Biochemical and Biophysical Research Communications 426 (2012) 317–323

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

NFAT5 regulates the canonical Wnt pathway and is required for cardiomyogenic differentiation

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ARTICLE INFO

Article history:
Received 31 July 2012
Available online 23 August 2012

Keywords:
P19CL6 cells
Cardiomyogenesis
Mesodermal induction
NFAT5
Canonical Wnt signaling

ABSTRACT

While nuclear factor of activated T cells 5 (NFAT5), a transcription factor implicated in osmotic stress response, is suggested to be involved in other processes such as migration and proliferation, its role in cardiomyogenesis is largely unknown. Here, we examined the role of NFAT5 in cardiac differentiation of P19CL6 cells, and observed that it was abundantly expressed in undifferentiated P19CL6 cells, and its protein expression was significantly downregulated by enhanced proteasomal degradation during DMSO-induced cardiomyogenesis. Expression of a dominant negative mutant of NFAT5 markedly attenuated cardiomyogenesis, which was associated with the inhibition of mesodermal differentiation. TOPflash reporter assay revealed that the transcriptional activity of canonical Wnt signaling was activated prior to mesodermal differentiation, and this activation was markedly attenuated by NFAT5 inhibition. Pharmacological activation of canonical Wnt signaling by [2′Z, 3′E]-6-bromoindirubin-3′-oxime (BIO) restored Brachyury expression in NFAT5DN-expressing cells. Inhibition of NFAT5 markedly attenuated Wnt3 and Wnt3a induction. Expression of Dkk1 and Cerberus1, which are secreted Wnt antagonists, was also inhibited by NFAT5 inhibition. Thus, endogenous NFAT5 regulates the coordinated expression of Wnt ligands and antagonists, which are essential for cardiomyogenesis through the canonical Wnt pathway. These results demonstrated a novel role of NFAT5 in cardiac differentiation of stem cells.

1. Introduction

The heart is the first organ that becomes functional in vertebrate embryonic development, and cardiac differentiation of stem cells is a multistep process consisting of commitment of undifferentiated cells to mesoderm, and differentiation into cardiac mesodermal cells, and subsequently into cardiac myocytes [1,2]. Recent advances in stem cell biology not only provide unique opportunities to study the development of cardiac myocytes, but also suggest the potential use of stem cell-derived cardiac myocytes for many medical applications such as cell transplantation therapy against various heart diseases and pharmacological testing on cardiac myocytes. However, the molecular mechanisms governing differentiation of stem cells into specific lineages are poorly understood, and their comprehension would improve the efficiency of differentiation into specific cell types.

Nuclear factor of activated T cells 5 (NFAT5), also referred to as tonicity-response element-binding protein (TonEBP), is a Rel family transcription factor, which also comprises the NF-κB and calcineurin-dependent NFATc proteins [3,4]. Although its DNA-binding domain shows a hybrid nature between those of NFAT and NF-κB proteins, there is no recognizable similarity outside the Rel homology region between NFAT5 and the other Rel family members. While NFAT5 DNA-binding domain is more similar to those of NFATc transcription factors than those of NFkB proteins, NFAT5 lacks the N-terminal NFAT homology region containing the calcineurin regulatory motif. Therefore, its activity is regulated in a calcium/calcineurin-independent manner. NFAT5 is most known to be involved in the adaptation of mammalian cells to hypertonic stress [3]. NFAT5 is activated by hypertonicity and, in turn, stimulates the transcription of target osmoprotective genes responsible for the metabolism of organic osmolytes. In addition to its critically important roles in mediating compensatory transcriptional responses to extracellular hypertonic stresses, NFAT5 is also shown to be involved in other processes independent of the hypertonic responses such as cellular migration and

Abbreviations: NFAT5, nuclear factor of activated T cells 5; BIO, [2′Z, 3′E]-6-bromoindirubin-3′-oxime; BrdU, 5-bromodeoxyuridine; cTnI, cardiac troponin I; αMHC, α myosin heavy chain; Cer, cerberus.

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http://dx.doi.org/10.1016/j.bbrc.2012.08.069

proliferation [4]. NFAT5 is expressed in embryonic stem cells and in multiple developing organs in mouse embryos [5]. Mice lacking NFAT5, which show renal atrophy and impairment of cardiac development and immune function, have dramatically reduced embryonic viability [6–8]. While NFAT5 is implicated in cell survival, migration, proliferation, vascular remodeling, carcinoma invasion, and angiogenesis [3,4], its role in cardiomyogenesis remains to be fully clarified.

In this study, we analyzed the role of NFAT5 in cardiomyogenesis using P19CL6 cells. P19CL6 cells are a clonal derivative of murine teratocarcinoma P19 cells and a very useful model to study cardiac myocyte differentiation. By using this cell line, we found a novel role of NFAT5 in cardiac differentiation of stem cells.

2. Materials and methods

2.1. Cell culture and differentiation

P19CL6 cells were cultured essentially as described previously [9,10]. In brief, the cells were grown in a 100-mm dish under adherent conditions with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) (growth medium), and were maintained in a 5% CO_2 atmosphere at 37 °C. To induce differentiation under adherent conditions, P19CL6 cells were plated at a density of 1.8×10^5 cells in a 6-well plate with the growth medium containing 1% dimethyl sulfoxide (DMSO) (differentiation medium). The medium was changed every two days. Days of differentiation were numbered consecutively after the first day of DMSO treatment as day 0. To activate Wnt/β-catenin signaling in cultured P19CL6 cells, the culture medium was supplemented with 2 μM [2′Z, 3′E]-6-bromoindirubin-3′-oxime (BIO; Biosciences) as described previously [11].

