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Coordinated regulation of differentiation and proliferation of embryonic cardiomyocytes by a jumonji (Jarid2)-cyclin D1 pathway

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SUMMARY

In general, cell proliferation and differentiation show an inverse relationship, and are regulated in a coordinated manner during development. Embryonic cardiomyocytes must support embryonic life by functional differentiation such as beating, and proliferate actively to increase the size of the heart. Therefore, progression of both proliferation and differentiation is indispensable. It remains unknown whether proliferation and differentiation are related in these embryonic cardiomyocytes. We focused on abnormal phenotypes, such as hyperproliferation, inhibition of differentiation and enhanced expression of cyclin D1 in cardiomyocytes of mice with mutant jumonji (*Jmj*, *Jarid2*), which encodes the repressor of cyclin D1. Analysis of *Jmj*/cyclin D1 double mutant mice showed that *Jmj* was required for normal differentiation and normal expression of GATA4 protein through cyclin D1. Analysis of transgenic mice revealed that enhanced expression of cyclin D1 decreased GATA4 protein expression and inhibited the differentiation of cardiomyocytes in a CDK4/6-dependent manner, and that exogenous expression of GATA4 rescued the abnormal differentiation. Finally, CDK4 phosphorylated GATA4 directly, which promoted the degradation of GATA4 in cultured cells. These results suggest that CDK4 activated by cyclin D1 inhibits differentiation of cardiomyocytes by degradation of GATA4, and that initiation of *Jmj* expression unleashes the inhibition by repression of cyclin D1 expression and allows progression of differentiation, as well as repression of proliferation. Thus, a Jmj-cyclin D1 pathway coordinately regulates proliferation and differentiation of cardiomyocytes.

KEY WORDS: Mouse, Heart, Cell cycle, GATA4, Cyclin D1, CDK4, Jumonji (Jarid2)

INTRODUCTION

Regulation of both cell proliferation and differentiation is essential for normal organogenesis in order to obtain appropriate size, morphology and function. Proliferation and differentiation are closely related to each other, and are regulated in a coordinated manner. In general, the levels of proliferation and differentiation show an inverse relationship. Although the mechanisms of proliferation and differentiation have each been studied extensively, the mechanisms that link these two events and their coordination remain largely unknown. It is unclear whether proliferation and differentiation inhibit each other, and if they do, by what molecular mechanisms.

The heart begins to function in the early stages of development and is indispensable for embryonic survival and development. Cardiomyocytes in the heart begin to differentiate and beat at an early stage of development before cardiac looping occurs, and then continue to pump the circulating blood, thus supplying oxygen and nutrients to the entire body. Although the myofilament structures in cardiomyocytes are immature in the early stages of development, afterwards the structure and cytoarchitecture undergo significant development to support the life of the growing embryo. If this differentiation does not subsequently occur or progress, the embryo cannot survive. However, active proliferation of the cardiomyocytes is also necessary for embryonic development. As the size of the embryo increases substantially each day, heart size must also increase to circulate more and more blood. During embryonic development of the mammalian heart, the proliferation of cardiomyocytes results in an increase in the size of the heart. Therefore, both proliferation and functional differentiation progress simultaneously, and are indispensable for embryonic development. The situation is different from many other types of cells, such as skeletal muscle cells or neuronal cells, which are known to differentiate functionally after cell cycle arrest. In embryonic cardiomyocytes, which differ in proliferation and differentiation, the issue remains of whether proliferation and differentiation are related to each other, and whether one inhibits the other.

In the present study, we focused on abnormal phenotypes in the heart of jumonji (*Jmj* or *Jarid2*) mutant mice. The *Jmj* gene and the mutant mice were originally identified by a mouse gene trap strategy (Takeuchi et al., 1995). The *Jmj* mutant mice exhibit various abnormalities, such as neural tube, cardiac, neural and haematopoietic defects (Jung et al., 2005; Takahashi et al., 2007; Takeuchi et al., 2006), and die of cardiac or haematopoietic defects in utero (Kitajima et al., 1999; Takahashi et al., 2004). *Jmj* mutant mice with a C3H background exhibit hyperproliferation of cardiomyocytes on embryonic day 10.5 (E10.5), and die around E11.5 (Takeuchi et al., 1999). Expression of cyclin D1 is increased in these cardiomyocytes (Takeuchi et al., 2006; Toyoda et al.,

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Table 1. Antibodies used

Antigen	Antibody (source)	Methods used
α-Tubulin	DM1A (Sigma)	WB
Actinin	A7811 (Sigma)	IF
ANP	CHMICON AB5490 (Millipore)	IF
BrdU	M0744 (DACO)	IF
CDK4	sc-260 (Santa Cruz Biotechnology)	WB
CDK4	C-18720 (BD Transduction Laboratories)	WB
Cyclin D1	sc-450 (Santa Cruz Biotechnology)	IF, WB
Cyclin D1	553 (Medical & Biological Laboratories)	WB
GAPDH	sc-32233 (Santa Cruz Biotechnology)	WB
GATA4	sc-1237 (Santa Cruz Biotechnology)	IF, WB
HA	12CA5 (Roche Applied Science)	WB
MEF2	sc-313 (Santa Cruz Biotechnology)	IF
MHC	MF20	IF
Nkx2.5	sc-8697 (Santa Cruz Biotechnology)	IF, WB
pGATA4-S105	Ab-5245 (Abcam)	WB
Sarcomeric α-actin	5c5 (Sigma)	IF, IHC

IF, immunofluorescence; IHC, immunohistochemistry; WB, western blotting.

2003). D-type cyclins, including cyclin D1, are key components of the cell cycle machinery. The cyclin-dependent kinases, CDK4/6, are activated by D-type cyclins and trigger cell cycle progression by phosphorylating critical proteins, such as RB (Sherr, 1993; Sherr, 1994). Our studies showed that the level of proliferation of cardiomyocytes in normal mouse embryos exhibits a peak around E10-E12, following which the level decreases (Toyoda et al., 2003). We also showed that *Jmj* triggers the decrease in proliferation of cardiomyocytes by repression of cyclin D1 expression, and that Jmj shows transcriptional repressor activity by recruiting histone H3-K9 methyltransferases, G9a and GLP, to the cyclin D1 promoter (Shirato et al., 2009; Toyoda et al., 2003).

In addition to hyperproliferation, the differentiation of cardiomyocytes is also impaired in *Jmj* mutant mice (Takeuchi et al., 1999). We speculated that the abnormal differentiation of cardiomyocytes is related to hyperproliferation and/or enhanced expression of cyclin D1. In the present study, we examined this possibility and found that CDK4 activated by cyclin D1 inhibits differentiation of cardiomyocytes by degradation of GATA4, and that Jmj unleashes the inhibition by repression of cyclin D1 expression. Thus, our studies showed that proliferation inhibits differentiation of embryonic cardiomyocytes and revealed the mechanism by which the inhibition is unleashed during development.

MATERIALS AND METHODS

Vector construction

Three vectors for production of transgenic mice were constructed. All transgenes were driven by α-myosin heavy chain promoter (a kind gift from Dr J. Robbins, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA, GenBank U71441) (Subramaniam et al., 1991). We first produced two template vectors by insertion of internal ribosome entry site (IRES)-*EGFP* cDNA or IRES-*DsRED* cDNA into the cloning site of a plasmid containing the α-myosin heavy chain promoter (designated as pMIE or pMID, respectively), and then inserted a cDNA encoding cyclin D1 or cyclin D1K112E (a kind gift from Dr P. W. Hinds, Tufts Medical Center, Boston, MA, USA) into pMIE, and GATA4 (a kind gift from Dr M. Shirai, National Cardiovascular Center Research Institute, Suita, Japan) into pMID. These vectors were designated as MDE, MKE and MGD, respectively (Fig. 3A, Fig. 4A, Fig. 5A).

Mice

Mice with the C3H/HeJ Jcl (Clea Japan, Tokyo, Japan) genetic background were used. Original cyclin D1 mutant mice (Sicinski et al., 1995) were obtained from The Jackson Laboratory (Bar Harbor, ME) and were backcrossed with C3H/HeJ mice. The $Jmj^{trap/trap}$ or cyclin $D1^{-/-}$ mice were generated by intercrossing. Transgenic mice with MDE, MKE or MGD were produced as described previously (Hogan et al., 1994). *MDE(+)* and *MKE(+)* embryos expressed GFP in the heart. For an as yet unknown reason, Ds-Red fluorescence was not detected in *MGD(+)* embryos. Only founder embryos at E10.5 were analysed without establishing transgenic lines, because cyclin D1 expression in the heart causes embryonic lethality. Therefore, the sites of insertion in the genome would be different among the transgenic mice. As many embryos were dead or unhealthy owing to manipulation for making transgenic mice, we did not analyse these embryos to avoid any potential secondary effects. We could easily select transgenic embryos in which the heart strongly expressed the transgene using GFP fluorescence. All mice were genotyped by PCR. The presence of a vaginal plug was regarded as E0.5.

