(viii) Centrifuge for 15 min at 12,000*g* at 4 °C.
(ix) Transfer the aqueous phase (200–250 μl) of lysate to a new 1.5-ml microtube.
(x) Add 250 μl of isopropanol to the lysate transferred in Step 58 and mix well by pipetting several times.
(xi) Centrifuge for 5 min at 12,000*g* at 4 °C.
(xii) Remove the supernatant and add 500 μl of 70% (vol/vol) ethanol.
(xiii) Centrifuge for 2 min at 9,000*g* at 4 °C.
(xiv) Remove the ethanol completely and air-dry the pellet at room temperature for 5 min.
(xv) Resuspend the pellet in 50 μl of RNase-free water.
(xvi) Use 2 μl of the sample to determine the RNA concentration of samples by measuring with an optical spectrometer (NanoDrop), and adjust the concentration of each sample to 20 ng μl^{-1} by adding RNase-free water.
(xvii) Transfer 100 μl of lysate (which contains 2 μg of RNA) to a new 1.5-ml microtube.
(xviii) Add 250 μl of ethanol and 10 μl of 3 M sodium acetate, and then mix vigorously by shaking.
(xix) Refrigerate the lysate at −20 °C for 30 min.
(xx) Centrifuge for 15 min at 12,000*g* at 4 °C.
(xxi) Remove the supernatant and add 500 μl of 70% (vol/vol) ethanol.
(xxii) Centrifuge for 2 min at 9,000*g* at 4 °C.
(xxiii) Remove the ethanol completely and air-dry the pellet at room temperature for 5 min.
(xxiv) Resuspend the pellet in 16 μl of RNase-free water.
(xxv) Add 2 μl of 10× DNase I buffer and 2 μl of DNase I to the RNA lysate, mix gently by finger tapping and incubate for 15 min at room temperature.
(xxvi) Add 2 μl of 25 mM EDTA, mix gently by finger tapping and incubate for 10 min at 65 °C.
(xxvii) Transfer the tube onto ice.
(xxviii) Transfer 14 μl of RNA lysate to a new 1.5-ml microtube and then add 2 μl of Oligo dT and 8 μl of 2.5 mM dNTP mix.
(xxix) Incubate for 5 min at 65 °C.
(xxx) Place the tube on ice.
(xxxi) Add 8 μl of 5× first-strand buffer, 4 μl of 0.1 M DTT, 2 μl of 40 U μl^{-1} of RNase inhibitor and 2 μl of SuperScript II.
(xxxii) Mix gently by finger tapping and incubate for 50 min at 42 °C.
(xxxiii) Incubate for 15 min at 70 °C.
■ **PAUSE POINT** cDNA samples should be stored at −20 °C.
(xxxiv) Prepare 20 μl of real-time PCR mixture by mixing the reagents listed below in a 96-well PCR plate.

Real-time PCR mixture	Per tube (μl)
SYBR Premix ExTaq	10
Primer sense (10 μM)	0.4
Primer antisense (10 μM)	0.4
H_2O	4
Rox	0.2
DNA sample	5
Total	20

(xxxv) Perform a SYBR Green reaction in 96-well plates on an ABI 7500 real-time PCR instrument (95 °C, 30 s; 60 °C, 30 s; 72 °C, 30 s).
▲ **CRITICAL STEP** By using a cDNA sample obtained from infected PBMCs as positive control and standards, check the expression of transgenes that were normalized to the expression of GAPDH. Check the expression of TiPSC transgenes after 10–15 passages.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
12	The number of PBMCs is insufficient	Ficoll is mixed into the PBMC solution	If more than 1×10^5 cells were obtained, seed them in 400 µl of GT-T502 medium, incubate for 6–7 d, and go to Step 25. If not, repeat Steps 2–12 with new whole blood and collect PBMCs carefully without mixing Ficoll
35	No TiPSC colonies appear	Viral titer is low	Use new SeV solution. Avoid using SeV solution after repeated freeze-thaw cycles
		Most T cells are dead before infection	Ensure the density of T cells in each appropriate step. Try SeV infection on day 4 of activation
50	Signs of iPSC differentiation after expansion	Poor-quality human iPSC medium	Use new human iPSC medium or increase bFGF concentration in the medium

● TIMING
Steps 1–11, blood sampling: ~10 min
Steps 12–22, isolating PBMCs using Ficoll gradient: ~1 h
Steps 23 and 24, activating T cells: 5 d
Steps 25–31, SeV infection: ~1 d
Steps 32–38, plating MEFs: ~30 min
Steps 39–44, replating infected PBMCs: ~30 min
Steps 45 and 46, culturing infected PBMCs: ~3 weeks
Steps 47–62, picking up and expanding the TiPSC colonies: ~3 weeks

ANTICIPATED RESULTS

T cells make up approximately 70% or less of isolated PBMCs, and they are inefficiently infected by SeV vectors before activation. However, after a 5-d activation with IL-2 and anti-CD3 antibody, the T cell proportion increases to become >95% of PBMCs, and it can be effectively infected with SeV vectors. With proper activation, T cells are infected with SeV vectors at >80% efficiency at an MOI of 20 (**Fig. 3a–d**).

