3.2. Flk1⁺ mGS cells differentiated into cardiomyocytes and endothelial cells in AMI model mice heart

Cardiomyogenesis of the transplanted Flk1⁺ mGS cells in AMI model mouse hearts was evaluated by immunohiostochemistory. Transplanted Flk1⁺ mGS cells differentiated not only into cTn-I positive cardiomyocytes in the infarcted area but also endothelial cells that form tube-like structures (Fig. 2A and B). These differentiated cardiomyocytes and endothelial cells may partly contribute to the improvement of cardiac function.

To investigate another mechanism of the observed improved cardiac function with Flk1⁺ mGS cell transplantation, we quantified the number of vessels in the marginal zone. The marginal zone was defined as the area between infarcted area and normal (non-ischemic) area. The number of the vessels, defined as tube-like structures with CD31 positive cells, was significantly higher in the Flk1⁺ mGS cell transplanted group than that of the medium injected group (Fig. 2C). These results suggested that enhanced angiogenesis in the marginal zone should supply better blood flow in the Flk1⁺ mGS transplanted group than in the medium injected group. In the hearts transplanted Flk1⁻ mGS cells, we could not detect differentiated cardiomyocytes and endothelial cells derived from Flk1⁻ mGS cells.

3.3. Preserved LV wall thickness in the marginal zone by the Flk1⁺ mGS cells transplantation

In infarcted areas, we observed increased cardiomyocyte survival in the Flk1⁺ mGS cell transplanted group than the medium injected group (Fig. 3A–D). The wall thickness of infarcted area was much thicker in the Flk1⁺ mGS cell transplanted group than in the medium injected group (Fig. 3E). In contrast, the wall thickness of infarcted area in the Flk1⁻ mGS cell transplanted group was same as that of the medium injected group.

3.4. Apoptosis in the marginal zone was not suppressed by the Flk1⁺ mGS cells transplantation

To investigate the mechanism of cardiomyocyte survival, we first counted the number of apoptotic cells in the marginal zone of each group 0, 6, 12, and 24 h and 4 weeks by TUNEL staining after Flk1⁺ cell transplantation or medium injection (Fig. 3F–H). The number of apoptotic cells rapidly increased 6 h after infarction and then gradually decreased until 24 h. The ratio of apoptotic cells to non-apoptotic cells 24 h after Flk1⁺ mGS cell transplantation was almost the same as during the time course from 0 h to 4 weeks after Flk1⁺ cell transplantation or medium injection (Fig. 3I). These results revealed that the observed apoptosis after infarction was not suppressed by Flk1⁺ mGS cell transplantation compared to medium injection.

3.5. Senescence in the marginal zone was prevented and delayed by the Flk1⁺ mGS cells transplantation

Next, we investigated the association of Flk1⁺ mGS cell transplantation and senescence, a type of cell death [13,15,16]. Senescent cells were detected by a β-galactosidase assay 3 and 7 days after the Flk1⁺ mGS cell transplantation and the medium injection. Although there were no significant difference in β-galactosidase stained areas between the Flk1⁺ mGS cell transplanted group and

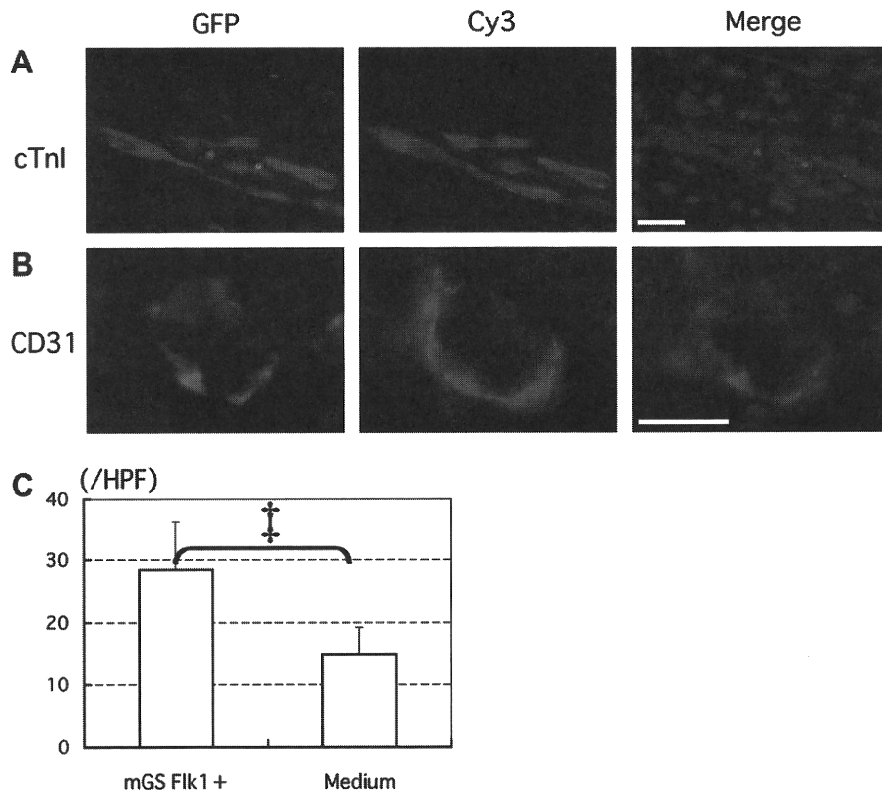

Fig. 2. (A) GFP⁺ mGS cells (left, green) were stained with cardiac troponin-I (cTn-I) (middle, red). Merged image (right). (B) GFP⁺ mGS cells with a tube structure (left panel, green) were stained with CD31 (middle panel, red). Merged image (right). The nuclei were counterstained with Hoechst 33342 (blue). (C) The number of CD31⁺ vessels in the marginal zone in each group. Scale bar: 100 μm. ‡$p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