2.2. Immunoblot analysis

Cell lysates containing equal amounts of protein were electrophoresed on SDS–polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore). Blots were performed with primary antibodies against NFAT5 (Affinity BioReagents), sarcomeric myosin (MF20; Developmental Studies Hybridoma Bank), ubiquitin (P4D1; Cell Signaling), or GAPDH (Chemicon), and horseradish peroxidase-conjugated anti-mouse or -rabbit IgG as a secondary antibody.

2.3. RNA analysis

Total RNA was extracted from cells using an RNeasy mini kit (QIAGEN). cDNA was synthesized from total RNA and analyzed by quantitative real-time PCR using the ABI Prism 7700 Sequence Detector system (Applied Biosystems) with SYBR premix Ex TaqII (Takara). Mouse GAPDH was used for normalization. Primers used were NFAT5-f: AAGGCAACTCAAAAGCTGGA, NFAT5-r: TGCAACACCACTGGTTCATT, Oct4-f: AGGCAGGAGCACGAGTGGAAAGCA, Oct4-r: GGAGGGCTTCGGGCACTTCAGAAA, Nanog-f: AAGTACCTCAGCCTCCAGCA, Nanog-r: GTGCTGAGCCCTTCTGAATC, Wnt3-f: GCGACTTCCTCAAGGACAAG, Wnt3-r: AAAGTTGGGGGAGTTCTCGT, Wnt3a-f: CCCTTTCCAGTCCTGGTGTA, Wnt3a-r: CTTGAAGAAGGGGTGCAGAG, Brachyury-f: AAGGAACCACCGGTCATC, Brachyury-r: GTGTGCGTCAGTGGTGTGTAATG, Mesp1-f: CCTTCGGAGGGAGTAGATCC, Mesp1-r: AAAGCTTGTGCCTGCTTCAT, GATA4-f: TCTCACTATGGGCACAGCAG, GATA4-r: CGAGCAGGAATTTGAAGAGG, Nkx2.5-f: CAGTGGAGCTGGACAAAGCC, Nkx2.5-r: TAGCGACGGTTCTGGAACCA, cardiac troponin I (cTnI)-f: TAAGATCTCCGCCTCCAGAA, cTnI-r: CGGCATAAGTCCTGAAGCTC, α myosin heavy chain (αMHC)-f: GAGGACCAGGCCAATGAGTA, αMHC-r: GCTGGGTGTAGGAGAGCTTG, Cerberus1 (Cer1)-f: AGGAGGAAGCCAAGAGGTTC, Cer1-r: CATTTGCCAAAGCAAAGGTT, Dkk1-f: GAGGGGAAATTGAGGAAAGC, Dkk1-r: GGTGCACACCTGACCTTCTT, GAPDH-f: TTGTGATGGGTGTGAACCACGAGA, and GAPDH-r: CATGAGCCCTTCCACAATGCCAAA.

2.4. Immunoprecipitation

Immunoprecipitation was performed using cell lysates from P19CL6 cells. Protein samples in lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0) containing 1% NP-40 and protease inhibitor mixture were incubated on ice and rotated at 4 °C for 2 h with mouse anti-ubiquitin or rabbit anti-NFAT5 antibody, and then protein G- or A-conjugated Sepharose beads (Amersham Biosciences) were added to samples and rotated at 4 °C for 1 h. The beads were collected by centrifugation at 15,000 rpm for 1 min and washed three times with the lysis buffer without protease inhibitor mixture. Samples were boiled at 95 °C for 5 min in Laemmli buffer for Western blotting.

2.5. Plasmids

An expression plasmid encoding a FLAG-tagged dominant negative mutant of NFAT5 (NFAT5DN) was kindly provided by Dr. Ben C.B. Ko (The Chinese University of Hong Kong) [12]. TOPflash and FOPflash plasmids were purchased from Upstate Biotechnology.

2.6. Stable transformants

To establish the permanent P19CL6 cell line expressing NFAT5DN (P19CL6-NFAT5DN), P19CL6 cells were transfected with the FLAG-tagged NFAT5DN expression vector by the lipofection method (Lipofectamine2000, PLUS Reagent; Invitrogen). Stable transformants were selected with 500 μg/ml neomycin (Gibco) for 7 days, and 3 independent clones were isolated.

2.7. Transfection and luciferase assays

P19CL6 cells were plated at a density of 8×10^5 cells in 60 mm dishes with the growth medium. Eighteen hr after plating, the cells were transfected with 1.0 μg of a TOPflash plasmid or a FOPflash plasmid, which was used as a control, using Lipofectamine2000 and PLUS reagent in Opti-MEM (Gibco). The medium was changed 3 h after transfection and the transfected cells were re-seeded at a density of 1.8×10^5 cells in 6-well plates 24 h after transfection. After 18 h, the cells were induced to undergo cardiomyogenesis by DMSO. Luciferase activity was measured using the Blight-Glo Luciferase Assay System (Promega).