From the 5×10^4 cells infected with SeV vectors encoding human OCT3/4, SOX2, KLF4 and c-MYC and seeded onto MEF feeder cells, 50–100 ESC-like colonies grow, on average (**Fig. 4a**). The TiPSC colonies clearly stain positive for alkaline phosphatase (ALP), a stem cell marker (**Fig. 4b**) and express ESC marker transcripts (**Fig. 4d**). The expression of TiPSC transgenes is not always detectable after 10–15 passages. They can also differentiate into three germ layer–derived tissues and generate teratomas *in vivo* (**Fig. 4e**) and embryoid bodies *in vitro*. These TiPSC lines will have TCR rearrangement in their genome, which is a hallmark of mature terminally differentiated T cells, and will show specific peaks for Dβ/Jβ and Vβ/Jβ recombination in capillary electrophoresis of the PCR products.

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AUTHOR CONTRIBUTIONS T.S. and S.Y. prepared most of the paper. K.F. provided advice and proofread the paper.

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CASE REPORT

Acute coronary syndrome or apical ballooning syndrome?

Yuichiro Maekawa · Akio Kawamura · Shinsuke Yuasa · Yohei Ohno · Takahide Arai · Keiichi Fukuda

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Abstract Apical ballooning syndrome (ABS) is uniquely characterized by the acute onset of transient extensive kinesis of the apical and mid portions of the left ventricle without significant epicardial coronary artery stenosis, accompanied by chest symptoms and electrocardiogram changes similar to those of acute coronary syndrome. We report a case of ABS with severe coronary artery stenosis presenting as acute coronary syndrome after emotional stress. ABS should be considered a cause of left ventricular wall motion abnormalities even if a coronary arteriogram shows severe coronary artery stenosis.

Keywords Apical ballooning syndrome · Acute coronary syndrome · Percutaneous coronary intervention · Coronary artery disease

Introduction

Apical ballooning syndrome (ABS), which mimics acute coronary syndrome (ACS), is characterized by acute left ventricular wall motion abnormalities in the absence of significant coronary artery stenosis. Some recent reports, however, indicate that ABS patients show incidental significant coronary artery disease, such as mild to moderate coronary artery stenosis [1, 2]. To our knowledge, however, there are no reports of an ABS patient with severe coronary artery stenosis.

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Case report

A 78-year-old woman developed chest discomfort after an altercation with her son and an electrocardiogram (ECG) revealed T wave inversions in leads V_{1-6}, suggesting severe ischemia. She had a history of hypertension and dyslipidemia as two coronary risk factors. Her serum troponin T level was slightly elevated (0.11 ng/ml, normal <0.09 ng/ml). As her Thrombolysis in Myocardial Infarction (TIMI) risk score, a method for predicting mortality and therapeutic decision-making, was 4 points [3], we performed an urgent left heart catheterization. A coronary angiogram (CAG) and left ventriculogram (LVG) revealed 99% stenosis of the mid-left anterior descending artery (LAD; Fig. 1a) and akinesis in the left ventricular mid segment in the anterior and inferior wall, with apical involvement (Fig. 2a, b). To exclude the possibility of coronary vasospasm, an intracoronary injection of isosorbide dinitrate was given. The injection did not relieve the LAD stenosis, and she therefore underwent percutaneous coronary intervention (PCI) of the LAD. Two bare metal stents were implanted (Fig. 1b). No remarkable complications occurred and her creatine phosphokinase levels remained within normal limits during the hospital stay. Dual anti-platelet therapy with aspirin and clopidogrel was continued after stent implantation. On day 5, she was discharged from our hospital, but later that same day she presented to the emergency room complaining of strong chest pain. ECG results suggested an ST elevation-type anteroseptal myocardial infarction. CAG showed occlusion of the mid-LAD due to subacute stent thrombosis (Fig. 1c) and PCI of the LAD was again performed (Fig. 1d). Creatine phosphokinase levels increased to 5,481 IU/l (normal <170 IU/l). Six months later, LVG showed recovered anterior and inferior wall motion except for the