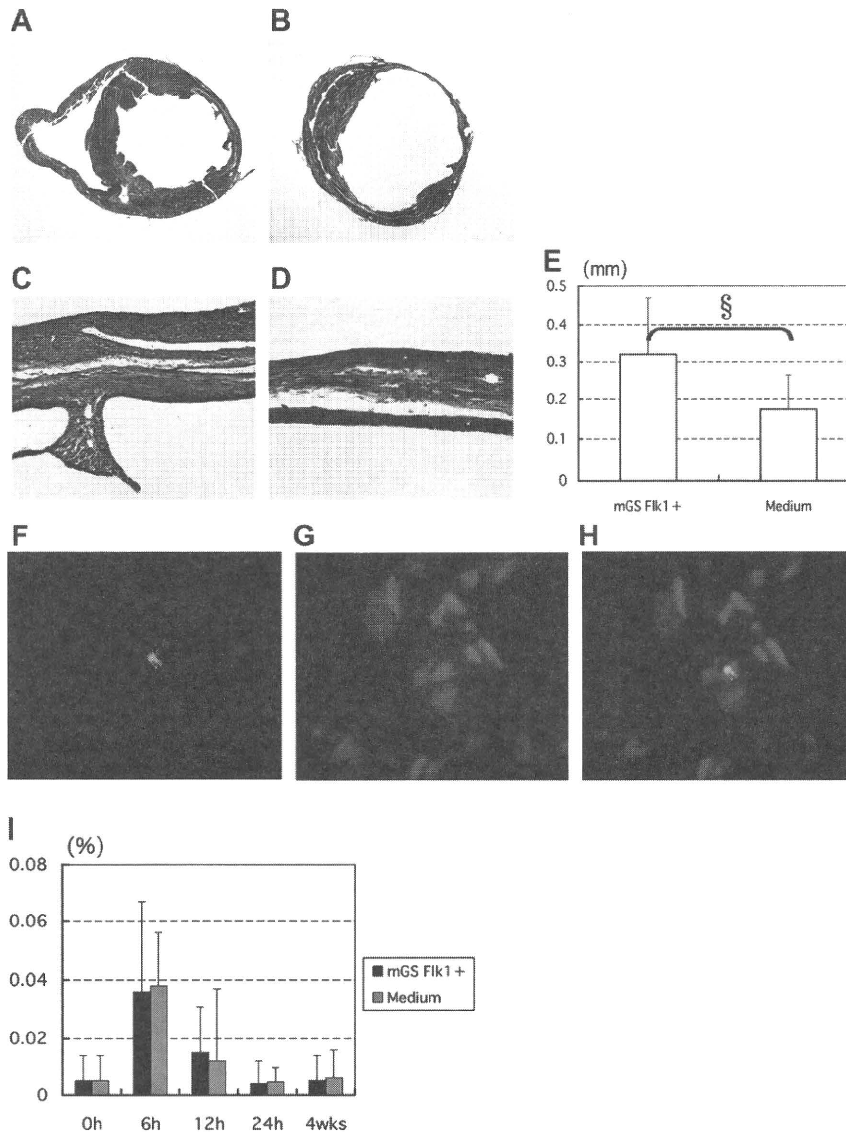

Fig. 3. (A) Coronal sections of Flk1^{+} mGS cell transplanted heart 4 weeks after the cell transplantation. This section was stained with Masson-Trichrome stain. (C) A magnified area of infarcted area from panel A. (B) The coronal section of a medium injected heart 4 weeks after the injection. This section was stained with Masson-Trichrome stain. (D) The magnification of infarcted area in the panel B. (E) The thickness of the infarcted anterior wall in each group. $^{\S}p < 0.05$. (F–H) TUNEL positive nucleus (F: left, green) was detected in the marginal zone. The nuclei were counterstained with Hoechst 33342 (G: middle, blue). Merged image (H: right). (I) The percentages of TUNEL positive nuclei were compared after Flk1^{+} mGS cell transplantation or control medium injection. There were no significant difference between the Flk1^{+} mGS cell transplanted group and the medium injected group over the 4-week time course. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the medium injected group 7 days after the transplantation, the β-galactosidase stained area was undoubtedly smaller 3 days after the transplantation in the Flk1^{+} mGS cell transplanted group than in the medium injection group (Fig. 4A–D). Especially in the Flk1^{+} mGS cell transplanted group, the β-galactosidase stained area on days 3 was located in the endocardium side of infarcted area. On the other hand, the epicardium side of infarcted area was not stained deeply by the β-galactosidase (Fig. 4A). These results revealed that the cell dearth via the cell senescence was delayed by the Flk1^{+} mGS cells transplantation. Finally, we investigated the cell signals, p21 and p53 that were elevated during the senescence pathway [17,18], to confirm that the senescence is actually prevented by the cells transplantation. Activated p21 and p53 were not clearly detected in the epicardium and endocardium sides of infarcted area in the Flk1^{+} mGS cell transplanted group on 3 days after the cell transplantation (Fig. 4F and G). As to the medium injected group, activated p21 and p53 were clearly detected all layers from endocardium to epicardium in the infarcted area (Fig. 4H and I).

4. Discussion

There were a lot of candidates of cell sources for the cell transplantation to heart failure model animals [2–4,19–21]. In these sources, we reported the Flk1^{+} mGS cells were one of the most convincing cell sources previously [10]. These Flk1^{+} mGS cells have enough potential in their expansion at the undifferentiated state and in their differentiation into cardiomyocytes and endothelial cells *in vitro*. Under this knowledge, we transplanted these cells into the hearts of AMI model mice. Stem cells derived from various tissues including adipose tissue and bone marrow can also differenti-

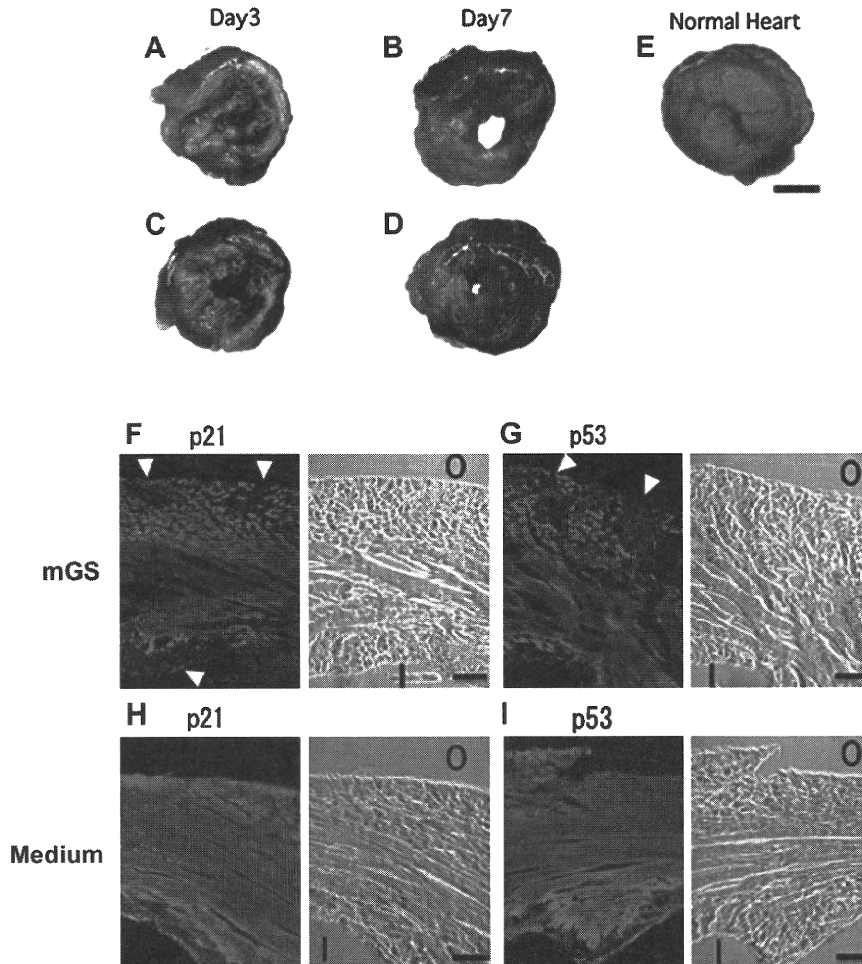

Fig. 4. (A–D) β-Galactosidase staining was detected 3 days (A) and 7 days (B) after Flk1$^+$ mGS cell transplantation. β-Galactosidase staining was also detected 3 days (C) and 7 days (D) after control medium injection. (E) β-Galactosidase staining was not detected in the non-ischemic normal heart. Scale bar: 1 mm. (F,G) Activated p21 and p53 were not clearly detected in the epicardium and endocardium of infarcted areas in Flk1$^+$ mGS cell-transplanted hearts (arrowheads). (H,I) Activated p21 and p53 were clearly detected in the epicardium and endocardium of the infarcted area in medium injected hearts. The nuclei were counterstained with Hoechst 33342 (blue). Bar; 50 μm. O, Outside the left ventricle. I, Inside the left ventricles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ate into cardiomyocytes and endothelial cells *in vitro* and *vivo* [22], indeed, but judging from *in vitro* differentiation capacity, mGS cells, which have equal potential in differentiation into endothelial cells to ES cells, have much priority.

Furthermore, the use of mGS cells in cardiac regeneration therapy for ischemic heart disease has much advantage. In the pluripotent or multipotent stem cells, ES cells have ethical problem, adult stem cells have less differentiate potential into cardiomyocyte and endothelial cells than ES cells or mGS cells *in vitro*, and induced pluripotent stem (iPS) cells are consist of transgenes and have a problem to be oncogenic.

Transplantation of the Flk1$^+$ mGS cells improved the left ventricular systolic function and a part of Flk1$^+$ mGS cells could differentiate into cardiomyocytes and endothelial cells *in vivo*. But we could not be sure that only a few numbers of these differentiated cells were sufficient to bear the results of this cardiac functional improvement. However; if we can improve the method of transplantation, it is surely expected that the differentiation potential of Flk1$^+$ mGS into cardiomyocytes and endothelial cells can be remarkably increased *in vivo* compared to other tissue stem cells. In contrast, the Flk1$^-$ mGS cell transplantation did not improve the cardiac function of AMI model mice. And more, we could not detect any differentiated cardiomyocyte or endothelial cell derived from Flk1$^-$ mGS cells in the infarcted area.