2.8. 5-Bromodeoxyuridine (BrdU) incorporation assay

Cell proliferation was analyzed by the measurement of BrdU incorporation into newly synthesized cellular DNA using Cell Proliferation ELISA, BrdU (colorimetric) (Roche Applied Science), as described previously [13].

2.9. Statistical analysis

All experiments were performed at least three times. Data are expressed as means ± SE and were analyzed by unpaired Student's *t*-test for comparisons between two groups or one-way ANOVA with post hoc analysis for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. NFAT5 protein expression is downregulated during cardiomyogenesis

We examined NFAT5 expression during cardiomyogenesis in P19CL6 cells. NFAT5 protein was abundantly expressed in undifferentiated P19CL6 cells, and upon induction of cardiomyogenesis by DMSO, its expression was gradually downregulated and then faintly detectable at day 10 (Fig. 1A). On the other hand, NFAT5 mRNA expression was not significantly altered during cardiomyogenesis (Fig. 1B). These results suggest that downregulation of NFAT5 protein expression during cardiomyogenesis is regulated by a post-transcriptional mechanism.

3.2. NFAT5 protein expression is downregulated by enhanced proteasomal degradation during cardiomyogenesis

We assessed NFAT5 protein stability using cycloheximide, an inhibitor of protein synthesis, during early and middle phases of cardiomyogenesis. Differentiating P19CL6 cells at day 4 or day 8 were exposed to cycloheximide (50 μM) for 24 h. NFAT5 protein stability was significantly reduced in cells treated with cycloheximide at day 8 compared with those at day 4 (Fig. 2A), indicating that increased proteolysis is induced during the middle phase of cardiomyogenesis. We then investigated whether the proteasome-mediated proteolysis is involved in the reduction of NFAT5 protein during DMSO-induced cardiomyogenesis. Differentiating P19CL6 cells at day 12 were exposed to a proteasome inhibitor, MG-132 (0.5 or 1.0 μM), for 24 h. Treatment with 1.0 μM MG-132 increased the protein level of NFAT5, while the mRNA level of NFAT5 was not altered by treatment with MG-132 (Fig. 2B), indicating that degradation of NFAT5 caused by DMSO-induced cardiomyogenesis is due to the activation of proteasome. To examine whether ubiquitination is involved in NFAT5 degradation during cardiomyogenesis, we performed immunoprecipitation assay using anti-ubiquitin or anti-NFAT5 antibody in undifferentiated P19CL6 cells and differentiating P19CL6 cells at day 10. In lysates from both cells, ubiquitinated protein was readily detectable, whereas ubiquitinated NFAT5 was not detected (Fig. 2C, left). We further verified that ubiquitinated NFAT5 was not observed even in lysates from differentiating P19CL6 cells at day 10 after treating for the last 24 h with MG-132, which increased NFAT5 protein (Fig. 2C, right). These data indicate that ubiquitin-independent proteasome proteolysis pathway is involved in NFAT5 degradation during cardiomyogenesis.

3.3. Inhibition of NFAT5 function impairs cardiomyogenesis

To examine the physiological role of NFAT5 in cardiomyogenesis, we established P19CL6 cells stably expressing NFAT5DN, which has the DNA-binding domain but lacks the transactivation domain [3]. Among 3 cell lines isolated, we used the highest expressing clone, termed P19CL6-NFAT5DN, for the following study (Fig. 3A). In P19CL6-NFAT5DN cells, mRNA expression of Oct3/4 and Nanog, undifferentiated markers, at day 0 was not different from that in P19CL6 cells stably expressing GFP (P19CL6-GFP), which were used as a control (Fig. 3B). The BrdU assay revealed

