is that CMs have a natural system inhibiting M-phase entry, is
more likely. The system would function to maintain cell cycle exit as well as silencing the cyclin D1 expression. As the mechanism of the system, deficiency in increases of Cdc25a and -b expression (Fig. 4, D and E) can explain the inhibition of CDK1 activation (Fig. 4, A–C), because both Cdc25a and -b are necessary for M-phase entry (29–31). If this is the case, the core of the system inhibiting M-phase entry is the silencing of the expression of Cdc25a and -b and its maintenance.

Previous transgenic studies have shown the effects of exogenous D-type cyclins driven by the Myh6 promoter in CMs; however, these results are markedly different from our data (42, 43). Reentry to the cell cycle by many adult CMs, which was observed in the present study (>40%, PCNA staining and cell cycle distribution patterns; Figs. 2 and 6), was not reported in these studies (at most, 0.3% incorporation of tritiated thymidine in intact mice) (42, 43). The most critical difference in methods is the developmental stages when exogenous D-type cyclins were expressed. D-type cyclins were expressed continuously from embryos to adults in the previous studies, because the Myh6 promoter works from early embryonic stages (18, 44). By contrast, we expressed cyclin D1 only at adult stages using an inducible Cre-loxP system (Fig. 1). The strong effects of cyclin D1 induction on cell cycle progression or main cell cycle regulators were transient (Figs. 2–4). Therefore, we speculate that the strong effects had transiently occurred only at embryonic or postnatal but not adult stages in the previous studies. In fact, the increase in expression of main cell cycle regulators was not high at adult stages. Especially, CDK1 expression was not detected (42, 43).

In the present study we analyzed the cell cycle distribution patterns of CMs in cyclin D1-induced mice by measurement of DNA content per nucleus and EdU pulse-chase experiments. The combined method can show completion of karyokinesis and cytokinesis by detecting an EdU-positive 2C nucleus in CMs. Thus, this method is a powerful tool with which analyze completion of mitosis. However, we could identify neither karyokinesis nor cytokinesis in the CMs of cyclin D1-induced adult mice, because no EdU-positive 2C nuclei were observed using this method. In addition, no pH3-S10 positive cells showed mitotic figures (Fig. 2C). These data indicated that mitosis of CMs, if any, would occur very rarely in cyclin D1-induced mice. Recent studies reported that a small number of CMs can complete mitosis at postnatal or adult stages (4, 5, 45, 46). The evaluation of karyokinesis and cytokinesis in some studies mainly depends on immunostaining against pH3-S10 or Aurora B in cardiac sections. However, it is impossible to conclude the completion of karyokinesis or cytokinesis only by these stainings. Evaluation of these results with methods such as ours will be more reliable.

The following two issues are important when considering further molecular mechanisms of cell cycle exit of CMs. First, what factors are required for maintenance of the silent state of cyclin D1? We speculate that epigenetic regulation maintains silencing of these genes because mRNA levels of these genes are down-regulated (data not shown). Second, what mechanisms are involved in the inhibition of cyclin B1-CDK activation in postnatal CMs? Deficiency in increases of Cdc25a and -b is more likely (Fig. 4, D and E). This needs to be proven, and how the silent state is maintained in an ATM/ATR independent manner should also be examined.

Elucidation of these issues will provide new insights to facilitate the proliferation of the CMs and will contribute to regenerative therapy by the proliferation of pre-existing CMs. It is very interesting that CMs have multiple systems for cell cycle exit (at least three systems; silencing the cyclin D1 expression, inhibition of M-phase entry, and a system inhibiting cytokinesis) in postnatal and adult mammals, which is distinct from non-mammalian model animals, which can regenerate their hearts at adult stages, such as zebrafish and newts (47, 48). Why do mammals have multiple inhibitory systems? We speculate that the robust cell cycle exit is necessary for the homeostasis of mammalian cardiac functions, and the multiple systems act as a backup mechanism to maintain the cell cycle exit robustly in postnatal and adult CMs. This could be examined and verified by canceling all inhibitory systems. If this is the case, careful manipulation of the reset of cell cycle exit is required for regenerative therapy. Future studies regarding cell cycle exit of CMs will be able to clarify the possibility of cardiac regeneration in mammals, significance of cell cycle exit in cardiac functions, and the property that determines the difference in CM proliferation and cardiac regeneration between mammalian and non-mammalian model animals.

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