Except for the cardiac and endothelial cells differentiation from the transplanted cells, the mechanisms of cardiac function improvement after the cell transplantation were reported previously. Some reported the mechanism was vasculogenesis [2,6,23], and others reported it was anti-apoptotic effect [2] after the cell transplantation for AMI models. In our experiments, the thickness of the infarcted area was significantly larger in the Flk1$^+$ mGS cell transplanted group than in the medium injected group. Simultaneously, angiogenesis in the marginal zone was more frequently detected in the Flk1$^+$ mGS cell transplanted group than that in the medium injected group. Most of these new vessels were not the result of regeneration from the transplanted Flk1$^+$ mGS cells, but the result of the angiogenesis from the host cells. These results indicate that the sufficient blood perfusion resulting from enough angiogenesis enables host cardiomyocytes to survive after Flk1$^+$ mGS cells transplantation. At this time, we questioned why angiogenesis is enhanced specifically after the Flk1$^+$ mGS cells transplantation and not after the medium injection. Furthermore, we investigated the cell death mechanism after cell transplantation. We first investigated the anti-apoptotic effect of cell transplantation. But the

anti-apoptotic effect was not clearly revealed by the TUNEL staining in our experiments. Gonzalez et al. previously reported that activation of cardiac progenitor cells reversed senescence and improved the cardiac function in the failing heart [16]. Because we think that the Flk1$^+$ mGS cells are similar to cardiac stem/progenitors cells, a similar phenomenon may occur after the Flk1$^+$ mGS cell transplantation. The reduction effect of senescence may occur in the transplantation of ES cells, adipose tissue stem cells or bone marrow derived cells, further examination is needed for selecting the most effective cells from these cell source and mGS cells. In the β-galactosidase assay, cell death via senescence was clearly inhibited at the early phase of AMI after the Flk1$^+$ mGS cells transplantation. In addition, the signals p21 and p53 were activated in senescent cells and both signals were especially inhibited around the epicardial layers in the infarcted area at the very early phase of AMI. From these results, Flk1$^+$ mGS cell transplantation first prevents cell senescence in infarcted areas at the very early phase of ischemia. Next, angiogenesis from transplanted cells was enhanced during this period. Finally, a number of original host cardiomyocytes survived in the Flk1$^+$ mGS cell transplanted group. We think that the cardiac function of the Flk1$^+$ mGS cell transplanted group was significantly improved through these hypothetical mechanisms. Above these insights, we expect both the effect of angiogenesis and the reduction effect of senescence simultaneously from Flk1$^+$ mGS transplantation *in vivo* when we can improve the method of the cell transplantation to ischemic heart.

In conclusion, the transplanted Flk1$^+$ mGS cells improved the cardiac function in the AMI model mice and we observed that these cells differentiate into cardiomyocytes and endothelial cells. In addition, they prevented host cardiomyocytes death mainly through inhibition of senescence. Of course, these mechanisms require further investigation. When these mechanisms become more clearly elucidated, we hope that in the pluripotent or multipotent stem cells which are available for clinical application of cardiac regeneration therapy, Flk1$^+$ mGS cell transplantation will have advantage in the therapy for AMI patients.

Conflict of Interest

None.

Acknowledgments

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References

[1] S.W. Etoch, S.C. Koenig, M.A. Laureano, et al., Results after partial left ventriculectomy versus heart transplantation for idiopathic cardiomyopathy, J. Thorac. Cardiovasc. Surg. 117 (5) (1999) 952–959.
[2] A.A. Kocher, M.D. Schuster, M.J. Szabolcs, et al., Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function, Nat. Med. 7 (4) (2001) 430–436.
[3] A.P. Beltrami, L. Barlucchi, D. Torella, et al., Adult cardiac stem cells are multipotent and support myocardial regeneration, Cell 114 (6) (2003) 763–776.
[4] F.D. Pagani, H. DerSimonian, A. Zawadzka, et al., Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation, J. Am. Coll. Cardiol. 41 (5) (2003) 879–888.
[5] S. Baba, T. Heike, M. Yoshimoto, et al., Flk1(+) cardiac stem/progenitor cells derived from embryonic stem cells improve cardiac function in a dilated cardiomyopathy mouse model, Cardiovasc. Res. 76 (1) (2007) 119–131.
[6] A.A. Kocher, M.D. Schuster, N. Bonaros, et al., Myocardial homing and neovascularization by human bone marrow angioblasts is regulated by IL-8/Gro CXC chemokines, J. Mol. Cell. Cardiol. 40 (4) (2006) 455–464.
[7] S.I. Nishikawa, S. Nishikawa, S. Hirashima, et al., Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages, Development 125 (9) (1998) 1747–1757.
[8] M. Iida, T. Heike, M. Yoshimoto, et al., Identification of cardiac stem cells with FLK1, CD31, and VE-cadherin expression during embryonic stem cell differentiation, FASEB J. 19 (3) (2005) 371–378.
[9] M. Kanatsu-Shinohara, K. Inoue, et al., Generation of pluripotent stem cells from neonatal mouse testis, Cell 119 (7) (2004) 1001–1012.
[10] S. Baba, T. Heike, K. Umeda, et al., Generation of cardiac and endothelial cells from neonatal mouse testis-derived multipotent germline stem cells, Stem Cells 25 (6) (2007) 1375–1383.
[11] M. Hirashima, H. Kataoka, S. Nishikawa, et al., Maturation of embryonic stem cells into endothelial cells in an *in vitro* model of vasculogenesis, Blood 93 (4) (1999) 1253–1263.
[12] M. Ogawa, M. Kizumoto, S. Nishikawa, et al., Expression of alpha4-integrin defines the earliest precursor of hematopoietic cell lineage diverged from endothelial cells, Blood 93 (4) (1999) 1168–1177.
[13] T. Minamino, T. Yoshida, K. Tateno, et al., Ras induces vascular smooth muscle cell senescence and inflammation in human atherosclerosis, Circulation 108 (18) (2003) 2264–2269.
[14] T. Minamino, H. Miyauchi, T. Yoshida, I. Komuro, Endothelial cell senescence in human atherosclerosis: role of telomeres in endothelial dysfunction, J. Cardiol. 41 (1) (2003) 39–40.
[15] C. Chimenti, J. Kajstura, D. Torella, et al., Senescence and death of primitive cells and myocytes lead to premature cardiac aging and heart failure, Circ. Res. 93 (7) (2003) 604–613.
[16] A. Gonzalez, M. Rota, D. Nurzynska, et al., Activation of cardiac progenitor cells reverses the failing heart senescent phenotype and prolongs lifespan, Circ. Res. 102 (5) (2008) 597–606.
[17] B.D. Chang, Y. Xuan, E.V. Broude, et al., Role of p53 and p21waf1/cip1 in senescence-like terminal proliferation arrest induced in human tumor cells by chemotherapeutic drugs, Oncogene 18 (34) (1999) 4808–4818.
[18] X. Zhang, J. Li, D.P. Sejas, Q. Pang, The ATM/p53/p21 pathway influences cell fate decision between apoptosis and senescence in reoxygenated hematopoietic progenitor cells, J. Biol. Chem. 280 (20) (2005) 19635–19640.
[19] G.V. Silva, E.C. Perin, H.F. Dohmann, et al., Catheter-based transendocardial delivery of autologous bone-marrow-derived mononuclear cells in patients listed for heart transplantation, Tex. Heart Inst. J. 31 (3) (2004) 214–219.
[20] N. Hattan, H. Kawaguchi, K. Ando, et al., Purified cardiomyocytes from bone marrow mesenchymal stem cells produce stable intracardiac grafts in mice, Cardiovasc. Res. 65 (2) (2005) 334–344.
[21] M.A. Laflamme, K.Y. Chen, A.V. Naumova, et al., Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts, Nat. Biotechnol. 25 (9) (2007) 1015–1024.
[22] Y. Yamada, S. Yokoyama, X. Wang, et al., Cardiac stem cells in brown adipose tissue express CD133 and induce bone marrow nonhematopoietic cells to differentiate into cardiomyocytes, Stem Cells 25 (2007) 1326–1333.
[23] M.D. Schuster, A.A. Kocher, T. Seki, et al., Myocardial neovascularization by bone marrow angioblasts results in cardiomyocyte regeneration, Am. J. Physiol. Heart Circ. Physiol. 287 (2) (2004) H525–H532.