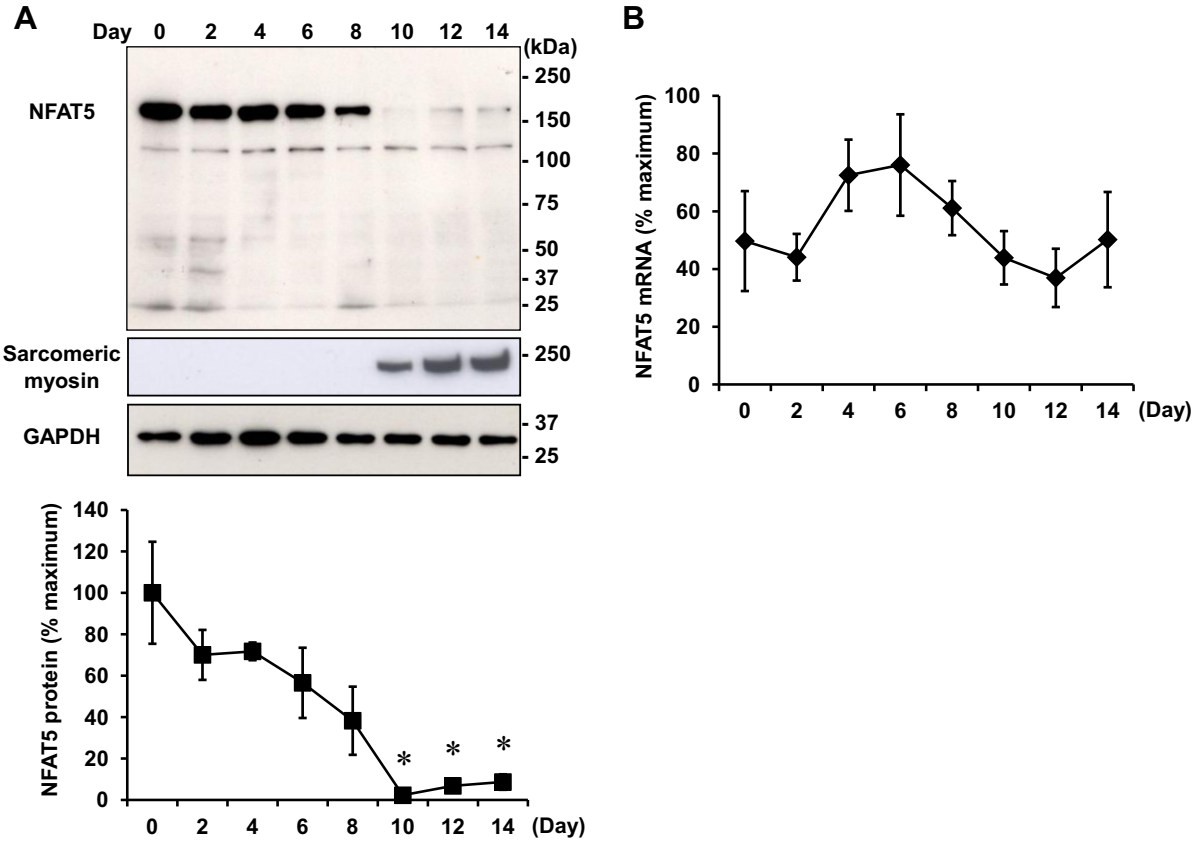

Fig. 1. NFAT5 protein expression during cardiomyogenesis. Cardiomyogenesis was induced by 1% DMSO in P19CL6. (A) Expression of NFAT5 and sarcomeric myosin protein was analyzed by Western blots using anti-NFAT5 and anti-sarcomeric myosin (MF20) antibodies, respectively, with cell lysates prepared from P19CL6 cells on the indicated days. GAPDH was used as a loading control. $^{*}P < 0.05$ compared with P19CL6 cells at day 0. (B) NFAT5 mRNA expression was analyzed by quantitative real-time PCR with total RNA isolated from P19CL6 cells on the indicated days. GAPDH was used as a control for assessing RNA loading.