Histology and immunostaining

The immunostaining, in situ hybridisation and transmission electron microscopy procedures were as described previously (Motoyama et al., 1997; Takeuchi et al., 1999; Takeuchi et al., 1995). For immunostaining, frozen sections were stained with the antibodies shown in Table 1. At least three embryos of each genotype were examined, and only data in which the same results were obtained are described. GFP fluorescence was not detected in sections derived from transgenic mice, most proabably because of the fixation procedure.

Table 2. Primers used

Gene	Forward primer	Reverse primer
α-cardiac actin	AGAGTATGATGAGGCAGGCC	ATGACTGATGAGAGATGGGG
α-MHC	AACTTAATGCGGCTGCAGGA	GCATCTTCTTGGCACCAATG
ANP	GGCCATATTGGAGCAAATCCTGTG	CATGACCTCATCTTCTACCGGCAT
β-MHC	GCCAACACCAACCTGTCCAAGTTC	TGCAAAGGCTCCAGGTCTGAGGGC
Cyclin D1	CTGGCCATGAACTACCTGGA	ATCCGCCTCTGGCATTTTGG
GAPDH	GGGTGG GCCAAACGGGTCATC	GCCAGTGAGCTTCCCGTTCAGC
GATA4	TGCGGCCTCTACATGAAGCT	GACAGCACTGGATGGATGGA
MLC-2v	AAAGAGGCTCCAGGTCCAAT	CCTCTCTGCTTGTGTGGTCA

Real-time RT-PCR

Real-time RT-PCR was performed as described previously (Toyoda et al., 2003). Primers are shown in Table 2. To standardise the amount of sample cDNA, *GAPDH* was used as an endogenous control.

Western blotting analysis

Western blotting analysis was performed as described previously (Toyoda et al., 2000). The endocardial cushion was removed from cardiac ventricles. Membranes to which proteins were transferred were probed with the antibodies shown in Table 1. Signal intensity was measured using Image J (http://rsbweb.nih.gov/ij/).

In vitro phosphorylation assay

Recombinant GATA4 and GATA4-S105A proteins were produced in *Escherichia coli* using a pCold expression vector (Takara Bio, Otsu, Japan) and purified using His-tag and TALON Superflow Metal Affinity Resin (Takara Bio). Recombinant RB protein was purchased from QED Bioscience. These recombinant proteins were incubated in 20 μl of kinase reaction buffer containing 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 2.5 mM EGTA, 20 μM DTT, 0.1 mM ATP and 5.4 μCi [γ-^{32}P] ATP (3000 Ci/mmol; Institute of Isotopes, Budapest, Hungary) with or without 0.1 μg of recombinant cyclin D1-CDK4 complex (Abcam, Cambridge, UK) at 30°C for 2 hours. For experiments using an antibody against GATA4 phosphorylated at S105 (α-pGATA4-S105; Abcam), [γ-^{32}P] ATP was not added. Reactants were subjected to SDS-PAGE and phosphorylated proteins were detected by autoradiography or western blotting analysis using α-pGATA4-S105. GATA4 proteins were detected with CBB staining or western blotting analysis using an antibody against HA because GATA4 proteins were tagged with HA.

In vivo phosphorylation assay

An expression vector for GATA4 was transfected into HeLa cells with or without expression vectors for cyclin D1/CDK4 (wild type or mutant). PD0332991 (0.5 μM, Axon Medchem) was used as a CDK4 inhibitor, and was added 6 hours after transfection. Cell lysates were analysed by western blotting 2 days after transfection.

Degradation assay

Expression vectors for GATA4 and GATA4-S105A were transfected into HeLa cells with or without expression vectors for cyclin D1/CDK4 (wild type or mutant). Cells were treated with 12.5 μg/ml cycloheximide with or without 20 μM MG132 1 day after transfection and then incubated for 0-4 hours. Cell lysates were subjected to western blotting analysis. The intensity of the bands was quantified using Image J, and the signal intensity of GATA4 or GATA4-S105A was normalised using the individual levels of α-tubulin.

Statistical analysis

The experimental data were analysed using Student's *t*-test. Tukey's multiple comparison test was also used when a significant difference was detected with one- or two-way analysis of variance (ANOVA) for multiple comparison tests.

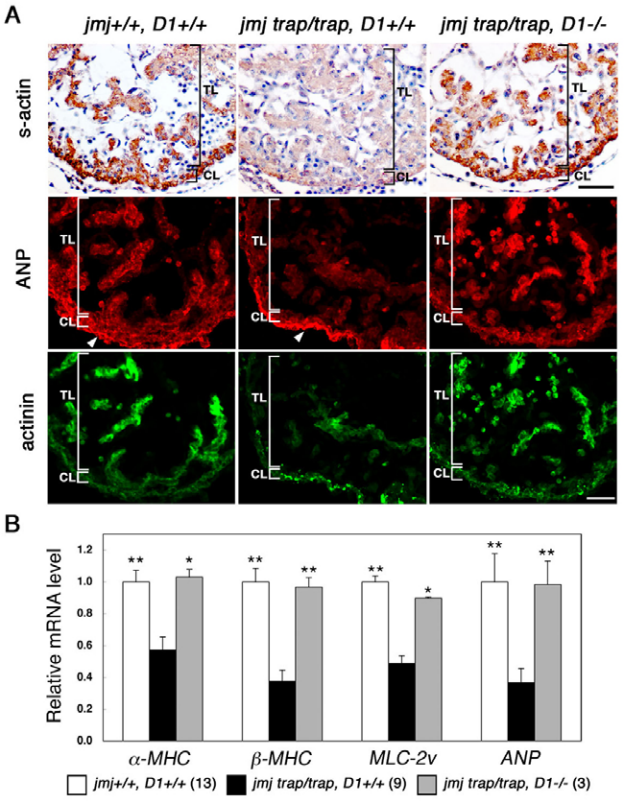

Fig. 1. Loss of cyclin D1 in *Jmj*$^{trap/trap}$ embryos rescues abnormal differentiation. (**A**) Morphology and expression of differentiated cardiomyocyte marker proteins in the left ventricle of wild-type (*Jmj*+/+, *D1*+/+), *Jmj* mutant (*Jmj trap/trap, D1*+/+) and *Jmj*/cyclin D1 double mutant (*Jmj trap/trap, D1*–/–) embryos at E10.5. Transverse sections were analysed by immunohistochemistry for α-sarcomeric actin and stained with Haematoxylin for counterstaining (s-actin) or by immunofluorescence staining (ANP and actinin). TL and CL, trabecular and compact layers, respectively. White arrowheads, the chest wall showing non-specific staining. Scale bars: 50 μm. (**B**) mRNA levels of differentiated cardiomyocyte markers in cardiac ventricles were analysed by real-time quantitative PCR. Genotypes are the same as those shown in A. mRNA levels relative to wild-type embryos are presented as the mean+s.e.m. Numbers in parentheses indicate numbers of mice examined. *P≤0.05 and **P≤0.01 versus *Jmj*$^{trap/trap}$, cyclin D1$^{+/+}$ embryos (Tukey's multiple comparison test after obtaining a significant difference with one-way ANOVA). No significant differences were observed between wild-type and *Jmj*/cyclin D1 double mutant embryos.

RESULTS

Jmj is required for normal differentiation of cardiomyocytes through cyclin D1

Previously, we have shown that proliferation was enhanced and differentiation was severely affected in trabecular cardiomyocytes of embryos lacking *Jmj* functions (*Jmj*$^{trap/trap}$ embryos) (Takeuchi et al., 1999), and that expression of cyclin D1 was enhanced in the trabecular cardiomyocytes (Toyoda et al., 2003). To examine whether enhanced expression of cyclin D1 causes abnormal differentiation of trabecular cardiomyocytes, as well as proliferation (Toyoda et al., 2003), we produced *Jmj* and cyclin D1 double mutant mice. Ventricular morphology of the double mutant embryos (*Jmj*$^{trap/trap}$ cyclin D1$^{-/-}$) appeared to be normal (Fig. 1A). Immunostaining with antibodies for the three differentiated cardiomyocyte markers α-sarcomeric actin (s-actin), atrial natriuretic peptide (ANP) and actinin indicated markedly decreased expression of these proteins in cardiomyocytes of *Jmj*$^{trap/trap}$ mice. However, no apparent differences were observed between the wild-type and double mutant embryos (Fig. 1A). The mRNA expression levels of the differentiated cardiomyocyte marker genes in the cardiac ventricles of *Jmj*$^{trap/trap}$ mice decreased significantly compared with wild-type controls and the double mutant embryos, but no significant differences were observed between wild-type and the double mutant embryos (Fig. 1B).