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Cyclin-Dependent Kinase 9 Forms a Complex With GATA4 and Is Involved in the Differentiation of Mouse ES Cells Into Cardiomyocytes

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The treatment of ES cells with trichostatin A (TSA), an HDAC inhibitor, induces the acetylation of GATA4 as well as histones, and facilitates their differentiation into cardiomyocytes. Recently, we demonstrated that cyclin-dependent kinase 9 (Cdk9), a core component of positive elongation factor-b, is a novel GATA4-binding partner. The present study examined whether Cdk9 forms a complex with GATA4 in mouse ES cells and is involved in their differentiation into cardiomyocytes. Mouse ES cells and Nkx2.5/GFP ES cells, in which green fluorescent protein (GFP) is expressed under the control of the cardiac-specific Nkx2.5 promoter, were induced to differentiate on feeder-free gelatin-coated plates. Immunoprecipitation/Western blotting in nuclear extracts from mouse ES cells demonstrated that Cdk9 as well as cyclin T1 interact with GATA4 during myocardial differentiation. TSA treatment increased Nkx2.5/GFP-positive cells and endogenous mRNA levels of Nkx2.5 and atrial natriuretic factor. To determine the role of Cdk9 in myocardial cell differentiation, we examined the effects of a dominant-negative form of Cdk9 (DN-Cdk9), which loses its kinase activity, and a Cdk9 kinase inhibitor, 5,6-dichloro-1-β-ribofuranosyl-benzimidazole (DRB) on TSA-induced myocardial cell differentiation. The introduction of the DN-Cdk9 inhibited TSA-induced increase in GFP expression in Nkx2.5/GFP ES cells. The administration of DRB into ES cells significantly inhibited TSA-induced increase of endogenous Nkx2.5 mRNA levels in ES cells as well as GFP expression in Nkx2.5/GFP ES cells. These findings demonstrate that Cdk9 is involved in the differentiation of mouse ES cells into cardiomyocytes by interacting with GATA4.

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During embryogenesis, cell type-specific gene expression plays a pivotal role in the determination of cell fate, including differentiation, proliferation, and apoptosis. In contrast to other cell types, cardiac muscle cells are highly organized and their developmental processes require a number of cell type-specific transcription factors (Sachnidis et al., 2003). Among these, the zinc finger protein GATA4, which is one of the pivotal DNA-binding transcription factors involved in myocardial differentiation, is expressed at the earliest stage during heart development (Grepin et al., 1997; Kuo et al., 1997; Molkentin et al., 1997).

Histone acetyltransferases (HATs) and histone deacetylases (HDACs), which are recruited to target genes in association with specific DNA-binding transcription factors, regulate gene expression patterns by affecting the chromatin structure (Johnson and Turner, 1999; Chan and La Thangue, 2001). One of HATs, p300, serves as a transcriptional coactivator of GATA4 and is able to acetylate GATA4 and enhance its DNA-binding activities (Shikama et al., 1997; Johnson and Turner, 1999; Chan and La Thangue, 2001; Yanazume et al., 2003; Kawamura et al., 2005; Takaya et al., 2008). During the differentiation of ES cells into cardiomyocytes, the treatment of ES cells with trichostatin A (TSA), an HDAC inhibitor, induces the acetylation of GATA4 as well as the expression of p300, and facilitates their differentiation into cardiomyocytes (Kawamura et al., 2005).

Positive elongation factor-b (p-TEFb) is a key regulator of the process controlling the processing of RNA polymerase II and possesses a kinase activity that can phosphorylate the carboxy-terminal domain of the largest subunit of RNA polymerase II (Zhu et al., 1997; Shim et al., 2002; Marshall and Grana, 2006). p-TEFb is composed of cyclin-dependent kinase 9 (Cdk9) and one of four C-type cyclin subunits (cyclin T1, cyclin T2a, cyclin T2b, and cyclin K). p-TEFb not only plays an important role in most

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RNA pol II-dependent transcription, but also is recruited to cellular promoters by interacting with a variety of transcription factors (Kanazawa et al., 2000; Barboric et al., 2001; Eberhardy and Farnham, 2002; Simone et al., 2002).

Recently, we demonstrated that by tandem affinity purification and mass spectrometric analyses, Cdk9 is one of the components of the p300/GATA4 complex in HeLa cells as well as cyclin T1 (Sunagawa et al., 2010). The present study examined whether Cdk9 forms a complex with GATA4 and is involved in the differentiation of ES cells into cardiomyocytes.

Materials and Methods

Cell line and cell culture of embryonic stem cells

The 129/Ola-derived ES cell lines we used in the present study were ht7 carrying the Oct3/4-hygromycin^r gene and its derivative (Nkx2.5/GFP ES cells), in which green fluorescent protein (GFP) is knocked into the Nkx2.5 locus (Hidaka et al., 2003). These cells were maintained on gelatinized dishes without feeder cells using culture medium containing Glasgow-modified Eagle's medium (GMEM), 1,000 U/ml leukemia inhibitory factor (LIF, Chemicon International, Billerica, MA), 100 mg/ml hygromycin (Invitrogen, Carlsbad, CA), 10% heat-inactivated fetal calf serum, 1 × non-essential amino acids, 1 mmol/L sodium pyruvate, 50 U/ml penicillin, 0.05 mg/ml streptomycin, and 0.1 mmol/L 2-mercaptoethanol. To induce differentiation, 3×10^4 ES cells were seeded in each well of the gelatinized 6-well plates without the formation of embryoid bodies using the above-mentioned medium lacking LIF and hygromycin. Differentiated ES cells were stimulated with TSA (Invitrogen) and/or 5,6-dichloro-1-β-ribofuranosyl-benzimidazole (DRB) (Invitrogen) on day 7 for 24 h.

Immunoprecipitation and Western blotting

Western blotting were performed as previously described (Yanazume et al., 2003) using rabbit polyclonal anti-GATA4 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-Cdk9 (Santa Cruz Biotechnology), rabbit polyclonal anti-cyclin T1 (Santa Cruz Biotechnology), mouse monoclonal anti-RNA polymerase II (Abcam, San Francisco, CA), mouse monoclonal anti-phosphorylated serine 2 of RNA polymerase II C-terminal domain (Abcam), and mouse monoclonal anti-β-actin (SIGMA, St Louis, MO) antibodies. To detect the physical interaction of GATA4 with Cdk9 in mouse ES cells, nuclear extracts were first immunoprecipitated with a goat polyclonal anti-GATA4 or anti-Cdk9 antibody on day 8, as previously described (Iwanaga et al., 1998; Wada et al., 2002; Hirai et al., 2004). Nuclear extracts were also immunoprecipitated with an anti-IgG antibody as a control. The precipitates were sequentially subjected to Western blotting using rabbit polyclonal anti-Cdk9, rabbit polyclonal anti-GATA4, and rabbit polyclonal anti-cyclinT1 antibodies.

Plasmid constructs

A lentivirus vector, pLenti6/V5-D-TOPO® (Invitrogen), was subcloned to contain the phosphoglycerate kinase (pgk) promoter–enhancer and used as a null expression vector (pLenti-Null) for lentiviral infection. pLenti-DN-Cdk9 was constructed by inserting human Cdk9 cDNA including a point mutation at nucleotide 563, which converts Asp into Asn, into pLenti-Null. pLenti-GFP was constructed by inserting enhanced-GFP cDNA into pLenti-Null.

Lentiviral infection

To generate the lentivirus vectors, 293FT packaging cells (Invitrogen) were seeded at 1.5×10^6 cells per 100-mm dish using Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum, 50 U/ml penicillin, and 0.05 mg/ml streptomycin. On the next day, pLenti6/V5-D-TOPO®-based lentivirus vectors were induced into 293FT cells by the calcium phosphate method. These cells were incubated at 37°C with 5% CO_2 for 8 h. Thereafter, the medium was replaced with GMEM-based medium for the differentiation culture of ES cells. After 12 h at 32°C with 5% CO_2, supernatants containing lentivirus produced by these 293FT cells were passed through a 0.45-μm filter and supplemented with 8 μg/ml polybrene. Thirty thousand ES cells were transferred into each well of 6-well plates including these supernatants and maintained for 12–24 h. Thereafter, the supernatant was replaced with the fresh medium. The day on which ES cells were transferred was designated as day 0. Then, ES cells were differentiated as previously described.

Flow cytometry

For the quantitative analysis of the number of GFP-positive cells, ES cells were dissociated into single cells and analyzed by flow cytometry (BD Biosciences, San Jose, CA) on day 8, as previously described (Hidaka et al., 2003).

Quantitative RT-PCR

Total RNAs from ES cells were isolated using TRIzol® reagent (Invitrogen), reverse transcribed, and amplified as previously described (Yanazume et al., 2003). Quantitative PCR was performed as previously described (Kawamura et al., 2005). Primer sequences of Nkx2.5 (Hidaka et al., 2003), VEGF (Ogawa et al., 2005), β-myosin heavy chain (β-MHC) (Hidaka et al., 2003), Cdk9 (Takaya et al., 2009), and GAPDH (Iwanaga et al., 1998) were described previously.

Statistical analysis

The results are presented as means ± SE. Statistical comparisons were performed using unpaired two-tailed Student's *t*-tests or ANOVA with Scheffe's test where appropriate, with a probability value <0.05 taken to indicate significance.