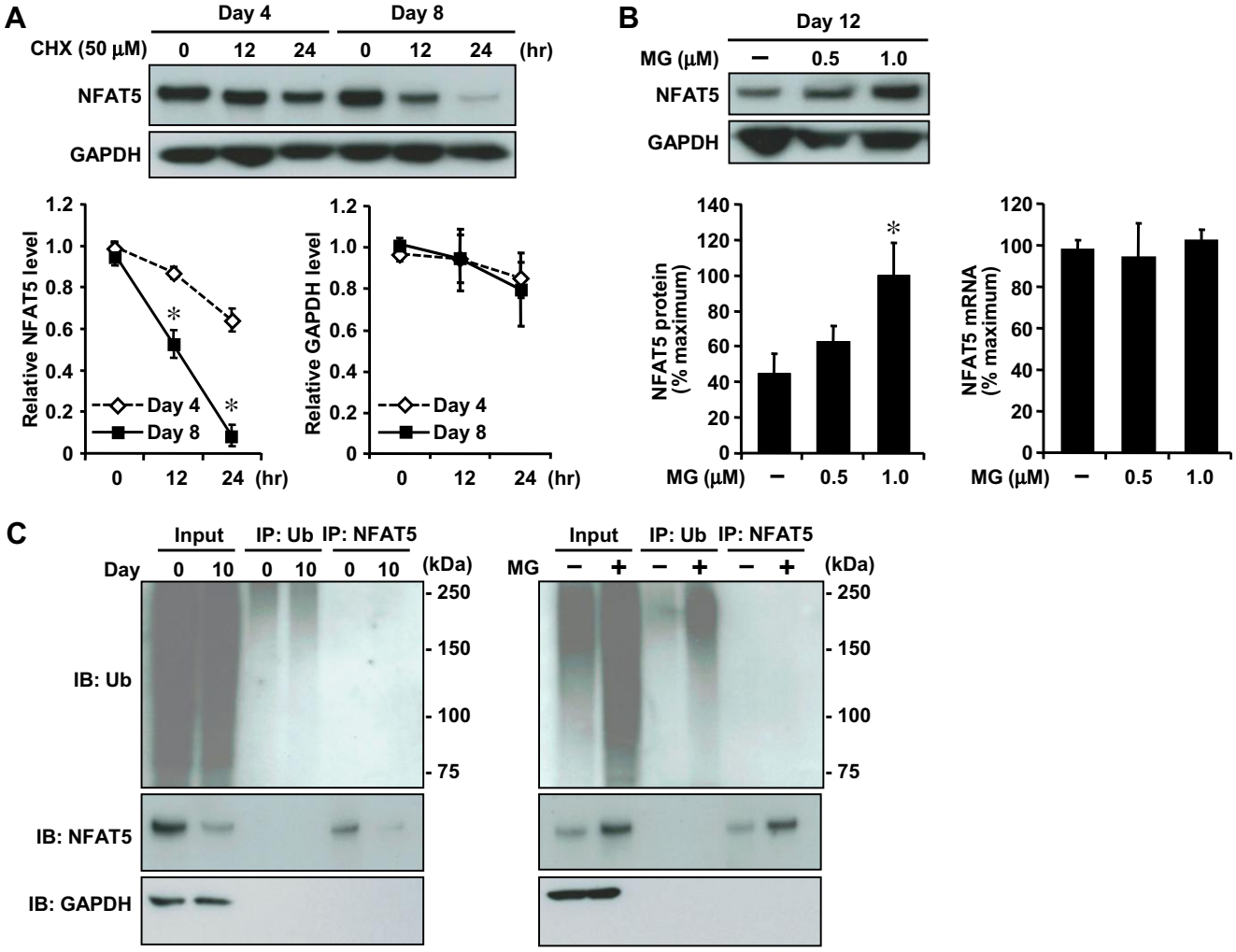

Fig. 2. Proteasomal degradation of NFAT5 protein during cardiomyogenesis. (A) Differentiating P19CL6 cells at day 4 or day 8 were treated with cycloheximide (CHX, 50 μM) for 24 h, and then expression of NFAT5 protein was analyzed by Western blots using an anti-NFAT5 antibody. $^{*}P < 0.05$ compared with P19CL6 cells at day 4. (B). Differentiating P19CL6 cells at day 12 were treated with MG-132 (0.5 or 1.0 μM) for 24 h. Western blots and quantitative real-time PCR were performed for NFAT5 protein and mRNA expression with cell lysates and total RNA isolated from P19CL cells, respectively. $^{*}P < 0.05$ compared with MG-132-untreated P19CL6 cells. GAPDH was used as a control for assessing RNA loading. (C) Immunoprecipitation assay was performed using anti-ubiquitin and anti-NFAT5 antibodies with cell lysates from undifferentiated P19CL6 cells and differentiating P19CL6 cells at day 10 (left panels). Differentiating P19CL6 cells at day 10 were treated with or without MG-132 for 24 h, and then immunoprecipitation assay was performed using anti-ubiquitin and anti-NFAT5 antibodies with the cell lysates (right panels). GAPDH was used as a loading control.

that the proliferative capacity of P19CL6-NFAT5DN cells did not differ from that in P19CL6-GFP cells (Fig. 3C). These observations indicate that P19CL6-NFAT5DN cells maintain an undifferentiated state.

Upon induction of cardiomyogenesis, expression of sarcomeric myosin protein was markedly impaired in P19CL6-NFAT5DN cells compared with that in P19CL6-GFP cells (Fig. 3D). Impaired protein expression of sarcomeric myosin was also observed in the other two cell lines stably expressing NFAT5DN (data not shown). mRNA expression of cardiomyocyte-specific transcription factors, such as Nkx2.5 and GATA4, and cardiac sarcomeric proteins, such as αMHC and cTnI, in P19CL6-NFAT5DN cells was significantly attenuated compared with that in P19CL6-GFP cells (Fig. 3E). To examine whether mesodermal differentiation is induced in P19CL6-NFAT5DN cells, we compared the mRNA expression of Brachyury, a panmesodermal marker gene, and Mesp1, a cardiogenic mesodermal marker gene, during the early phase of differentiation in P19CL6-GFP and P19CL6-NFAT5DN cells. Brachyury and Mesp1 mRNA expression in P19CL6-NFAT5DN cells was significantly reduced compared with that in P19CL6-GFP cells (Fig. 3F).

3.4. NFAT5 function is required for activation of the canonical Wnt pathway during cardiomyogenesis

Since Wnt/β-catenin signaling has been suggested to be required for mesodermal formation [14], we assessed Wnt/β-catenin signaling by performing the TOPflash reporter assay in P19CL6 cells after treatment with DMSO. The TOPflash reporter assay revealed that the activity of canonical Wnt signaling after induction of cardiogenesis was significantly increased at 24 h, peaked at 48 h, and disappeared at 72 h in P19CL6-GFP cells, while this activation was markedly attenuated by NFAT5DN expression (Fig. 4A). To investigate the role of the canonical Wnt pathway in NFAT5-mediated Brachyury expression, we used BIO, a selective GSK3α/β inhibitor. GSK-3β phosphorylates β-catenin and leads to its degradation, which decreases canonical Wnt signaling [14,15], and inhibition of GSK3β by BIO activates canonical Wnt signaling [16]. Pharmacological activation of the canonical Wnt pathway by BIO increased Brachyury expression in P19CL6-NFAT5DN cells (Fig. 4B).