These results indicated that the loss of cyclin D1 rescues deregulation of differentiation in *Jmj*$^{trap/trap}$ embryos, and that Jmj is required for normal differentiation of cardiomyocytes through cyclin D1.

Jmj regulates GATA4 expression in cardiomyocytes through cyclin D1

Next, we examined the mechanisms by which *Jmj* regulates the differentiation of cardiomyocytes by investigating the expression of transcription factors, such as GATA4, MEF2 and Nkx2.5, which regulate both cardiac development and differentiation of cardiomyocytes (Harvey, 1999; Olson, 2006).

Immunofluorescence staining showed that GATA4 expression in trabecular cardiomyocytes decreased compared with wild-type mice (Fig. 2A, parts d and j), whereas Nkx2.5 and MEF2 showed no apparent changes (Fig. 2A, parts e and k or f and l, respectively). In addition, expression of GATA4 appeared to be normal in *Jmj*/cyclin D1 double mutant mice (Fig. 2B). Western blotting analysis showed that the protein levels of GATA4 in the ventricles of *Jmj* mutant mice decreased markedly, whereas the levels in *Jmj*/cyclin D1 double mutant mice were comparable with those in wild-type controls (Fig. 2C).

These data showed that Jmj regulated GATA4 expression in the cardiomyocytes through cyclin D1.

Enhanced expression of cyclin D1 inhibits differentiation and decreases GATA4 protein, but not mRNA, levels in the cardiomyocytes

Our results obtained from *Jmj* and *Jmj*/cyclin D1 mutant embryos showed that enhanced expression of cyclin D1 decreases the GATA4 level and also inhibits the differentiation of cardiomyocytes. To confirm these results in a gain-of-function experiment, we produced and analysed transgenic mice [designated as *MDE(+)*] expressing cyclin D1 specifically in cardiomyocytes using the α-myosin heavy chain promoter (Subramaniam et al., 1991) (Fig. 3A). Specific and increased expression of the transgene was confirmed by monitoring the fluorescence of co-expressed GFP (Fig. 3B), immunostaining (Fig. 3C), real-time quantitative RT-PCR (Fig. 3E) and western blotting analysis (Fig. 3G). Only founder embryos were analysed in all transgenic experiments, including those using other transgenic mice (Figs 4, 5), because we expected it would be difficult to establish transgenic mouse lines due to embryonic lethality.

In *MDE(+)* mice, the spaces between trabeculae in the ventricles were very small (Fig. 3C,F). The numbers and mitotic indices of cardiomyocytes in the ventricles of *MDE(+)* mice increased significantly compared with non-transgenic littermates (*Non-Tg*) (Fig. 3D). The protein and mRNA expression levels of differentiated cardiomyocyte markers decreased in *MDE(+)* mice (Fig. 3C,E). These phenotypes were similar to those in trabecular cardiomyocytes of $Jmj^{trap/trap}$ embryos (Fig. 1) (Takeuchi et al., 1999).

We next found that the intensity of GATA4 decreased in cardiomyocytes compared with *Non-Tg* mice (Fig. 3F, parts d and j), whereas the intensities of Nkx2.5 (Fig. 3F, parts e and k) or MEF2 (Fig. 3F, parts f and l) showed no distinct differences. Western blotting analyses also showed marked decreases in GATA4 in *MDE(+)* mice (Fig. 3G). Next, we examined mRNA levels of *GATA4, MEF2c* and *Nkx2.5* in the cardiac ventricles by in situ hybridisation and quantitative RT-PCR. On in situ hybridisation, the intensities for all three genes showed no apparent differences in ventricular cardiomyocytes from *MDE(+)* mice at E10.5 compared with *Non-Tg* mice (see Fig. S1A in the supplementary material). Real-time quantitative PCR showed that there were no significant differences in the mRNA levels of *GATA4* compared with *Non-Tg* mice (see Fig. S1B in the supplementary material). We also found that the levels of *GATA4* were not significantly different between *Jmj* mutant and wild-type embryos (see Fig. S1C in the supplementary material).

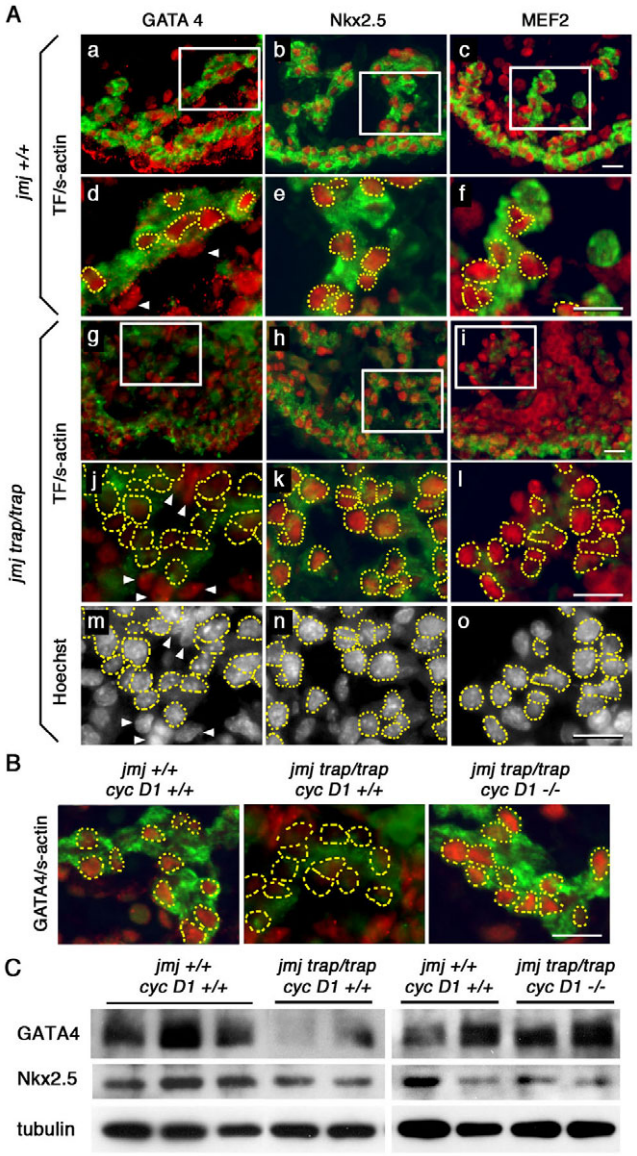

Fig. 2. Loss of function of a *Jmj*-cyclin D1 pathway decreases GATA4 expression in the cardiomyocytes. (**A**) Expression of transcription factors (TF; GATA4, Nkx2.5 or MEF2; red) and s-actin (green) in the left ventricle of wild-type (*Jmj* +/+) and *Jmj* mutant (*Jmj trap/trap*) embryos at E10.5. Transverse sections were analysed by immunofluorescence (a-l) and Hoechst (m-o) staining. (Panels d-f, j and m, k and n, and l and o are high-power views of the regions indicated by the rectangles in a-c, g, h and i, respectively.) Broken yellow lines show nuclei of trabecular cardiomyocytes. White arrowheads in d, j and m show endocardiac cells, in which GATA4 expression levels were not different between genotypes. Scale bars: 20 μm. (**B**) Expression of GATA4 (red) and s-actin (green) in the left ventricle of *Jmj*/cyclin D1 double mutant embryos at E10.5. (See Fig. 1 and A for genotype abbreviations and broken yellow lines. respectively.) Scale bar: 20 μm. (**C**) Expression of GATA4 and Nkx2.5 in cardiac ventricles was examined by western blotting analysis. Cardiac ventricles from one embryo were analysed in each lane. α-Tubulin (tubulin) was used as the control protein.

These results indicated that enhanced expression of cyclin D1 affects not only the proliferation but also the differentiation of cardiomyocytes, and decreases the GATA4 protein but not the mRNA levels in cardiomyocytes.