Results

Expression patterns of genes involved in myocardial differentiation in ES cells

To investigate patterns regarding the spontaneous differentiation of ES cells, these cells were seeded on gelatin-coated plates with no feeder cells. Differentiation was induced without the formation of EBs by removing LIF and antibiotics from the media. In this system, ES cells exhibited myogenic contractions on day 10. These beating cells were positive for both cardiac troponin T and cardiac myosin heavy chain, and exhibited sarcomere formation and myofibril organization, typical features of cardiomyocytes (Kaichi et al., 2010). During the induction of the spontaneous differentiation of ES cells, we examined the time course of the mRNA levels of genes involved in myocardial differentiation as well as that of Cdk9, a major component of p-TEFb. The day on which we started differentiation induction was designated as day 0. Total RNAs were isolated from ES cells on days 0, 3, 5, and 8 and synthesized cDNAs were subjected to quantitative PCR. We examined the mRNA level of a zinc finger protein, GATA4, and a cardiac-specific transcription factor, Nkx2.5. As shown in Figure 1Aa,b, the levels of both GATA4 and Nkx2.5 increased from days 3 to 5. Then, we examined their mRNA expression levels on day 4. The GATA4 levels increased from days 3 (100 ± 9) to 4 (354 ± 42). Meanwhile, the Nkx2.5 levels were similar between days 3 (100 ± 13) and 4 (97 ± 31). These findings demonstrate that GATA4 mRNA increases from days 3 to 5 and that Nkx2.5 mRNA increases after day 4. Next, we examined the expression of a gene encoding the cardiac structure protein, β-

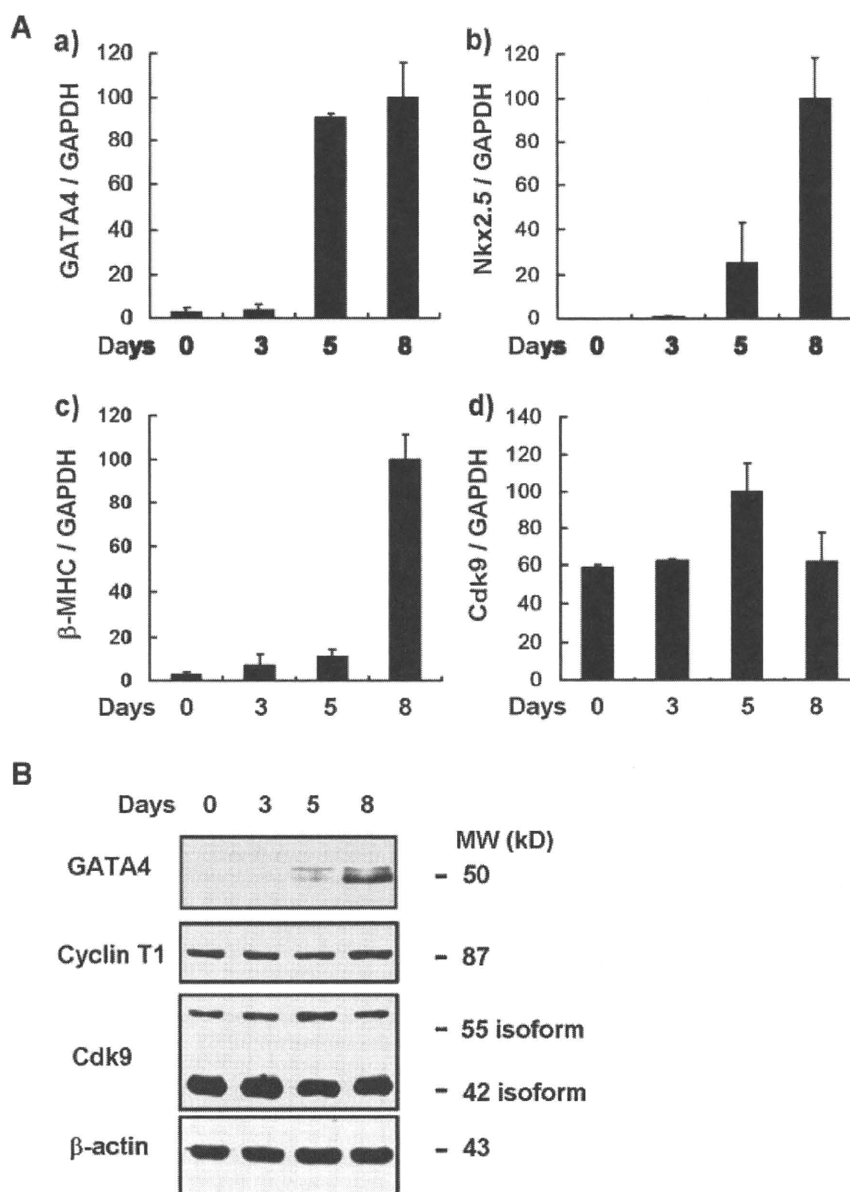

Fig. 1. A: Total RNAs were extracted from ES cells on days 0, 3, 5, and 8, and reverse-transcribed into cDNAs. Using quantitative PCR, expressions of cardiac-specific genes, transcription factors, and Cdk9 were analyzed. Levels of GAPDH transcripts were used to normalize cDNA levels. The maximal mRNA level of specific targets relative to GAPDH at different time points was set at 100. Data are presented as the means ± SE of 3–5 independent experiments. (a) GATA4, (b) Nkx2.5, (c) β-MHC, and (d) Cdk9. B: Nuclear extracts from differentiated ES cells on days 0, 3, 5, and 8 were subjected to Western blotting with anti-GATA4, anti-Cdk9, anti-cyclin T1, and anti-β-actin antibodies at the indicated time points. The photographs are representative. We performed three or more independent experiments and obtained similar results.

myosin heavy chain (β-MHC). A significant level of β-MHC mRNA was detected only on day 8 (Fig. 1Ac). The Cdk9 gene was abundantly expressed at the undifferentiated stage and maintained at almost similar levels throughout the stages of differentiation (Fig. 1Ad).

Then, we examined the protein levels of GATA4, Cdk9, and cyclin T1. Nuclear extracts from ES cells were isolated on days 0, 3, 5, and 8 and subjected to Western blotting. The signal of GATA4 protein was first detected on day 5 (Fig. 1B). In contrast, the signals of Cdk9 and cyclinT1 proteins were already marked at the undifferentiated stage and maintained at relatively constant levels. The expression patterns of these proteins were compatible with those of their endogenous mRNA.

Trichostatin A, an HDAC inhibitor, up-regulates the expression of cardiac-specific genes

ES cells, in which GFP is expressed under the control of the cardiac-specific Nkx2.5 promoter (Nkx2.5/GFP ES cells), were treated with TSA, an HDAC inhibitor, at concentrations of 0, 10, 50, and 100 ng/ml on day 7 for 24 h. These cells were dissociated into single cells by trypsin on day 8 and subjected to analysis by flow cytometry. The rate of Nkx2.5/GFP-positive

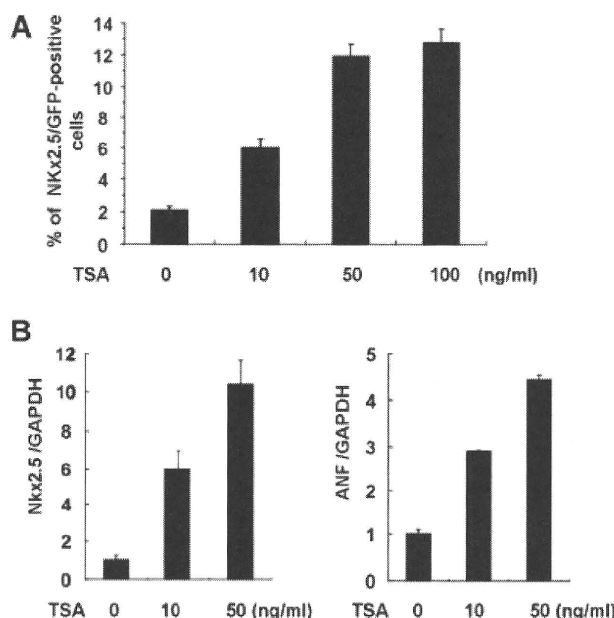

Fig. 2. A: Nkx2.5/GFP ES cells were stimulated with TSA (10, 50, or 100 ng/ml) or a corresponding amount of its solvent, ethanol, on day 7 for 24 h. Differentiated ES cells were dissociated into single cells with trypsin on day 8 and subjected to analysis by flow cytometry. Data represent the rate of Nkx2.5/GFP-positive cells to total extant cells at the indicated TSA concentrations. B: ES cells on day 7 were stimulated with TSA (10 or 50 ng/ml) or its solvent for 24 h. Total RNAs were isolated on day 8 from theses cells, and synthesized cDNAs were subjected to quantitative PCR. Levels of GAPDH transcripts were used to normalize specific cDNA levels. The relative mRNA levels of Nkx2.5 and ANF in ES cells stimulated with 50 ng/ml of TSA were set at 100. Data are presented as the means ± SE of 3–5 independent experiments.

cells to total extant cells before TSA stimulation was around 2%. Treatment with TSA increased Nkx2.5/GFP-positive cells by 3- to 6.5-fold in a concentration-dependent manner (Fig. 2A).