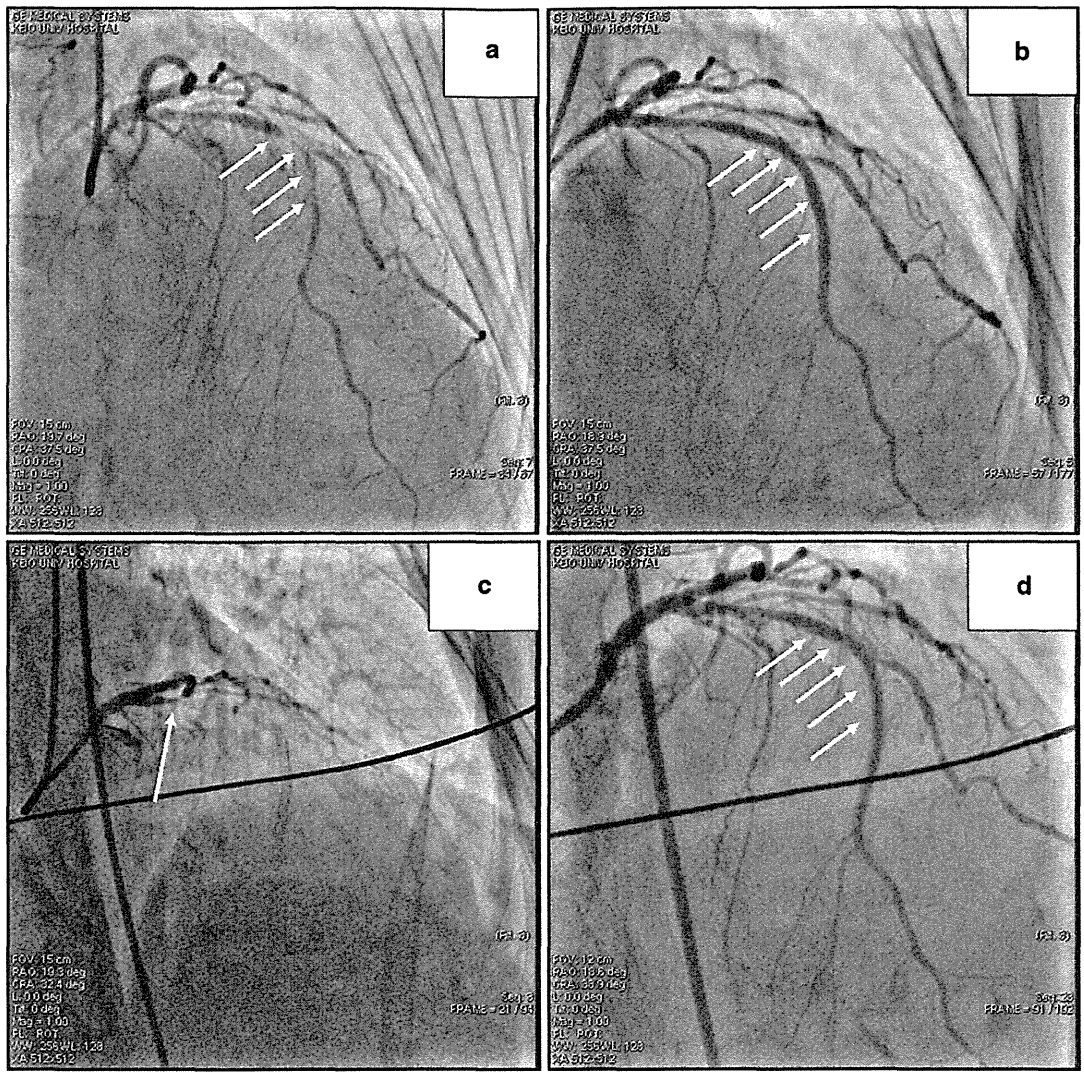

Fig. 1 Coronary angiograms of the LAD **a** before PCI and **b** after PCI. RAO cranial view showing severe stenosis of the mid-LAD (*white arrows*). Coronary angiograms of the occluded LAD due to stent thrombosis (**c**) and after PCI (**d**). RAO cranial view showing occluded LAD (*white arrows*). *LAD* left anterior descending artery, *PCI* percutaneous coronary intervention, *RAO* right anterior oblique

apical segment (Fig. 2c, d). In retrospect, the finding of the first LVG indicated myocardial wall motion abnormalities due to emotional stress, suggesting ABS rather than ACS.

Discussion

There are currently no established diagnostic criteria for ABS. Mayo Clinic diagnostic criteria propose that ABS is accompanied by regional wall motion abnormalities that extend beyond the LAD distribution in the absence of obstructive coronary disease or angiographic evidence of acute plaque rupture [4]. A previous European study, however, demonstrated that ABS can coincide with mild to moderate coronary artery disease, but it is unclear whether patients with ABS can have severe stenosis of the LAD [2]. The incidence of ABS is 1–2% in patients receiving previous left heart catheterization for suspected ACS [5]. ECG findings and clinical symptoms in patients with ABS are sometimes indistinguishable from those in patients with ACS, although previous reports demonstrated that ECG findings of ABS are different from those of ACS [6, 7]. In the present case, we did not immediately recognize that the wall motion abnormalities observed in the first LVG were due to emotional stress. The complete recovery of the anterior and inferior wall motion in the LVG 6 months after the onset, however, suggested that the original wall motion abnormalities were due to emotional stress. In ABS patients