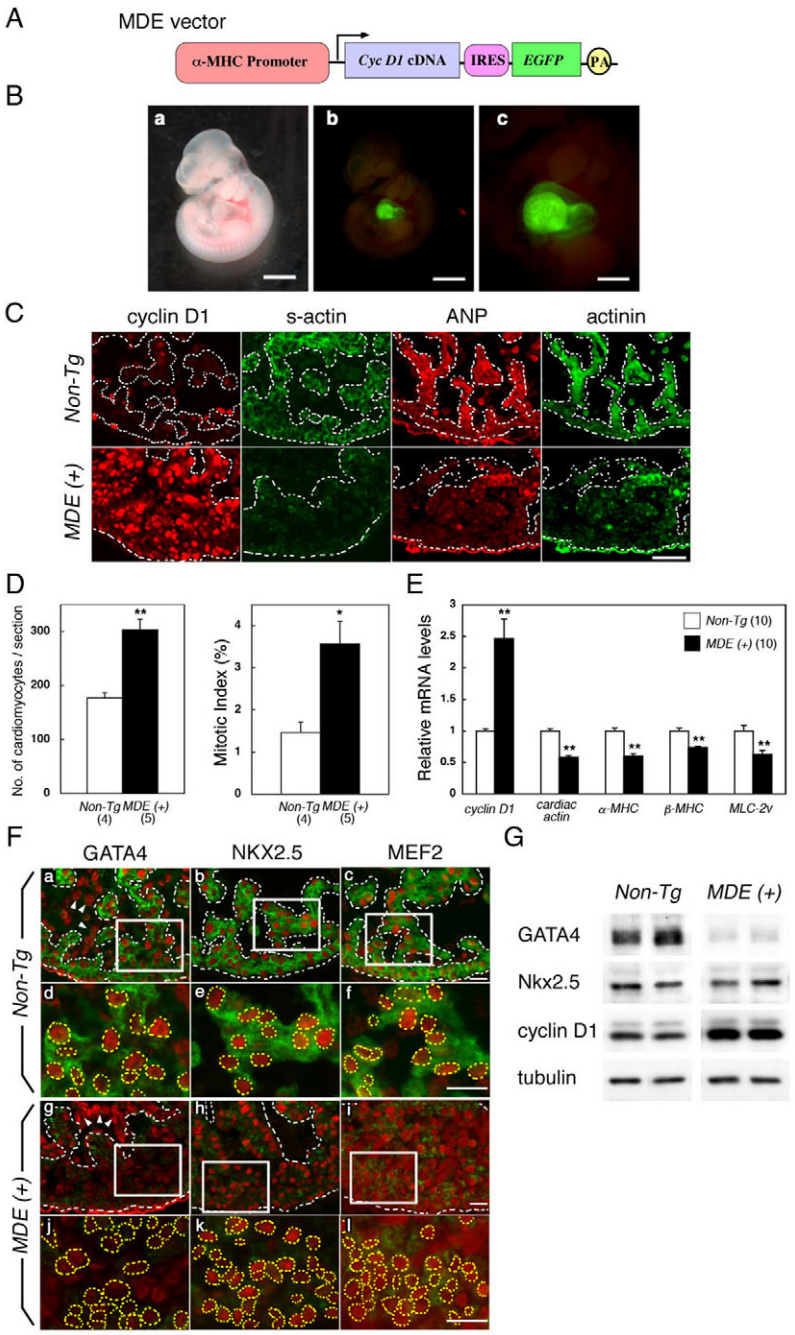

Fig. 3. Enhanced expression of cyclin D1 inhibits differentiation and decreases GATA4 protein level. (**A**) MDE vector. α-MHC promoter, α-myosin heavy chain promoter (cardiomyocyte-specific promoter); *cyc D1*, cyclin D1 cDNA; IRES, internal ribosomal entry sequence; *EGFP*, enhanced GFP cDNA; PA, polyadenylation signal. (**B**) Transgene expression in transgenic mice [*MDE(+)*] was monitored using GFP fluorescence. a, bright field; b and c, dark field for GFP observation; c, high-magnification view of b. EGFP was strongly expressed only in the heart of *MDE(+)* embryos. Scale bars: 1 mm in a and b; 400 μm in c. (**C**) Morphology and expression of cyclin D1 (red), s-actin (green), ANP (red) and actinin (green) in the left ventricles at E10.5. Protein expression was analysed by immunofluorescence staining. *Non-Tg*; non-transgenic mice. Broken white lines represent the region where cardiomyocytes were localised. Although there were several endocardiac cells in the region of *MDE(+)* embryos, lines within the regions are abbreviated for simplicity. Scale bar: 50 μm. (**D**) Numbers per section (left graph) and mitotic indices (right graph) of cardiomyocytes in left ventricles at E10.5 are presented as the mean+s.e.m. (**E**) mRNA levels of cyclin D1 and differentiated cardiomyocyte markers were analysed by real-time quantitative PCR and the levels relative to *Non-Tg* littermates are presented as the mean+s.e.m. (D,E) $*P \leq 0.05$; $**P \leq 0.005$ versus *Non-Tg* embryos (Student's *t*-test). Numbers in parentheses indicate the numbers of mice examined. (**F**) GATA4, Nkx2.5, MEF2 (red) and s-actin (green) expression were analysed by immunofluorescence staining. (Panels d-f, and j-l are high-magnification views of regions indicated by the rectangles in a-c and g-i, respectively.) White and yellow broken lines indicate the region where cardiomyocytes are localised and nuclei of cardiomyocytes, respectively. Scale bars: 20 μm. White arrowheads indicate endocardiac cells, in which GATA4 expression levels were not different between genotypes. (**G**) Protein levels in cardiac ventricles from one embryo were analysed in each lane by western blotting.

Cyclin D1 decreases GATA4 expression and inhibits the differentiation of cardiomyocytes in a CDK4/6-dependent manner

D-type cyclins bind and activate the cyclin-dependent kinases CDK4 and CDK6, and these activated CDKs then promote the cell cycle progression by phosphorylation of retinoblastoma family proteins. In addition, D-type cyclins have several functions other than activation of CDKs. In particular, D-type cyclins can modulate the activity of many transcription factors in a CDK4/6-independent manner (Bienvenu et al., 2010; Fu et al., 2004). Therefore, we next investigated whether activation of CDK by cyclin D1 inhibited cardiomyocyte differentiation, decreased the level of GATA4 protein and increased cell proliferation.

A cyclin D1 point mutant protein, cyclin D1K112E, cannot activate the catalytic activity of CDK4/6, but has CDK-independent functions (Baker et al., 2005; Hinds et al., 1994; Neuman et al., 1997; Zwijsen et al., 1997). Therefore, we analysed transgenic mice [designated as *MKE(+)*] expressing cyclin D1K112 specifically in cardiomyocytes using α-myosin heavy chain promoter (Fig. 4A) and compared the results with those of *MDE(+)* mice expressing intact cyclin D1 (Fig. 3). Specific and strong expression of the transgene in cardiomyocytes was confirmed using similar methods in *MDE(+)* mice (Fig. 4B,D,F).

The cardiomyocytes of *MKE(+)* mice showed no apparent morphological abnormalities at E10.5 (Fig. 4C). Immunofluorescence staining and quantitative RT-PCR revealed