Next, differentiated ES cells were treated with TSA on day 7 for 24 h. Total RNAs were isolated from these cells on day 8, and synthesized cDNAs were subjected to quantitative PCR. Treatment with TSA at the concentrations of 10–50 ng/ml increased the endogenous mRNA level of Nkx2.5 by 6- to 10.5-fold and that of a cardiac-specific peptide hormone, atrial natriuretic factor (ANF), by 3- to 4.5-fold (Fig. 2B).

GATA4 physically interacts with Cdk9/cyclin T1 in ES cells

We demonstrated that Cdk9 forms a physical complex with GATA4 as well as p300 by in vivo GST-binding assay (Sunagawa et al., 2010). To examine the physical interaction of GATA4 with Cdk9 in ES cells, nuclear extracts were isolated on day 8 and subjected to immunoprecipitation/Western blotting (Fig. 3). First, these nuclear extracts were immunoprecipitated with goat polyclonal anti-GATA4 antibody or anti-IgG antibody as a control and sequentially subjected to Western blotting using rabbit polyclonal anti-Cdk9 and rabbit polyclonal anti-GATA4 antibodies. The Cdk9 protein was immunoprecipitated with GATA4. Next, these extracts were immunoprecipitated with goat polyclonal anti-Cdk9 antibody and sequentially subjected to Western blotting using rabbit polyclonal anti-GATA4 and rabbit polyclonal anti-Cdk9 antibodies. The GATA4 protein was also immunoprecipitated with Cdk9. Finally, the precipitates formed by anti-GATA4 antibody and those by anti-Cdk9 antibody were subjected to Western blotting using rabbit polyclonal anti-cyclin T1 antibody. The cyclin T1 protein was immunoprecipitated with GATA4 as well as Cdk9. These results demonstrate that GATA4 physically interacts with the Cdk9/cyclin T1 complex in ES cells.

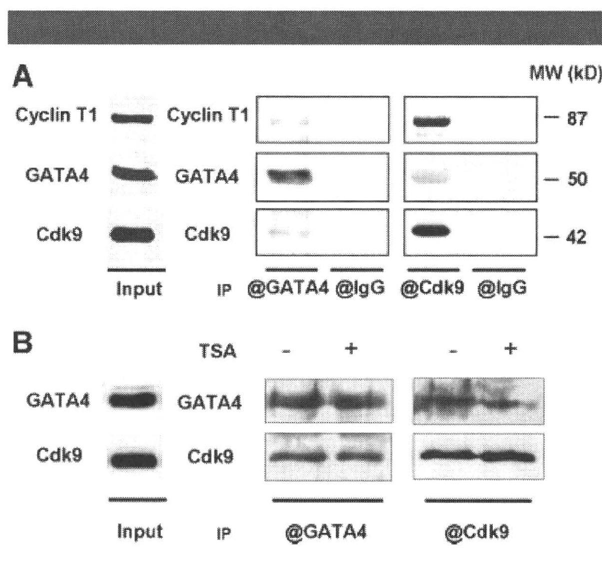

Fig. 3. A: Nuclear extracts from ES cells on day 8 were immunoprecipitated with indicated antibodies and sequentially subjected to Western blotting with anti-GATA4, anti-cyclin T1, and anti-Cdk9 antibodies. The photographs are representative. We performed three independent experiments and obtained similar results. B: Differentiated ES cells were treated with TSA or untreated on day 7 and nuclear extracts were isolated on day 8. These extracts were immunoprecipitated with the indicated antibodies and sequentially subjected to Western blotting with anti-GATA4 and anti-Cdk9 antibodies. The photographs are representative. We performed three or more independent experiments and obtained similar results.

Next, to examine whether TSA treatment alters the interaction of GATA4 with Cdk9, the differentiated ES cells were treated with TSA or untreated on day 7 and the nuclear proteins were isolated on day 8. The nuclear extracts were immunoprecipitated with a goat polyclonal anti-GATA4 or anti-Cdk9 antibody. The precipitates were sequentially subjected to Western blotting using a rabbit polyclonal anti-Cdk9 or anti-GATA4 antibody. The signal levels of GATA4 and Cdk9 were similar between the precipitates treated with TSA and those left untreated (Fig. 3B). These results demonstrate that TSA treatment did not alter the interaction of GATA4 with Cdk9.

A dominant-negative form of Cdk9 inhibited the TSA-induced increase in Nkx2.5/GFP-positive cells

To examine the effect of a dominant-negative form of Cdk9 (DN-Cdk9), which loses its kinase activity, on myocardial differentiation, pLenti-DN-Cdk9 or pLenti-Null was introduced into ES cells at the undifferentiated stage by lentivirus-mediated gene transfer. These cells were induced to differentiate and stimulated with TSA at a concentration of 10 ng/ml on day 7. On the next day, differentiated ES cells were isolated, and the nuclear extracts were subjected to Western blotting (Fig. 4A). The signals of RNA polymerase II protein (RNA pol II a) were similar in DN-Cdk9- and Null-introduced ES cells. However, the signal of the phosphorylated form of RNA polymerase II protein (RNA pol II o) was inhibited by the introduction of pLenti-DN-Cdk9. Treatment with TSA had no effect on the signal levels of both RNA pol II a and RNA pol II o. The signals of the proteins, GATA4, Cdk9, and cyclin T1 were not affected by treatment with TSA or by the introduction of DN-Cdk9.

Next, pLenti-DN-Cdk9 or pLenti-Null was introduced into Nkx2.5/ES cells. Then, these cells were introduced to