We further examined the expression of Wnt signaling mediators. Wnt3 and Wnt3a mRNA expression in P19CL6-GFP cells was

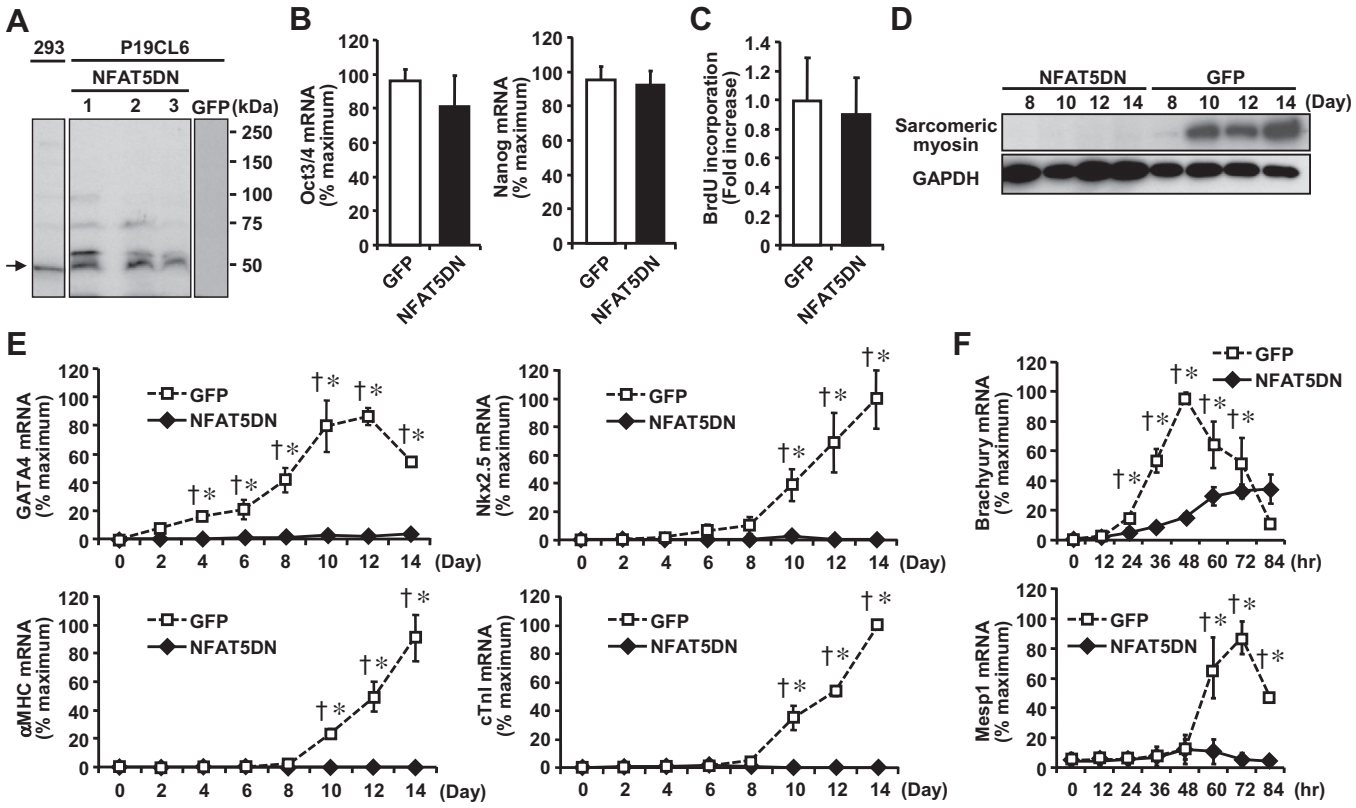

Fig. 3. Impaired cardiomyogenesis due to inhibition of NFAT5 function. (A) Expression of exogenous FLAG-tagged NFAT5DN protein in P19CL6-NFAT5DN cells was analyzed by Western blots with an anti-FLAG antibody. 293, 293 cells transfected with the FLAG-tagged NFAT5DN expression vector. GFP, P19CL6 cells stably expressing GFP. An arrow indicates exogenous FLAG-tagged NFAT5DN protein. (B) Oct3/4 and Nanog mRNA expression was analyzed by quantitative real-time PCR in undifferentiated P19CL6-NFAT5DN cells. (C) BrdU incorporation was assessed in undifferentiated P19CL6-NFAT5DN cells. (D) Expression of sarcomeric myosin protein was analyzed in P19CL6-NFAT5DN and -GFP cells on the indicated days by Western blots with an anti-sarcomeric myosin (MF20) antibody. GAPDH was used as a loading control. (E) Expression of GATA4, Nkx2.5, αMHC, and cTnI mRNA was analyzed in P19CL6-NFAT5DN and -GFP cells on the indicated days by quantitative real-time PCR. †$P < 0.05$ compared with P19CL6-GFP cells at 0 h. *$P < 0.05$ compared with P19CL6-NFAT5DN cells at the corresponding times. (F) Expression of Brachyury and Mesp1 mRNA was analyzed in P19CL6-NFAT5DN and -GFP cells at the indicated hours by quantitative real-time PCR. GAPDH was used as a control for assessing RNA loading. †$P < 0.05$ compared with P19CL6-GFP cells at 0 h. *$P < 0.05$ compared with P19CL6-NFAT5DN cells at the corresponding times. GFP, P19CL6-GFP cells. NFAT5DN, P19CL6-NFAT5DN cells.

upregulated and peaked at 48 h and 72 h after induction of cardiomyogenesis, respectively, whereas their mRNA expression in P19CL6-NFAT5DN cells was not upregulated (Fig. 4C). We then assessed the mRNA expression of Cer1 and Dkk-1, which are known to be secreted Wnt antagonists [17]. Bimodal expression of Cer1 mRNA at 0 h and 48 h after induction of cardiomyogenesis was observed in P19CL6-GFP cells, while in P19CL6-NFAT5DN cells, upregulation of Cer1 mRNA expression was markedly impaired at the corresponding times. Dkk1 mRNA expression peaked around 48–60 h after DMSO treatment in P19CL6-GFP cells, whereas its expression in P19CL6-NFAT5DN cells was significantly attenuated.