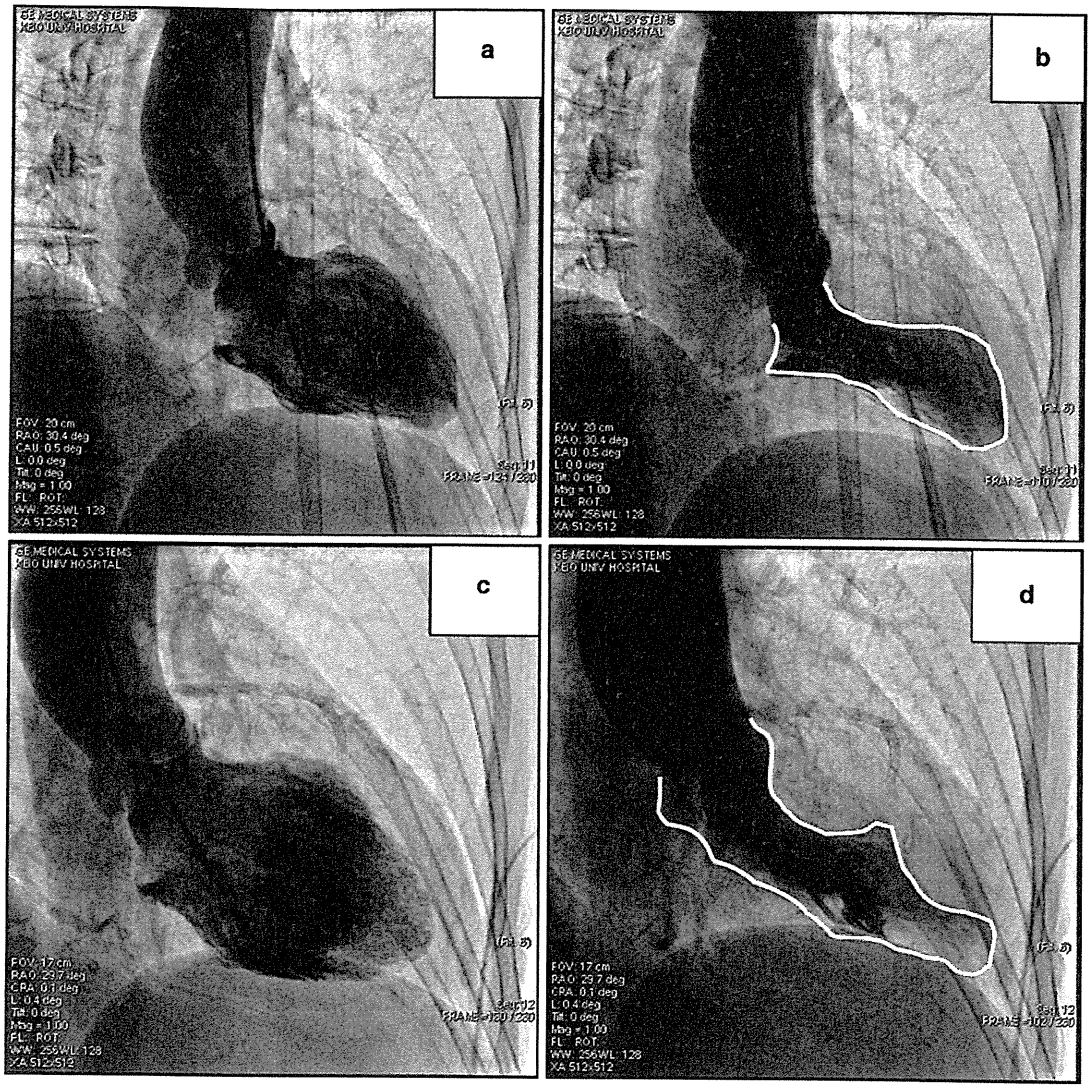

Fig. 2 The first left ventriculogram in **a** diastole and **b** systole, showing akinesis of the mid and apical ventricle with hypercontractility of the base. The left ventriculogram at 6 months in **c** diastole and **d** systole showing akinesis of the apical segment and normal anterior and inferior wall

with severe stenosis of the LAD, it is very difficult to determine whether to perform PCI. As we recognized that ABS could not coexist with severe stenosis of the LAD, we performed PCI for the LAD. Cardiovascular magnetic resonance (CMR) imaging at the time of initial clinical presentation could provide valuable information on functional and tissue characteristics of ABS patients to exclude ACS or myocarditis [8–10]. In addition to transthoracic echocardiogram and LVG, CMR imaging could be a promising diagnostic tool to confirm the diagnosis of ABS, because ABS has a considerably broad spectrum of clinical presentation [8, 11, 12]. In elderly female patients presenting with symptoms of ACS after emotional stress, ABS should be considered a cause of left ventricular wall motion abnormalities even if CAG shows severe stenosis of the LAD.

Conflict of interest None.

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Direct Comparison of Takotsubo Cardiomyopathy between Japan and USA: 3-year Follow-up Study

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Abstract

Objective Takotsubo cardiomyopathy (TC) mimics myocardial infarction and is well defined and known to not only Japan but also western countries. However, whether or not there are differences in the characteristics of TC between Japan and USA remains unknown.

Patients Data for patients who had undergone urgent left heart catheterization for suspected acute coronary syndrome were retrospectively retrieved from Keio University School of Medicine (KUSM) database in Japan and Lahey Clinic Medical Center (LCMC) database in USA between 2002 and 2007.

Results During the study period, 626 coronary angiographies were performed in KUSM and 1,880 coronary angiographies were performed in LCMC. Twelve patients in Japan and 34 patients in USA met the inclusion criteria. Mean age of patients in Japan was 75 years where 92% were women, compared to 67 years and 94% women in USA. Although the prevalence of hypertension, dyslipidemia and diabetes mellitus were similar between Japan and USA, there was a trend towards fewer patients in Japan displaying a history of coronary revascularization. Surprisingly, a family history of premature coronary artery disease (CAD) was present in 21% of USA patients, whereas no patients were present in Japan. There were no differences in the incidence of readmission for heart failure, cardiac death and TC recurrence during the follow-up period.

Conclusion Patients with TC in Japan have fewer prior overt CAD and fewer family history of premature CAD, but no significant differences were found in the long-term prognosis and the recurrence rate between patients in Japan and USA.

Key words: cardiomyopathies, coronary artery disease, heart failure

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Introduction

Dote et al first described a syndrome consisting of an acute onset of transient extensive akinesia of the apical and mid portions of the left ventricle without significant epicardial coronary artery stenosis, accompanied by chest symptoms, ECG changes, and minimal enzymatic release. They named this syndrome "Takotsubo" shaped cardiomyopathy because the systolic left ventricular image bears resemblance to the Japanese "takotsubo" (octopus trap or pot) (1). Thereafter, Japanese researchers have reported similar cases in the Japanese population (2). Therefore, researchers have considered Takotsubo cardiomyopathy (TC) to have a high prevalence in Japanese population. Recently a syndrome with the same characteristics as TC was observed and reported in other countries including USA and Europe (3-7). However, the differences in the characteristics have not yet been precisely clarified. To identify the differences in TC characteristics originating from Japan and USA, we have directly compared the database obtained from Japan and USA.