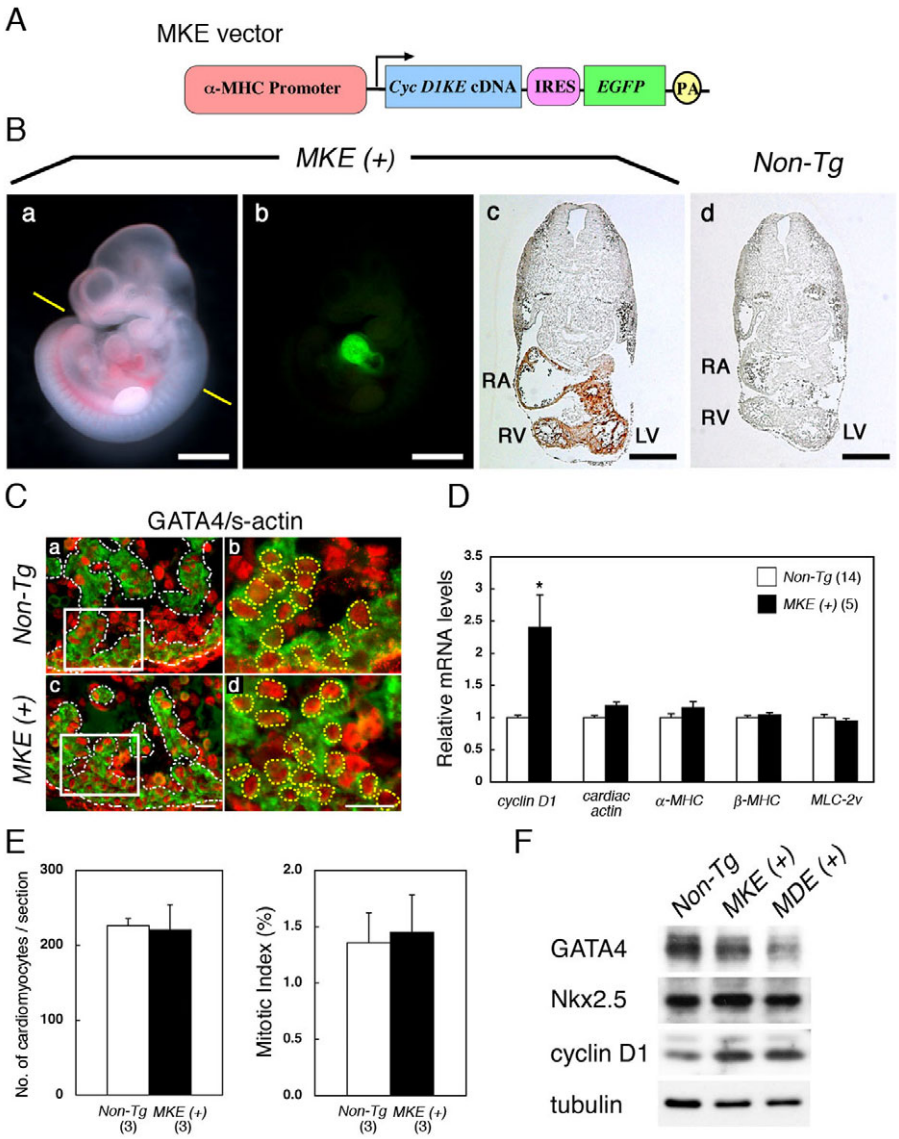

Fig. 4. Cyclin D1 decreases GATA4 expression and inhibits the differentiation of cardiomyocytes in a CDK4/6-dependent manner. (**A**) MKE vector. *cyc D1KE*, the cDNA encoded a point mutant cyclin D1; cyclin D1K112E, which cannot activate the catalytic activity of CDK4/6. (**B**) (a-c) Transgene expression in transgenic mice [*MKE(+)*]; (d) *Non-Tg* mice. a, bright field; b, dark field for GFP observation; c, expression of the cyclin D1KE protein (brown) was visualized in the transverse section with immunohistochemistry using an antibody against HA because the cyclin D1KE protein was tagged with HA. The position of the section is shown in yellow bars in a. (d) A section of *Non-Tg* mice at a similar position to c was stained. RA, right atrium; RV, right ventricle; LV, left ventricle. Scale bars: 1 mm in a and b; 300 μm in c and d. EGFP and cyclin D1KE protein were strongly expressed only in the heart of *MKE(+)* embryos. (**C**) Morphology and expression of GATA4 (red) and s-actin (green). Transverse sections were analysed by immunofluorescence staining. (b,d) High-magnification views of regions indicated by the rectangles in a,c, respectively. *MKE(+)* and *Non-Tg* show transgenic mice expressing cyclin D1K112E and non-transgenic mice, respectively. White and yellow broken lines indicate the region where cardiomyocytes are localised and nuclei of cardiomyocytes, respectively. Scale bars: 20 μm. (**D**) mRNA levels of cyclin D1 and differentiated cardiomyocyte markers were analysed by real-time quantitative PCR and the levels relative to *Non-Tg* littermates are presented as the mean+s.e.m. *$P \leq 0.005$ versus *Non-Tg* embryos (Student's *t*-test). (**E**) Numbers per section (left graph) and mitotic indices (right graph) of cardiomyocytes in left ventricles at E10.5 are presented as the mean+s.e.m. No significant differences were detected. (D,E) Numbers in parentheses indicate the numbers of mice examined. (**F**) Protein levels in cardiac ventricles from one embryo were analysed in each lane by western blotting.

that protein and mRNA levels of differentiated cardiomyocyte markers (Fig. 4C,D) and GATA4 protein expression (Fig. 4C, parts b and d) in cardiomyocytes of *MKE(+)* mice did not show any apparent differences compared with *Non-Tg* mice. In addition, no significant differences were observed in the numbers and mitotic indices of cardiomyocytes in ventricles between *MKE(+)* and *Non-Tg* mice (Fig. 4E). Western blotting analyses showed that the GATA4 level decreased in *MDE(+)* mice, while that in *MKE(+)* mice did not show a marked difference when compared with *Non-Tg* mice (Fig. 4F).

These results were completely different from those of *MDE(+)* mice (Fig. 3), suggesting that activation of CDK4/6 by cyclin D1 is necessary for inhibition of cardiac differentiation, decreased GATA4 expression and increased cell proliferation.

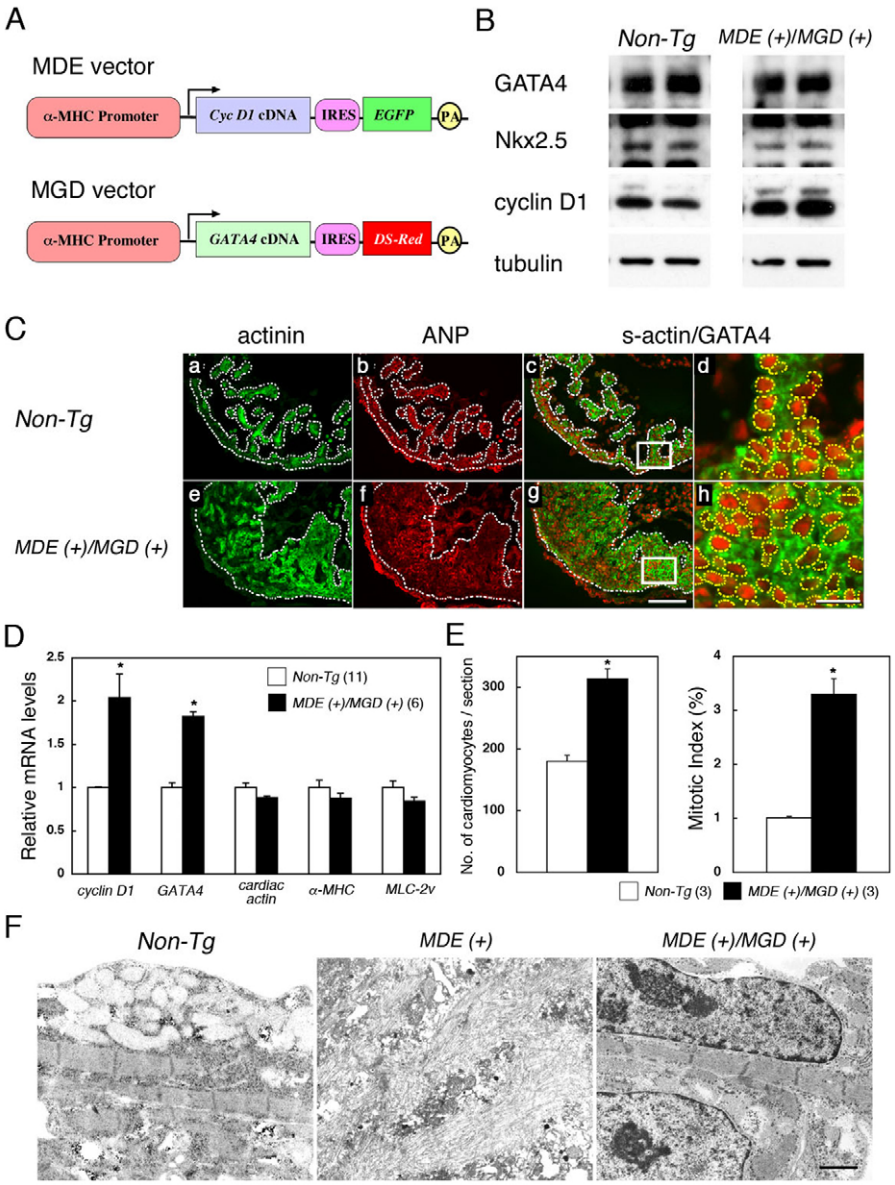

Fig. 5. GATA4 expression rescues abnormalities in differentiation caused by enhanced expression of cyclin D1. (**A**) MDE and MGD vectors. (**B**) The protein levels in cardiac ventricles from one embryo were analysed in each lane by western blotting. *MDE(+)/MGD(+)* and *Non-Tg* indicate transgenic mice expressing both exogenous cyclin D1 and GATA4 in the cardiomyocytes [*MDE(+)/MGD(+)*] and non-transgenic mice, respectively. (**C**) Morphology and expression of actinin (a and e, green), ANP (b and f, red), s-actin (c,d,g,h, green) and GATA4 (c,d,g,h, red). d and h are high-magnification views of regions indicated by the rectangles in c and g, respectively. Transverse sections were analysed by immunofluorescence staining. Broken white and yellow lines indicate the region where cardiomyocytes are localised and nuclei of cardiomyocytes, respectively. Although there were several endocardiac cells in the region of *MDE(+)/MGD(+)* embryos, broken white lines within the regions are abbreviated for simplicity. Scale bar: 100 μm in a-c,e-g; 20 μm in d and h. (**D**) mRNA levels of cyclin D1, *GATA4* and differentiated cardiomyocyte markers were analysed by real-time quantitative PCR and the levels relative to *Non-Tg* littermates are presented as the mean+s.e.m. (**E**) Numbers per section (left graph) and mitotic indices (right graph) of cardiomyocytes in left ventricles at E10.5 are presented as the mean+s.e.m. (D,E) *$P \leq 0.005$ versus *Non-Tg* embryos (Student's *t*-test). Numbers in parentheses indicate the numbers of mice examined. (**F**) Ultrastructural analysis of cardiac myocytes in left ventricles of *Non-Tg*, *MDE(+)* and *MDE(+)/MGD(+)* embryos at E10.5. Typical photographs of cardiomyocytes in *Non-Tg*, *MDE(+)* and *MDE(+)/MGD(+)* embryos are shown. Scale bar: 2 μm.