4. Discussion

Wnt signaling plays multiple roles during early cardiac development. Canonical Wnt signaling during induction of mesoderm is a prerequisite for the formation of cardiac progenitor cells, while its downregulation is essential during specification of cardiac precursor cells and noncanonical Wnt signaling supports this step [14]. Brachyury is a direct transcriptional target for canonical Wnt signaling and is required for mesodermal formation [14,18–20]. Mesp1, whose expression is positively controlled by Wnt signaling, is the earliest marker of cardiovascular progenitors and promotes cardiovascular differentiation during embryonic development and pluripotent stem cell differentiation [21]. We demonstrated that the increase in TOPflash activity was concurrent with the expression of Brachyury mRNA and preceded the expression of Mesp1 mRNA in P19CL6-GFP cells, confirming that canonical Wnt signaling is activated prior to mesodermal differentiation in P19 CL6 cells. Inhibition of NFAT5 function impaired the increase in TOPflash activity and expression of Brachyury and Mesp1 mRNA. BIO, which enhances the stability and the accumulation of cytosolic β-catenin, partly restored Brachyury mRNA expression in P19CL6-NFAT5DN cells. These observations indicate that NFAT5 function is required for the mesodermal induction through the canonical Wnt pathway.

Proteasomes are essential for many aspects of cellular function including protein quality control, DNA repair, transcription, cell-cycle regulation, signal transduction, and antigen presentation [22]. We showed that degradation of NFAT5 was enhanced during the middle phase of cardiomyogenesis, which was mediated by a proteasome-dependent proteolytic process in a ubiquitin-independent manner. Since TOPflash activity was downregulated after cardiac mesodermal induction, enhanced degradation of NFAT5 during cardiomyogenesis may partly contribute to the downregulation of canonical Wnt signaling.

Wnt3a mostly couples to β-catenin signaling [14], and Wnt3 and Wnt3a play critical roles in mesodermal development [23–26]. Considering that increased TOPflash activity was accompanied by elevated expression of Wnt3 and Wnt3a in P19CL6-GFP cells, reduced TOPflash activity in P19CL6-NFAT5DN cells is likely attributable to suppressed expression of Wnt3 and Wnt3a. Cer is an antagonist of Wnt, Nodal, and BMP signals, and initiates cardiogenesis in Xenopus [17,27]. We showed that Cer1 mRNA