Materials and Methods

Between January 2002 and December 2007, we retrospectively enrolled 626 consecutive patients in Keio University School of Medicine (KUSM) and 1,880 consecutive patients in Lahey Clinic Medical Center (LCMC) who had under-

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gone urgent left heart catheterization for suspected acute coronary syndrome (ACS). Of these, 12 patients in Japan and 34 patients in USA fulfilled the Mayo Clinic diagnostic criteria for the diagnosis of TC (8). Medical records, including medical history, physical examination, laboratory tests, coronary angiography and left ventriculography, 12-lead ECG, and, when available, echocardiographic findings were carefully reviewed. The following data were obtained: age; gender; coronary risk factors including cigarette smoking, hypertension as defined by the Joint National Committee VII (9), diabetes mellitus as defined by the World Health Organization study group (10), dyslipidemia was considered present if the total cholesterol concentration on admission was higher than 220 mg/dL or the low-density lipoprotein-cholesterol concentration on admission was higher than 140 mg/dL and a family history of premature coronary artery disease (CAD) was defined as myocardial infarction or sudden death in a first relative, male <55 and female <65 years; possible triggering factors, concomitant medications before and after hospitalization including aspirin, beta-blockers, angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers, statins and warfarin. Cardiac enzymes [creatine kinase (CK) and troponin T] and brain natriuretic peptide (BNP) levels were measured and 12-lead ECG findings (ST shifts, T-wave changes and Q-wave formation in all leads) were examined in each database. All coronary angiograms were evaluated by two independent experienced angiographers who were blinded to all other clinical data. Normal coronary artery was defined as stenosis of <50% in the diameter of the left main coronary artery or stenosis of <75% in 1 or more of the major epicardial vessels. In-hospital complications included pump failure (a grade of class 2 or greater according to Killip's classification or subset II or greater according to Forrester's classification), malignant ventricular arrhythmia (sustained ventricular tachycardia or ventricular fibrillation), cardiac death and cardiogenic shock (11). Follow-up data, including the history of readmission for heart failure, recurrent TC, and cardiac deaths related to sudden deaths and thromboembolic events such as cerebral infarction were obtained through direct contact at an outpatient clinic, telephone interview or reviewing medical records of surviving patients. Major adverse cardiac and cerebrovascular events (MACCE) were defined as cardiac death, recurrence of TC, readmission for heart failure or cerebrovascular accident. The coronary angiograms and left ventriculograms were analyzed by two independent angiographers without knowledge of the patient's background. Global left ventricular ejection fraction (LVEF) was estimated from the right anterior oblique projection of contrast left ventriculography. The study protocol was in agreement with the guidelines of the ethics committee of our institution.

Statistical analyses

All of the data are expressed as mean ± SD. Distributions of continuous variables in the 2 groups were compared with either the unpaired *t* test or the Mann-Whitney U-test according to whether data followed the normal distribution. Comparison between two groups was performed by using the chi-square test for categorical variables. A p value of 0.05 was considered to be statistically significant. All statistical analyses were performed using Statview 5.0 software (SAS Institute Inc.) and Microsoft Office Excel (Microsoft).

Results

Patient characteristics

We retrospectively enrolled 626 consecutive patients in KUSM and 1,880 consecutive patients in LCMC who had undergone urgent left heart catheterization for suspected acute coronary syndrome. Of these, 12 patients in Japan and 34 patients in USA were diagnosed as TC. Baseline characteristics for both groups are listed in Table 1. The mean age of the 12 patients in Japan was 75±8 years (range 61 to 84), and that of the 34 patients in USA was 67±15 years (range 36 to 88). The number of women was greater in both groups.

Although the prevalence of cigarette smoking, hypertension, diabetes mellitus and dyslipidemia were similar in 2 groups, there was a trend towards fewer patients in Japan displaying a history of coronary revascularization including percutaneous coronary intervention and coronary artery bypass graft. The number of patients with a family history of premature CAD was significantly higher in USA compared to Japan. Triggers of TC such as physical and emotional stress were found to be similar in patients from Japan and USA. Table 2 shows triggers of TC in both groups. Warfarin was used more frequently in TC patients in Japan when patients left the hospital.

Peak CK and BNP levels

There was no significant difference in the peak CK levels between Japan and USA. The BNP levels on admission were also similar between the two groups (Fig. 1).

Coronary angiographic findings

There were no significant differences in LVEF and left ventricular end-diastolic pressure between Japan and USA. There tended to be more patients with normal coronary artery in Japan compared to USA (Table 3).

In-Hospital complications

The number of patients with pump failure was not significantly different between Japan and USA. The prevalence of fatal arrhythmia including ventricular tachycardia and/or ventricular fibrillation and cardiogenic shock was similar between the 2 groups. No cardiac deaths were observed while patients were in the hospital in both groups (Fig. 1).