GATA4 expression rescues abnormalities in differentiation caused by enhanced expression of cyclin D1

Next, we examined whether the decrease in GATA4 level in mice overexpressing cyclin D1 contributes to the inhibition of cardiac differentiation. For this purpose, we produced double transgenic mice [designated as *MDE(+)/MGD(+)* mice] expressing both cyclin D1 and *GATA4*. The MGD vector encodes GATA4 (Fig. 5A). We then examined whether additional GATA4 expression rescued the abnormalities in differentiation but not proliferation.

The levels of expression of both cyclin D1 and *GATA4* mRNA were increased significantly compared with *Non-Tg* littermates (Fig. 5D). Western blotting analysis (Fig. 5B) and immunofluorescence staining (Fig. 5C, parts d and h) indicated that the GATA4 levels in the ventricles of *MDE(+)/MGD(+)* mice were similar to those of *Non-Tg* mice and were markedly increased compared with those of *MDE(+)* mice (Fig. 3F,G). These results showed that the decreased level of GATA4 was rescued in *MDE(+)/MGD(+)* mice.

The morphology of ventricular cardiomyocytes of *MDE(+)/MGD(+)* mice was similar to that of *MDE(+)* mice (Fig. 3C, Fig. 5C). The numbers and mitotic indices of cardiomyocytes in *MDE(+)/MGD(+)* mice were increased significantly (Fig. 5E). The protein expression levels of differentiated cardiomyocyte markers, actinin, ANP and s-actin were similar to those of *Non-Tg* mice (Fig. 5C). Quantitative RT-PCR showed that there were no significant differences in the levels of differentiated cardiomyocyte markers compared with *Non-Tg* mice (Fig. 5D).

We also examined the ultrastructure of cardiomyocytes in *MDE(+)* and *MDE(+)/MGD(+)* mice (Fig. 5F). Although some of the ventricular cardiomyocytes of *MDE(+)* embryos had a normal striated sarcomeric structure, many cells showed disorganised and loose structures without Z-discs [Fig. 5F, *MDE(+)*]. The abnormal structures were quite similar to those observed in trabecular cardiomyocytes of *Jmj* mutant mice (Takeuchi et al., 1999). These abnormal sarcomeric structures were observed in a few cardiomyocytes of *MDE(+)/MGD(+)* mice, but many cells showed normal myofilaments [Fig. 5F, *MDE(+)/MGD(+)*].

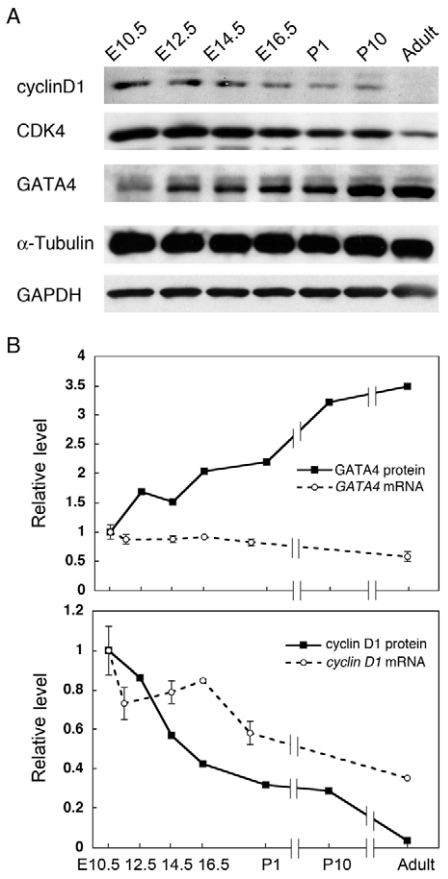

Fig. 6. Negative relationship between cyclin D1 and GATA4 protein expression during cardiac development. (**A**) Cyclin D1, CDK4 and GATA4 expression in cardiac ventricles were examined during development by western blotting analysis. α-Tubulin and GAPDH were analysed as controls. P1 and P10 indicate postnatal days 1 and 10, respectively. (**B**) Protein levels of GATA4 (upper panel) and cyclin D1 (lower panel) in A are shown after densitometric analysis and normalisation. In addition, mRNA levels of *GATA4* (upper panel) and cyclin D1 (lower panel) are presented as mean±s.e.m. of three embryos. All data are shown as levels relative to that at E10.5.

These data suggest that exogenous expression of *GATA4* rescued abnormal differentiation, and that a decrease in GATA4, caused by enhanced expression of cyclin D1, inhibited cardiac differentiation.

Negative relationship between cyclin D1 and GATA4 protein expression during cardiac development

Based on the data described above indicating that cyclin D1-CDK4 decreases GATA4 protein levels, it was expected that the levels of cyclin D1 and GATA4 expression would be negatively correlated during development. Western blotting analysis showed that the levels of cyclin D1 and CDK4 protein decreased (Fig. 6A), whereas those of GATA4 increased during development. Three independent experiments using different samples yielded the same results. The results of densitometric analyses of cyclin D1 and GATA4 are shown in Fig. 6B. Quantitative RT-PCR of ventricles revealed that the GATA4 mRNA level did not change markedly, especially during the embryonic stages (Fig. 6B). The cyclin D1 mRNA tended to decrease (Fig. 6B).

These data indicated a negative relationship between cyclin D1 and GATA4 protein expression during cardiac development, and suggested that GATA4 expression is regulated at the protein level.

CDK4 phosphorylates GATA4 directly and the phosphorylation promotes degradation of GATA4

The results described above suggest that enhanced expression of cyclin D1 in the heart of *Jmj*$^{trap/trap}$ or *MDE(+)* mice decreases GATA4 protein levels by post-translational regulation. We assumed that cyclin D1 overexpression elevated CDK4/6 activity and induced cyclin D1-CDK4/6-dependent phosphorylation of GATA4, which was involved in the downregulation of GATA4 protein expression.

To confirm this hypothesis, we first examined whether CDK4 phosphorylates GATA4 in vitro. Recombinant GATA4 (tagged with Trigger Factor) was incubated with or without cyclin D1-CDK4 complex. [γ-^{32}P] incorporation into GATA4 (Fig. 7B) was detected clearly when cyclin D1-CDK4 was added. No incorporation into Trigger Factor alone was detected (data not shown). These data indicate that activated CDK4 phosphorylates GATA4 directly. A positive control, RB protein, was phosphorylated more strongly, most likely because RB is phosphorylated at many sites by CDK4 (Kitagawa et al., 1996).

Previously, we reported a consensus motif for phosphorylation by cyclin D1-CDK4 (Kitagawa et al., 1996) and searched for candidates in GATA4. Serine 105 (S105) in the PPVSPR sequence in GATA4 was a possible site of phosphorylation by cyclin D1-CDK4 (Fig. 7A). We performed two experiments to investigate this possibility, i.e. incorporation of [γ-^{32}P] into mutant GATA4 (GATA4-S105A) (Fig. 7B) in which S105 was substituted with alanine (Fig. 7A), and detection with a specific antibody against GATA4 phosphorylated at S105 (Fig. 7C). [γ-^{32}P] incorporation into GATA4-S105A decreased markedly in comparison with wild-type GATA4 (Fig. 7B). Quantitative analysis showed that the intensity of the phosphorylation signal of GATA4-S105A was 18.9% of the wild-type protein. Phosphorylation at S105 was detected strongly with the antibody specific for phospho-S105 in wild-type GATA4 but not GATA4-S105A when cyclin D1-CDK4 was added (Fig. 7C). These results indicated that S105 is a major phosphorylation site in GATA4. In addition, we examined phosphorylation at S105 by cyclin D1-CDK4 in vivo. Expression vectors for GATA4 with or without vectors for cyclin D1 and CDK4 expression were transfected into HeLa cells, and the levels of signals for phosphorylation at S105 were examined (Fig. 7D). The relative level for phosphorylation at S105 (intensity of signal for phosphorylation at S105/intensity of signal for GATA4) increased markedly when cyclin D1 and CDK4 were co-expressed, whereas the increases were inhibited when both mutant proteins of cyclin D1 (D1KE) and CDK4 (CDK4 D158N, a catalytically inactive mutant) or wild-type cyclin D1 and CDK4 D158N were co-expressed (Fig. 7D, graph). In addition, the CDK4 inhibitor PD0332991 inhibited the phosphorylation (Fig. 7D, graph). These results showed that phosphorylation at S105 was promoted by cyclin D1-CDK4 in vivo.