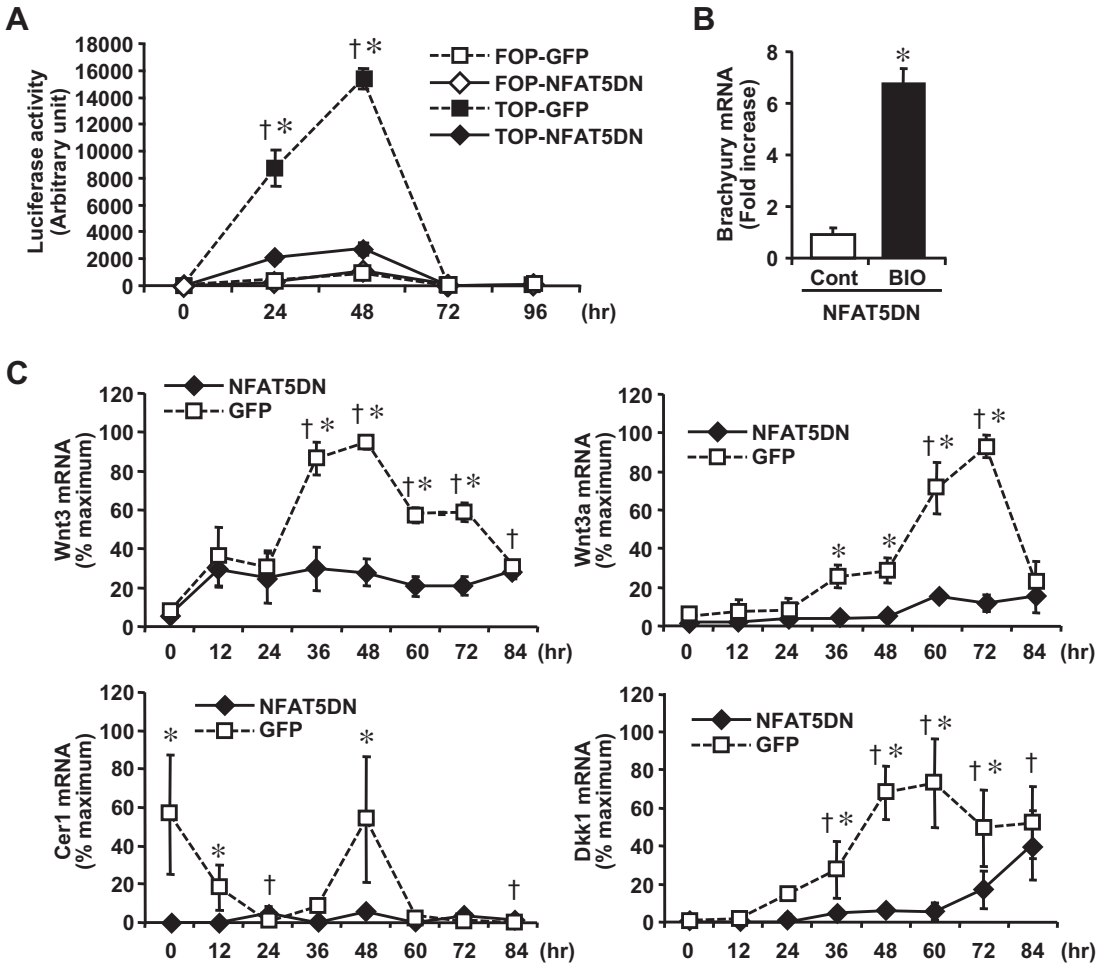

Fig. 4. Requirement of NFAT5 function for activation of the canonical Wnt pathway during cardiomyogenesis. (A) P19CL6-GFP and -NFAT5DN cells were transfected with TOPflash or FOPflash plasmid. Cells at the indicated hours were used to measure luciferase activity. FOP-GFP, P19CL6-GFP cells transfected with the FOPflash plamid. FOP-NFAT5DN, P19CL6-NFAT5DN cells transfected with the FOPflash plamid. TOP-GFP, P19CL6-GFP cells transfected with the TOPflash plamid. TOP-NFAT5DN, P19CL6-NFAT5DN cells transfected with the TOPflash plamid. †$P < 0.05$ compared with TOP-GFP cells at 0 h. *$P < 0.05$ compared with TOP-NFAT5DN cells at the corresponding times. (B) Expression of Brachyury mRNA in BIO-treated or -untreated P19CL6-NFAT5DN cells at 48 h after induction by DMSO was analyzed by quantitative real-time PCR. *$P < 0.05$ compared with BIO-untreated P19CL6-NFAT5DN cells. (C) Expression of Wnt3, Wnt3a, Cer1, and Dkk1 mRNA was analyzed in P19CL6-NFAT5DN and -GFP cells on the indicated days by quantitative real-time PCR. GAPDH was used as a control for assessing RNA loading. †$P < 0.05$ compared with P19CL6-GFP cells at 0 h. *$P < 0.05$ compared with untreated P19CL6-NFAT5DN cells at the corresponding times. GFP, P19CL6-GFP cells. NFAT5DN, P19CL6-NFAT5DN cells.

expression was reduced in undifferentiated P19CL6-NFAT5DN cells, suggesting that Cer1 expression depends on NFAT5 function in undifferentiated P19CL6 cells. Dkk1 antagonizes Wnt signaling and initiates cardiogenesis in Xenopus [17]. In P19CL6-GFP cells, upregulation of Cer1 and Dkk1 mRNA expression during cardiomyogenesis preceded downregulation of TOPflash activity, which probably contributes to downregulation of canonical Wnt signaling. In P19CL6-NFAT5DN cells, mRNA expression of Cer1 and Dkk1 was suppressed during cardiomyogenesis. Cer transcription is cooperatively activated by the Wnt and Nodal pathways [28]. Dkk1 expression is upregulated by Mesp1, although whether it is directly regulated by Mesp1 is controversial [14]. These findings support the idea that impaired expression of Cer1 and Dkk1 in P19CL6-NFAT5DN cells is due to lack of the preceding activation of canonical Wnt signaling and mesodermal induction. Alternatively, NFAT5 might directly regulate expression of Cer1 and Dkk1 during cardiomyogenesis.

A recent report demonstrated that NFAT5-deficient mice showed thinner ventricular wall with reduced cell density at the compact and trabecular areas of myocardium, and died after 14.5 days of embryonic development [8], which differs from our finding regarding the extent of cardiomyogenic differentiation. The dissimilarity between these two results might be explained by different experimental systems. With regard to the extent of cardiomyogenic differentiation, our findings correspond to a previous study showing that inhibition of Wnt/β-catenin signaling impaired cardiomyogenesis in P19CL6 cells [29]. In mouse embryos, signals from anterior endoderm and non-neutral ectoderm work to promote cardiac development in concert with signals from the anterior lateral mesoderm itself [30]. The cell culture-based system using P19CL6 cells is insufficient to replicate the signals from endoderm or ectoderm in vivo, which might account for the difference of the findings. In cardiac myocytes, inhibition of NFAT5 was reported to enhance cell death [31]. Endogenous NFAT5 was degraded during cardiomyogenesis but detectable in differentiated P19CL6 cells, while NFAT5DN was constitutively expressed and inhibits the function of its target genes during cardiomyogenesis in P19CL6-NFAT5DN cells. Impaired cardiomyogenesis in P19CL6-NFAT5DN cells might reflect NFAT5DN-mediated attenuation of both cell viability in differentiated cells and mesodermal differentiation in differentiating cells.

Our study demonstrated that cardiomyogenesis using P19CL6 cells exhibited a novel role of NFAT5 in cardiac differentiation of stem cells. Endogenous NFAT5 regulates cardiomyogenesis

through the temporal and coordinated expression of Wnt ligands and antagonists. These findings might contribute to improvement of the efficiency of differentiation into specific cell types.

Acknowledgments

We thank M. Nakata for her expert technical assistance. This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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