Long-term prognosis

Mean follow-up period was 37±8 months. During the

Table 1. Baseline Characteristics

Variable	USA (n=34)	Japan (n=12)	p value
Age	67 ± 15	75 ± 8	0.10
Female, gender	94%	92%	0.77
Hypertension	76%	58%	0.23
Dyslipidemia	44%	42%	0.88
Diabetes Mellitus	18%	25%	0.58
Current Smoking	15%	17%	0.87
Physical or emotional stress	53%	50%	0.86
Family history of CAD	21%	0%	0.047
History of coronary revascularization	15%	0%	0.16
Onset of symptom			
Chest pain	74%	50%	0.14
Dyspnea	59%	42%	0.31
ECG findings			
ST elevation	65%	58%	0.69
Abnormal Q wave	12%	17%	0.66
CLBBB	3%	0%	0.55
CRBBB	3%	0%	0.55
Therapy			
IABP insertion	18%	8%	0.44
Discharge medication			
Aspirin	32%	33%	0.95
Beta-blockers	47%	50%	0.86
ACEI/ARB	32%	50%	0.37
Statins	29%	25%	0.77
Warfarin	3%	42%	0.001

CAD = coronary artery disease; CLBBB = complete left bundle branch block; CRBBB = complete right bundle branch block; IABP = intra-aortic balloon pumping; ACEI = angiotensin-converting enzyme inhibitor; ARB = angiotensin receptor blocker.

Table 2. Triggers (USA,n=34, Japan,n=12)

Physical stress	
Severe illness	7
Injury	4
Operation	1
Severe pain	1
Emotional stress	
Car accident	1
Death of a family member or friend or pet	4
Receiving bad news	2
Financial loss	2
Altercation	2
Unknown	22

follow-up period, the incidence of cardiac death tended to be greater in USA patients than Japan patients, whereas the incidences of congestive heart failure and recurrence of TC were similar between Japan and USA patients (Fig. 2). No patients suffered thromboembolic events during the follow-up period in Japan and USA. The major adverse cardiac and cerebrovascular events were not different between Japan and USA patients (Fig. 3).

Discussion

This is the first retrospective study to directly compare TC characteristics displayed by patients from Japan and USA. We demonstrated that there tended to be more patients with TC have a history of coronary revascularization in USA compared to Japan. We also found that the number of patients with a family history of premature CAD was significantly higher in USA compared to Japan. However, the long-term clinical outcomes were satisfactory in both groups.

In the present study, we demonstrated the possibility of a trend towards fewer TC patients that have undergone coronary revascularization in Japan compared to USA. Although a previous report also showed that TC could coincide with CAD in European population (12), our finding is the first to demonstrate that fewer TC patients in Japan have had coronary revascularization compared to those in USA.

Data from a study in Japan demonstrated that few TC patients in Japan had a family history of premature CAD (2). On the other hand, almost 20% of TC patients in western countries had a family history of premature CAD (5, 14). The present study showed that there was a significantly higher percentage of TC patients who had a family history of premature CAD in USA as compared to in Japan. This finding may be a clue to clarify the pathophysiology of TC. A previous paper demonstrated that CAD patients in USA had a greater prevalence of myocardial infarction and family history of CAD (13). Therefore, there is also a possibility that our conclusions may be associated with the differences of prevalence of CAD between Japan and USA though little has been reported about the prevalence of CAD in Japan.

We demonstrated that TC patients in Japan more fre-

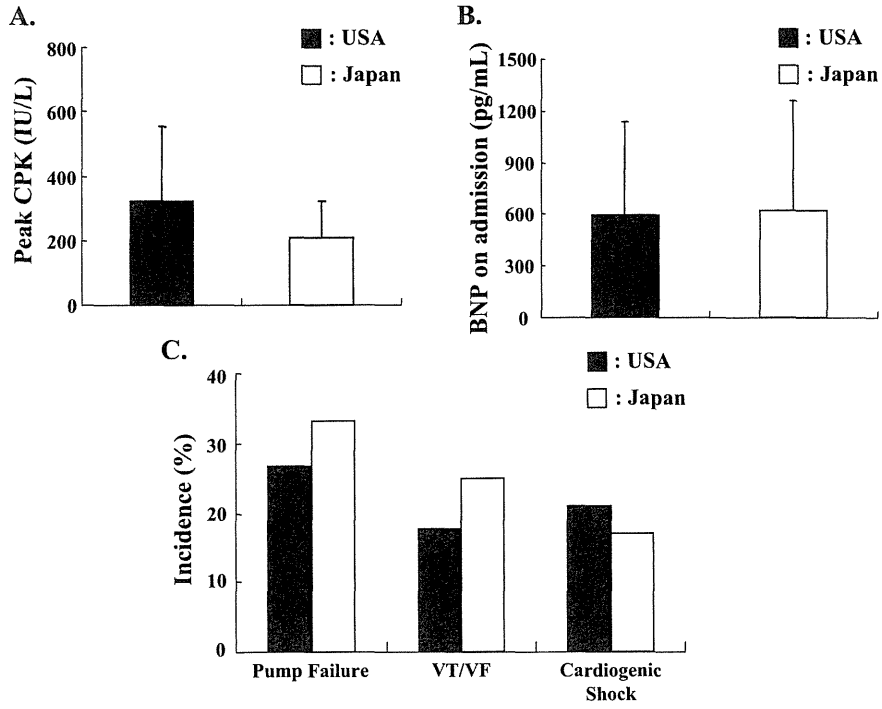

Figure 1. (A) Peak CK levels in patients with TC in USA or Japan. (B) Plasma BNP concentrations in patients with TC in USA or Japan on admission. (C) In-Hospital Complications. The incidence of pump failure or ventricular tachycardia/fibrillation or cardiogenic shock in patients with TC in USA or Japan when the patients were in the hospital.