Next, to examine whether cyclin D1-CDK4 promotes the degradation of GATA4, the effects of cyclin D1-CDK4 expression on the turnover of GATA4 were investigated by measuring GATA4 levels after blocking protein synthesis with cycloheximide (CHX). The co-expression of cyclin D1-CDK4 significantly promoted the degradation of GATA4 protein (Fig. 7E and see Fig. S2 in the supplementary material). However, the degradation of GATA4 S105A was markedly inhibited, especially at the early stages (Fig. 7E and see Fig. S2 in the supplementary material). In the absence of

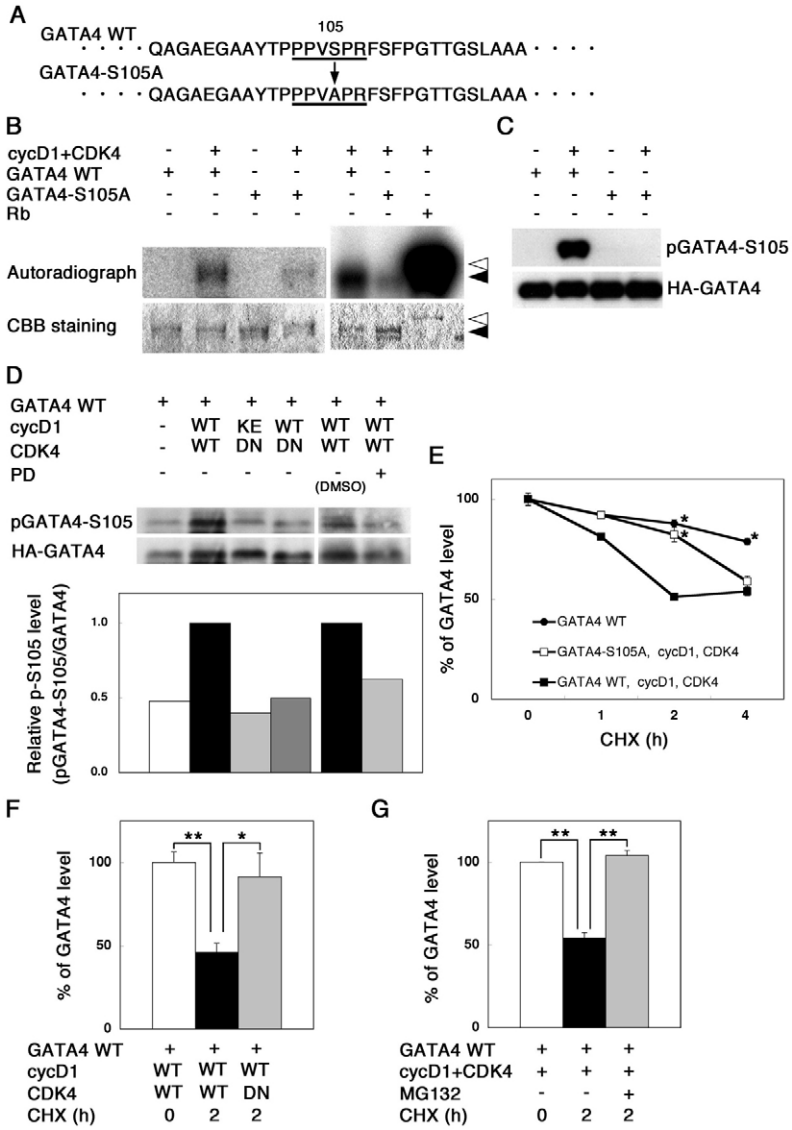

Fig. 7. CDK4 phosphorylated GATA4 directly and the phosphorylation promoted degradation of GATA4. (**A**) Amino acid sequence around the putative phosphorylation site (S105) by CDK4. The underlined sequence indicates the consensus motif for phosphorylation by CDK4. GATA4 WT, wild-type GATA4. The protein in which S105 was substituted with alanine (GATA4-S105A) was used in (B,C,E). (**B**,**C**) Phosphorylation of GATA4 by CDK4 was examined by in vitro phosphorylation assay using [γ-^{32}P] ATP (B) or western blotting analysis with an antibody against GATA4 phosphorylated at S105 (pGATA4-S105) after in vitro phosphorylation assay (C). GATA4, GATA4-S105A or RB (a positive control) recombinant proteins were incubated in kinase reaction buffer with or without recombinant cyclin D1-CDK4 complex. GATA4 proteins were tagged with TF (Trigger Factor) and HA. Black and white arrowheads represent GATA4 and RB, respectively. (**D**) In vivo phosphorylation assay using western blotting analysis with an anti-pGATA4-S105 antibody. The proteins indicated were transiently expressed in HeLa cells. WT, wild-type; KE, cyclin D1K112E; DN, CDK4 D158N. PD, the CDK4 inhibitor PD0332991. A control experiment for PD was performed by addition of only solvent (DMSO). Relative intensities for phosphorylation at S105 (intensity for phosphorylation at S105/intensity for GATA4) are indicated in the graph. (**E**) Effects of cyclin D1-CDK4 on protein turnover of wild-type GATA4 (GATA4 WT) or GATA4-S105A. Proteins were transiently expressed in HeLa cells and protein turnover was investigated by measuring GATA4 levels after blocking protein synthesis with cycloheximide (CHX). Cell lysates at the indicated times after CHX treatment were analysed by western blotting (see Fig. S2 in the supplementary material). The percentage of GATA4 protein level is shown as the mean+s.e.m. from three independent samples. *P≤0.01 versus GATA4 WT, cycD1, CDK4 (Tukey's multiple comparison test after obtaining a significant difference with two-way ANOVA). (**F**,**G**) Effects of CDK4 D158N (F) or the proteasome inhibitor MG132 (G) on protein turnover of wild-type GATA4. The average percentage of GATA4 protein level +s.e.m. (three to five independent samples) is shown. *P≤0.05; **P≤0.01 (Tukey's multiple comparison test after obtaining a significant difference with one-way ANOVA).

cyclin D1-CDK4, the degradation pattern of GATA4 S105A showed no apparent changes compared with that of wild-type GATA4 (data not shown). The degradation of wild-type GATA4 protein was inhibited significantly when mutant CDK4 was expressed (Fig. 7F) or by addition of the proteasome inhibitor MG132 (Fig. 7G).

Wild-type GATA4 protein levels decreased when cyclin D1 and CDK4 were co-expressed, compared with wild-type GATA4 protein levels when only GATA4 was expressed (see Fig. S2 in the supplementary material, CHX 0 hours). However, that of GATA4-S105A did not decrease (see Fig. S2

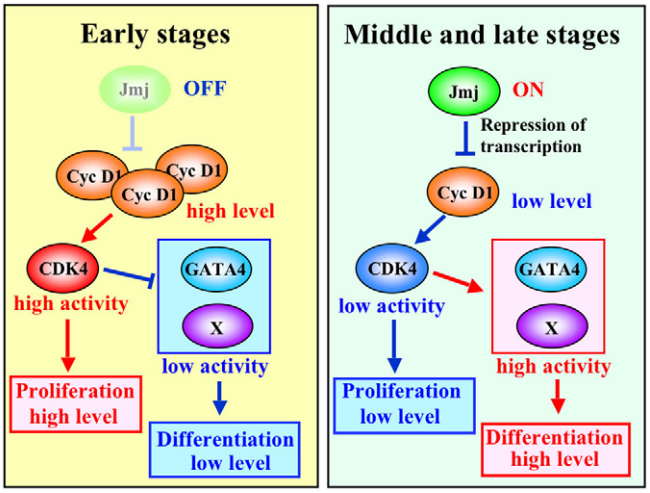

Fig. 8. Model of mechanisms regulating proliferation and differentiation by Jmj, cyclin D1, CDK4 and GATA4. Before *Jmj* expression starts, cyclin D1 is not repressed and shows a high level of expression. Consequently, CDK4/6 activity is high, and the protein levels of GATA4 and other factor(s), which regulate the differentiation of cardiomyocytes, are repressed. Factor(s) other than GATA4 may be involved. Therefore, cardiomyocytes show high proliferation and low differentiation levels (left). After *Jmj* expression starts, CDK4/6 activity decreases by repression of cyclin D1 and the decrease results in increased protein levels of GATA4 and other factor(s). Therefore, cardiomyocytes show low proliferation and high differentiation levels (right).

in the supplementary material). These data also suggested cyclin D1-CDK4- and S105-dependent degradation of GATA4.