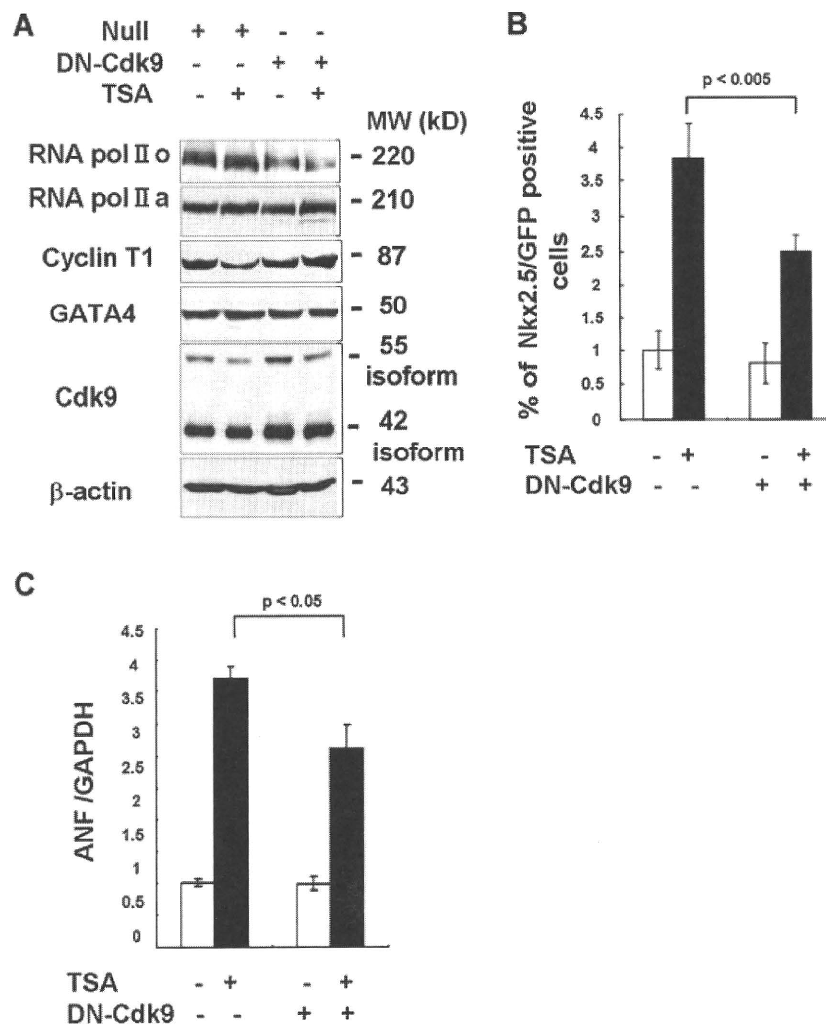

Fig. 4. A: ES cells were transformed with pLenti-Null or pLenti-DN-Cdk9 on day 0. These cells were induced to differentiate and stimulated with TSA (10 ng/ml) or its solvent on day 7 for 24 h. Nuclear extracts were isolated on day 8 and subjected to Western blotting using the indicated antibodies. The photographs are representative. We performed three independent experiments and obtained similar results. B: Nkx2.5/GFP ES cells were transformed with pLenti-Null or pLenti-DN-Cdk9 on day 0, induced to differentiate, and stimulated with TSA or its solvent on day 7 for 24 h. These cells were dissociated into single cells on day 8 and subjected to flow cytometry. These data represent the rate of Nkx2.5/GFP-positive cells to total extant cells and are expressed as means ± SE of three or more independent experiments. C: ES cells were transformed with pLenti-Null or pLenti-DN-Cdk9 on day 0. Then, these cells were induced to differentiate and stimulated with TSA (50 ng/ml) or its solvent on day 7 for 24 h. Total RNAs were isolated by TRIzol® reagent on day 8 and subjected to quantitative RT-PCR.

differentiate and treated with TSA on day 7 for 24 h. On the next day, these cells were dissociated into single cells by trypsin and subjected to flow cytometry. DN-Cdk9 significantly inhibited the TSA-induced increase in Nkx2.5/GFP-positive cells (Fig. 4B).

Furthermore, pLenti-DN-Cdk9 or pLenti-Null was introduced into Nkx2.5/ES cells on day 0. Then, these cells were induced to differentiate and stimulated with TSA on day 7. On day 8, total RNAs were isolated, and the synthesized cDNAs were subjected to quantitative PCR. Treatment with TSA increased the level of ANF mRNA more than 3.5-fold in differentiated ES cells into which pLenti-Null was introduced (Fig. 4C). The introduction of pLenti-DN-Cdk9 into ES cells significantly suppressed the TSA-induced increase in the ANF mRNA level. These results demonstrate that a dominant-negative Cdk9 inhibited TSA-induced ANF expression.

DRB inhibited TSA-induced expression of Nkx2.5

Differentiated ES cells were treated with TSA and/or Cdk9 inhibitor DRB on day 7. On day 8, these cells were isolated and their nuclear extracts were subjected to Western blotting. While the signal levels of RNA pol II a were not affected by treatment with TSA and/or DRB, the signal of RNA pol II o was inhibited by treatment with DRB. The signals of GATA4, Cdk9, and cyclin T1 proteins were not affected by treatment with either agent (Fig. 5A).

Next, Nkx2.5/GFP ES cells were induced to differentiate and treated with TSA and/or DRB on day 7 for 24 h. Then, these cells were dissociated into single cells and subjected to flow cytometry. While DRB did not affect the basal rate of Nkx2.5/GFP-positive cells relative to total extant cells, DRB almost completely inhibited the TSA-induced increase in Nkx2.5/GFP-positive cells (Fig. 5B). ES cells were similarly treated with these agents on day 7 and the total RNAs were isolated by TRIzol® reagent on day 8. The synthesized cDNAs were subjected to quantitative PCR. While treatment with TSA increased the expression of endogenous Nkx2.5 mRNA by more than 12-fold, additional DRB treatment markedly inhibited the TSA-induced increase in the endogenous Nkx2.5 mRNA level (Fig. 5Ca). On the other hand, treatment with TSA did not increase the endogenous mRNA level of VEGF, a maker of vascular endothelial cells. Treatment with DRB did not affect the endogenous mRNA expression of VEGF either (Fig. 5Cb).

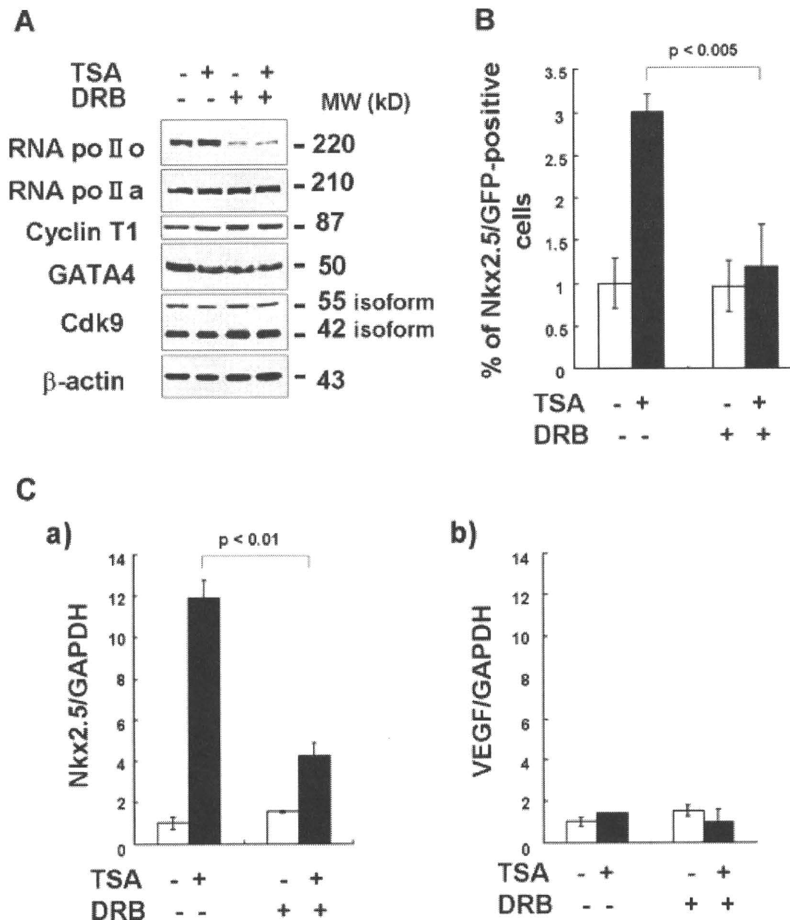

Fig. 5. A: Differentiated ES cells were stimulated with TSA and/or DRB on day 7 for 24 h and collected on day 8. Nuclear extracts from these cells were subjected to Western blotting using the indicated antibodies. The photographs are representative. We performed three independent experiments and obtained similar results. B: Differentiated Nkx2.5/GFP ES cells stimulated with TSA (50 ng/ml) and/or DRB (50 μM) were dissociated into single cells and subjected to flow cytometry. These data show the rate of Nkx2.5/GFP-positive cells to total extant cells. C: Total RNAs from differentiated ES cells treated with TSA (50 ng/ml), and/or DRB (50 μM), or both were isolated, and synthesized cDNAs were subjected to quantitative PCR. Levels of GAPDH transcripts were used to normalize cDNA levels. The mRNA level of Nkx2.5 (a) or VEGF (b) relative to GAPDH in ES cells treated with a solvent, ethanol, was set at 1.0. The data are expressed as means ± SE of three or more independent experiments.

Discussion

In this study, we examined whether Cdk9 forms a complex with GATA4 and is involved in the TSA-induced differentiation of mouse ES cells into cardiomyocytes. To induce the differentiation of ES cells, we used feeder-free gelatin-coated plates without the formation of EBs because it is simple and reproducible. Within this system, we initially examined the expression profiles of genes involved in cardiogenesis, and demonstrated that these profiles were similar to those in experiments involving EB formation (Kawamura et al., 2005). The treatment of ES cells with TSA, an HDAC inhibitor, increased Nkx2.5/GFP-positive cells and endogenous mRNA levels of Nkx2.5 and ANF in a concentration-dependent manner. We also confirmed that TSA increased endogenous mRNA levels of these genes in mouse-induced pluripotent stem cells (Kaichi et al., 2010).