Table 3. Angiographic Findings

	USA (n=34)	Japan (n=12)	p value
LVG			
LVEF (%)	28.1±10.6	34.3±7.8	0.05
LVEDP (mmHg)	23.3±6.2	19.8±4.1	0.10
CAG			
Normal coronary artery	79%	92%	0.35

LVEF = left ventricular ejection fraction; LVEDP = left ventricular end-diastolic pressure.

quently received warfarin compared to patients in USA. However, there were no differences in the number of patients experiencing thromboembolic events including cerebral infarction in both USA and Japan during the follow-up period. In patients with TC, left ventriculograms and echocardiographic findings showed akinesis of apical portion of the left ventricle. Therefore, physicians concern about the risk of thrombus formation in the left ventricle and the occurrence of which may result in cerebral infarction. We did not observe cardioembolic complications in TC patients from Japan and USA , despite a report that demonstrated high incidences of cardioembolic complications in TC patients (15). In addition, there was a significant difference in the use of warfarin in TC patients between Japan and USA in our study. Further investigation is necessary to evaluate the morbidity of patients with TC and cardioembolic complications, and to determine whether warfarin should be routinely administered to patients with TC.

TC is known to be a "stress-induced disease". In a previous report, 30-40% of patients with TC have experienced emotional stress (16). In the present study, more than 50% of patients experienced a preceding psychological or physical condition perceived as "stress". This is consistent with the results of the other study. Cathecholamines may be closely associated with the induction of TC (8). Plasma catecholamine concentrations at the onset were higher in patients with TC when compared to patients with myocardial infarction (17). During stress, the sympathetic nervous system is activated which may influence myocardial contraction. The distribution of sympathetic nerves in the myocardium may also relate to wall motion abnormality. In addition to an excess of catecholamines, vasospasm and myocarditis may also be related to the onset of TC, although the mechanism of TC is largely unknown.

Sympathetic activation is closely related to coronary microvascular dysfunction (18). According to previous reports, left ventricular wall motion abnormalities in TC patients might be associated with coronary microvascular impair-

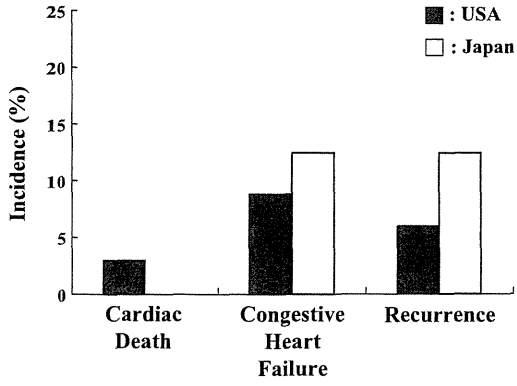

Figure 2. Long-term prognosis. Comparison of the incidences of cardiac death, congestive heart failure and recurrence in patients with TC between USA and Japan.

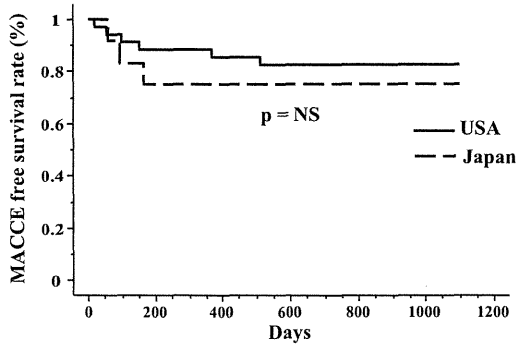

Figure 3. MACCE free survival rates. Comparison of the Kaplan-Meyer curves of the incidence of MACCE (Major Adverse Cardiac and Cerebrovascular Events) in patients with TC between USA and Japan.

ment (19-21). Coronary flow velocity reserve, reflecting the degree of coronary microvascular function, is decreased in patients with takotsubo-like transient left ventricular dysfunction (20). The differences of prevalence of history of coronary revascularization and the number of patients with a family history of premature CAD between Japan and USA might be associated with the difference of coronary microvascular dysfunction.

Patients with TC have relatively favorable long-term outcomes. The recurrence of TC has been observed, but the morbidity rate is not so high (22). In accord with previous reports, we recorded approximately 10% of patients with TC recurrences during the follow-up period in both databases. In addition, the occurrences of heart failure and cardiac death were rare and a comparison showed no differences in the percentage of incidences between Japan and USA. Further detailed studies are necessary to determine whether the differences of the short-and long-term prognosis of TC patients between Japan and USA would be identified in clinical studies which have a large number of patients.

Study Limitations

First, the sample size was small, therefore, the statistical power might not be strong enough for any negative data to be conclusive. Second, our institutes are most likely not representative of Japan and USA, respectively.

Conclusion

This is the first direct comparative study of TC between Japan and USA based on database assessment. Patients with TC in Japan have fewer prior overt CAD and there is a lower prevalence of a family history of premature CAD, but there were no differences in long-term prognosis and the recurrence rate between patients in Japan and USA.

The authors state that they have no Conflict of Interest (COI).