Taken together, these results suggest that cyclin D1-CDK4 facilitates the degradation of GATA4 by direct phosphorylation in a proteasome-dependent manner.

DISCUSSION

One of the crucial issues in developmental biology is how proliferation and differentiation are regulated in a coordinated manner. Progression of both proliferation and differentiation of cardiomyocytes is indispensable for development of the heart and survival of the embryo. It is largely unknown how the proliferation and differentiation are regulated. We examined this issue by focusing on abnormal phenotypes (enhanced proliferation and inhibited differentiation) in cardiomyocytes of *Jmj* mutant embryos and analysing many transgenic mice. We showed that CDK4/6 activated by cyclin D1 inhibits differentiation of cardiomyocytes through a decrease in GATA4 levels. In addition, Jmj unleashes the inhibition and further promotes differentiation by repression of cyclin D1 expression. Thus, our studies show the mechanisms by which proliferation inhibits differentiation of embryonic cardiomyocytes and how the inhibition is unleashed during development.

The proliferation levels of ventricular cardiomyocytes start to decrease around E10-E12 (Toyoda et al., 2003). We have shown that Jmj is a trigger for the decrease (Toyoda et al., 2003). Therefore, Jmj regulates both proliferation and differentiation at the same time. Based on these results, we proposed a model explaining coordinated regulation of proliferation and differentiation of cardiomyocytes by the *Jmj*-cyclin D1 pathway (Fig. 8). Before *Jmj* expression starts, cyclin D1 is not repressed and shows a high level of expression. Consequently, CDK4/6 activity is high, and the protein levels of GATA4 and other factor(s), which regulate the differentiation of cardiomyocytes, are repressed. Factor(s) other than GATA4 may be involved (see below). Therefore, cardiomyocytes show high proliferation and low differentiation levels (Fig. 8, left panel). However, after *Jmj* expression starts, CDK4/6 activity decreases by repression of cyclin D1 and the decrease results in increased protein levels of GATA4 and other factor(s). Therefore, cardiomyocytes show low proliferation and high differentiation levels (Fig. 8, right panel).

The model shown above is based on the idea that cyclin D1 decreases the GATA4 level, and that this decrease in GATA4 inhibits differentiation. We mapped the expression levels of cyclin D1 and GATA4, and GATA4 and s-actin, as a differentiated cardiomyocyte marker, of individual embryos in transgenic experiments. A strong and significant negative correlation between cyclin D1 and GATA4, and a strong and significant positive correlation between GATA4 and s-actin were observed (see Fig. S3 in the supplementary material). These data suggest that cyclin D1 and GATA4 linearly affect GATA4 and differentiation, respectively. In addition, the cyclin D1 protein level decreases during development, whereas that of GATA4 increases (Fig. 6). These results are consistent with our model.

In GATA4 mutant mice with null or hypomorphic alleles, abnormalities in the differentiation of cardiomyocytes were not reported (Kuo et al., 1997; Molkentin et al., 1997; Pu et al., 2004), with the exception of a decrease in the expression of α-*MHC* in the case of the null allele (Zeisberg et al., 2005). These previous results suggest that factors other than GATA4 were also affected in *Jmj* mutant or *MDE(+)* mice. Known proteins that are strong candidates include the GATA4-related protein GATA6. Analysis of GATA4/6 double heterozygous or homozygous mice showed that these two proteins have redundant functions and are required for cardiomyocyte differentiation (Xin et al., 2006; Zhao et al., 2008), suggesting that decreases in both GATA4/6 proteins result in abnormal differentiation in *Jmj* mutant and *MDE(+)* embryos. In fact, our observations suggested that GATA6 expression decreased in the cardiomyocytes of *MDE(+)* mice (K.N., M. Kojima and T.T., unpublished).

In addition to its role in differentiation, GATA4 has been suggested to be involved in cell proliferation. Conditional deletion of *GATA4* in cardiomyocytes resulted in marked myocardial thinning with decreased cardiomyocyte proliferation. These phenotypes were observed mainly in cells derived from the anterior heart field (AHF), such as cardiomyocytes in the right ventricle (Rojas et al., 2008; Zeisberg et al., 2005). Rojas et al. also reported that GATA4 activates cyclin D2 and *Cdk4* directly (Rojas et al., 2008). These reports are inconsistent with our results. In the hearts of *Jmj* mutant mice, the GATA4 level decreased (Fig. 2), but the expression of cyclin D2 and *Cdk4* did not decrease (Toyoda et al., 2003). Moreover, proliferation was not inhibited but rather enhanced (Toyoda et al., 2003). The discrepancy with regard to proliferation can be explained by the enhanced expression of cyclin D1 in *Jmj* mutant or *MDE(+)* mice (Toyoda et al., 2003). It is likely that strong expression of cyclin D1 would overcome the inhibition of proliferation by decreasing GATA4. There are two possible explanations for the discrepancy regarding expression of cyclin D2 and *Cdk4*. First, expression of GATA4 decreased but it was not completely blocked in *Jmj* mutant or *MDE(+)* mice (Figs 2, 3). It is likely that *Gata4* must be completely or markedly inactivated in cardiomyocytes to decrease the expression of cyclin D2 and *Cdk4*. Second, the regions and stages at which *Gata4* was influenced were different. Cyclin D2 and *Cdk4* decreased in AHF-derived cells (Rojas et al., 2008) in which *Gata4* was inactivated

from earlier stages using *Nkx2.5^{cre}* or AHF-specific *Cre* (from E7.5) (Ai et al., 2007; Zeisberg et al., 2005). Based on the expression pattern of *Jmj* and the stages at which the phenotypes were detected (Takeuchi et al., 1999), GATA4 may begin to decrease from E10.0 at the earliest in the trabeculae of the left ventricle in *Jmj* mutant mice. These differences in regions and stages may have been responsible for the different results. In fact, later inactivation of *GATA4* using α-myosin heavy chain-Cre (from E9.5), which was the same promoter used in *MDE(+)* mice, did not result in a decrease in cyclin D2 expression (Zeisberg et al., 2005). Interestingly, the levels of cyclin D1, cyclin D2 and cyclin D3 expression were increased. These results suggest that the functions of GATA4 for proliferation and activation of cell cycle genes are different among cardiac regions and stages.

Our studies suggested that serine 105 of GATA4 (S105) is a major site of phosphorylation by CDK4 and is involved in the degradation of GATA4 (Fig. 7). It has been reported that MEK1-ERK1/2 and RhoA-p38 MAPK signalling phosphorylate S105 of GATA4 and this phosphorylation enhances the DNA binding and transcriptional activity of GATA4 (Charron et al., 2001; Liang et al., 2001). The authors suggested that this signalling regulates cardiomyocyte hypertrophic growth. In these papers, the enhancement of GATA4 degradation by phosphorylation was not reported. One possible explanation is that differences in the target sites of these kinases other than S105 result in differences in the overall functions. GATA4 contains at least six other potential sites for MAPK-mediated serine phosphorylation. It is possible that phosphorylation at these other sites blocks GATA4 degradation.

The consensus motif for phosphorylation by cyclin D1-CDK4 is (P)-P-Z-S/T-P-(X)-K/R (Z is an aliphatic amino acid; P and X in parenthesis are not always necessary) (Kitagawa et al., 1996). The consensus motif is rarely found in mammalian proteins. Therefore, substrates of CDK4 other than RB family proteins and functions other than cell cycle progression are little known. Our studies revealed a new substrate of CDK4 and a new function that regulates differentiation. Further studies on the functions of CDK4/6 in regulation of GATA4 or other factors crucial for differentiation will provide important insights into the mechanisms controlling proliferation and differentiation.

In the present study, we showed that activation of CDK4/6 inhibited the differentiation of cardiomyocytes by decreasing the GATA4 protein level in vivo. It would be worthwhile examining whether similar mechanisms are active in other tissues, and conversely whether factors known to be differentiation regulators, such as GATA4, can inhibit cell proliferation.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.059295/-/DC1

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