To examine whether Cdk9 is involved in the differentiation of ES cells into cardiomyocytes, we ablated the Cdk9 kinase activity using either DN-Cdk9 or a Cdk9 inhibitor, DRB. The suppression of Cdk9 kinase activity inhibited the TSA-induced expression of the cardiac-specific transcription factor Nkx2.5. While DRB almost completely inhibited TSA-induced myocardial differentiation, inhibition by the introduction of DN-Cdk9 was partial. Cdk9 was abundantly expressed throughout stages of undifferentiation to differentiation, and the efficiency of gene transduction by lentivirus-mediated gene transfer might vary in each transfection. These may have attributed to the discrepancy in the inhibitory effects between DRB and DN-Cdk9.

Studies using either RNA interference or highly specific p-TEFb inhibitors have implicated p-TEFb as an important factor in global transcriptional elongation (Shim et al., 2002). In fact, Cdk9 and cyclinT1 were abundantly expressed from at the undifferentiated stage to differentiated stage. One of the key questions is how p-TEFb is recruited to its target genes in myocardial differentiation. At least, there are two mechanisms for the recruitment of p-TEFb. One is a less specific recruitment through the interaction between p-TEFb and Brd4, and the other is a gene-specific recruitment through an interaction between p-TEFb and transactivators (Kanazawa et al., 2000; Barboric et al., 2001; Eberhardy and Farnham, 2002; Simone et al., 2002). A previous report demonstrated that MyoD recruits p-TEFb onto the promoters and enhancers of muscle-specific genes during skeletal myogenesis (Giacinti et al., 2006). Another reported that recruitment of p-TEFb is a critical step for stimulation of MEF2-dependent transcription in skeletal muscle cells (Nojima et al., 2008). We presented that Cdk9 is recruited onto the ANF promoter by interacting with GATA4/p300 complex in cardiomyocyte hypertrophy, and that Cdk9 kinase activity was required for the p300-induced transcriptional activities, DNA-binding, and acetylation of GATA4 (Sunagawa et al., 2010). We have demonstrated that the Cdk9/cyclin T1 complex combines with GATA4 during myocardial differentiation in ES cells. These findings raise a hypothesis that Cdk9 kinase activity is involved in the TSA-induced activation of GATA4 in ES cells as well as in the pathway of cardiomyocyte hypertrophy. Therefore, it would be interesting to examine the detailed functional association between GATA4 and Cdk9 during myocardial differentiation.

Acknowledgments

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Literature Cited

Barboric M, Nissen RM, Kanazawa S, Jabrane-Ferrat N, Peterlin BM. 2001. NF-kappaB binds P-TEFb to stimulate transcriptional elongation by RNA polymerase II. Mol Cell 8:327–337.

Chan HM, La Thangue NB. 2001. p300/CBP proteins: HATs for transcriptional bridges and scaffolds. J Cell Sci 114:2363–2373.

Eberhardy SR, Farnham PJ. 2002. Myc recruits P-TEFb to mediate the final step in the transcriptional activation of the cad promoter. J Biol Chem 277:40156–40162.

Giacinti C, Bagella L, Puri PL, Giordano A, Simone C. 2006. MyoD recruits the cdk9/cyclin T2 complex on myogenic-genes regulatory regions. J Cell Physiol 206:807–813.

Grepin C, Nemer G, Nemer M. 1997. Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor. Development 124:2387–2395.

Hidaka K, Lee JK, Kim HS, Ihm CH, Iio A, Ogawa M, Nishikawa S, Kodama I, Morisaki T. 2003. Chamber-specific differentiation of Nkx2.5-positive cardiac precursor cells from murine embryonic stem cells. FASEB J 17:740–742.

Hirai M, Ono K, Morimoto T, Kawamura T, Wada H, Kita T, Hasegawa K. 2004. FOG-2 competes with GATA-4 for a transcriptional coactivator p300 and represses hypertrophic responses in cardiac myocytes. J Biol Chem 279:37640–37650.

Iwanaga Y, Kihara Y, Hasegawa K, Inagaki K, Yoneda T, Kaburagi S, Araki M, Sasayama S. 1998. Cardiac endothelin-1 plays a critical role in the functional deterioration of left ventricles during the transition from compensatory hypertrophy to congestive heart failure in salt-sensitive hypertensive rats. Circulation 98:2065–2073.

Johnson CA, Turner BM. 1999. Histone deacetylases: Complex transducers of nuclear signals. Semin Cell Dev Biol 10:179–188.

Kaichi S, Hasegawa K, Takaya T, Yokoo N, Mima T, Kawamura T, Morimoto T, Ono K, Baba S, Doi D, Yamanaka S, Nakahata N, Heike T. in press. Cell line-dependent differentiation of induced pluripotent stem cells into cardiomyocytes in mice. Cardiovasc Res.

Kanazawa S, Okamoto T, Peterlin BM. 2000. Tat competes with CIITA for the binding to P-TEFb and blocks the expression of MHC class II genes in HIV infection. Immunity 12:61–70.

Kawamura T, Ono K, Morimoto T, Wada H, Hirai M, Hidaka K, Morisaki T, Heike T, Nakahata T, Kita T, Hasegawa K. 2005. Acetylation of GATA-4 is involved in the differentiation of embryonic stem cells into cardiac myocytes. J Biol Chem 280:19682–19688.

Kuo CT, Morrisey EE, Anandappa R, Sigrist K, Lu MM, Parmacek MS, Soudais C, Leiden JM. 1997. GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. Genes Dev 11:1048–1060.

Marshall RM, Grana X. 2006. Mechanisms controlling CDK9 activity. Front Biosci 11:2598–2613.

Molkentin JD, Lin Q, Duncan SA, Olson EN. 1997. Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. Genes Dev 11: 1061–1072.

Nojima M, Huang Y, Tyagi M, Kao HY, Fujinaga K. 2008. The positive transcription elongation factor b is an essential cofactor for the activation of transcription by myocyte enhancer factor 2. J Mol Biol 382:275–287.

Ogawa S, Tagawa Y, Kamiyoshi A, Suzuki A, Nakayama J, Hashikura Y, Miyagawa S. 2005. Crucial roles of mesodermal cell lineages in a murine embryonic stem cell-derived in vitro liver organogenesis system. Stem Cells 23:903–913.

Sachinidis A, Fleischmann BK, Kolossov E, Wartenberg M, Sauer H, Hescheler J. 2003. Cardiac specific differentiation of mouse embryonic stem cells. Cardiovasc Res 58:278–291.

Shikama N, Lyon J, Thangue NB. 1997. The p300/CBP family: Integrating signals with transcription factors and chromatin. Trends Cell Biol 7:230–236.

Shim EY, Walker AK, Shi Y, Blackwell TK. 2002. CDK-9/cyclin T (P-TEFb) is required in two postinitiation pathways for transcription in the C. elegans embryo. Genes Dev 16:2135–2146.

Simone C, Stiegler P, Bagella L, Pucci B, Bellan C, De Falco G, De Luca A, Guanti G, Puri PL, Giordano A. 2002. Activation of MyoD-dependent transcription by cdk9/cyclin T2. Oncogene 21:137–148.

Sunagawa Y, Morimoto T, Takaya T, Kaichi S, Wada H, Kawamura T, Fujita M, Shimatsu A, Kita T, Hasegawa K. 2010. Cyclin-dependent kinase-9 is a component of the p300/GATA4 complex required for phenylephrine-induced hypertrophy in cardiomyocytes. J Biol Chem 285:9556–9568.

Takaya T, Kawamura T, Morimoto T, Ono K, Kita T, Shimatsu A, Hasegawa K. 2008. Identification of p300-targeted acetylated residues in GATA4 during hypertrophic responses in cardiac myocytes. J Biol Chem 283:9828–9835.

Takaya T, Ono K, Kawamura T, Takanabe R, Kaichi S, Morimoto T, Wada H, Kita T, Shimatsu A, Hasegawa K. 2009. MicroRNA-1 and MicroRNA-133 in spontaneous myocardial differentiation of mouse embryonic stem cells. Circ J 73:1492–1497.

Wada H, Hasegawa K, Morimoto T, Kakita T, Yanazume T, Abe M., Sasayama S. 2002. Calcineurin-GATA-6 pathway is involved in smooth muscle-specific transcription. J Cell Biol 156:983–991.

Yanazume T, Hasegawa K, Morimoto T, Kawamura T, Wada H, Matsumori A, Kawase Y, Hirai M, Kita T. 2003. Cardiac p300 is involved in myocyte growth with decompensated heart failure. Mol Cell Biol 23:3593–3606.

Zhu Y, Peery T, Peng J, Ramanathan Y, Marshall N, Marshall T, Amendt B, Mathews MB, Price DH. 1997. Transcription elongation factor P-TEFb is required for HIV-1 tat transactivation in vitro. Genes Dev 15:2622–2632.