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Original article

Wnt2 accelerates cardiac myocyte differentiation from ES-cell derived mesodermal cells via non-canonical pathway

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ABSTRACT

The efficient induction of cardiomyocyte differentiation from embryonic stem (ES) cells is crucial for cardiac regenerative medicine. Although Wnts play important roles in cardiac development, complex questions remain as to when, how and what types of Wnts are involved in cardiogenesis. We found that Wnt2 was strongly up-regulated during cardiomyocyte differentiation from ES cells. Therefore, we investigated when and how Wnt2 acts in cardiogenesis during ES cell differentiation. Wnt2 was strongly expressed in the early developing murine heart. We applied this embryonic Wnt2 expression pattern to ES cell differentiation, to elucidate Wnt2 function in cardiomyocyte differentiation. Wnt2 knockdown revealed that intrinsic Wnt2 was essential for efficient cardiomyocyte differentiation from ES cells. Moreover, exogenous Wnt2 increased cardiomyocyte differentiation from ES cells. Interestingly, the effects on cardiogenesis of intrinsic Wnt2 knockdown and exogenous Wnt2 addition were temporally restricted. During cardiomyocyte differentiation from ES cells, Wnt2 didn't activate canonical Wnt pathway but utilizes JNK/AP-1 pathway which is required for cardiomyocyte differentiation from ES cells. Therefore we conclude that Wnt2 plays strong positive stage-specific role in cardiogenesis through non-canonical Wnt pathway in murine ES cells.

1. Introduction

Cardiomyocytes derived from ES cells are a promising source of cells for cardiac regenerative medicine and models of early development of the heart [1]. Recent studies showed that induced pluripotent stem (iPS) cells are good candidates for regenerative medicine, as iPS cells can be generated from patients' somatic cells and have almost the same characteristics as ES cells [2,3]. Thus, understanding ES cell differentiation not only elucidates in vivo cardiac development, but facilitates the application of ES cells and iPS cells to cardiac myocyte differentiation for cell transplantation therapy [4,5]. Although methods for the generation of cardiomyocytes from ES cells have been tested, their efficiencies have proven insufficient for clinical application. Several key developmental factors have strong effects on in vitro cardiomyocyte differentiation from ES cells, and various signaling pathways have been investigated. The Wnt proteins constitute a family of highly conserved secreted glycoproteins, and the signals are transmitted via several pathways that are dependent upon a variety of intracellular molecules, such as the canonical Wnt/β-catenin pathway and the non-canonical Wnt/Ca^{2+} and JNK pathways [6,7]. Regarding the role of Wnt signaling in cardiogenesis, strong effects of Wnt signaling on cardiac development have been reported, with complexities related to differences in experimental models and developmental time periods.

In the canonical pathway, activation of Wnt signaling decreases the intracellular degradation of β-catenin, which allows translocation to the nucleus and transcription factor activation in conjunction with the co-transcriptional factors Lefs and Tcfs. In the beginning of Wnt study in the heart, canonical Wnt was implicated in the inhibition of cardiomyocyte induction, and Wnt antagonists were shown to enhance heart muscle formation [8–12]. In murine ES cells, hyperactivation of Wnt/β-catenin signaling reduces cardiac differentiation [13]. Furthermore, deletion of *β-catenin* in the murine embryonic endoderm results in the formation of multiple ectopic hearts [14]. These data reveal that Wnt/β-catenin signaling inhibits cardiogenesis. Conversely, Wnt/β-catenin signaling induced cardiomyocyte differentiation in the murine embryonal carcinoma (EC) cell line P19CL6 and in ES cells in a developing stage-dependent manner [15–18]. On the

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other hand, Wnt members that act through non-canonical pathways also promote cardiomyocyte differentiation. Wnt11 is expressed in the precardiac regions, and exogenous Wnt11 promotes cardiac differentiation in noncardiogenic tissue, EC cells and ES cells through protein kinase C- and JNK-dependent pathways [19–22].

Although the Wnt pathway appears to play a crucial role in cardiogenesis, it remains unclear as to when, how and what types of Wnts are involved in cardiogenesis from ES cells. Since we found that Wnt2 was strongly up-regulated during cardiogenesis from ES cells, we analyzed how and when Wnt2 acts in cardiac development from ES cells. Initially, we confirmed that expression patterns of key genes in ES cells, which included mesodermal markers, cardiac specific markers and *Wnt2*, were similar to those of in vivo. We applied these findings to an ES cell differentiation culturing system. We performed *Wnt2* knockdown experiments and added Wnt2 protein to differentiating ES cells. Although our results reveal that Wnt2 plays a critical positive role in cardiogenesis from murine ES cells through non-canonical pathways, its effect is temporally limited.

2. Materials and methods

2.1. Mice

Pregnant ICR wild-type mice were purchased from Japan CLEA. The β-catenin reporter transgenic mice (ins-TOPEGFP mice) were generated [23]. All experiments were approved by the Keio University Ethics Committee for Animal Experiments.

2.2. Whole-mount RNA in situ hybridization and histologic analysis

ICR wild-type mice at embryonic Day (E) 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0 were used, and whole-mount in situ hybridization was performed using digoxigenin-labeled RNA probes, as described previously [24]. The cDNAs for mouse *wnt2* (accession number NM_023653) were kindly provided by Andrew P. McMahon (Harvard University, Boston). The full-length cDNAs for mouse *nkx2.5* (accession number NM_008700) and *wnt3a* (accession number NM_009522) were obtained by RT-PCR and subcloned into the pBluescript plasmid. The probes were transcribed with T3 or T7 RNA polymerase.

2.3. ES cell culture

Murine embryonic fibroblast-free ES cells were used. Undifferentiated ES cells (EB3 [25], R1 [26]) were maintained on gelatin-coated dishes in GMEM that was supplemented with 10% FBS (Equitechbio), 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, and 2000 U/ml murine LIF (Chemicon). EB3 cells, a subline derived from E14tg2a ES cells [27], (a kind gift from Hitoshi Niwa, Riken, Kobe, Japan), which carry the blasticidin S-resistant selection marker gene driven by the Oct3/4 promoter (active in the undifferentiated status), were maintained in medium that contained 20 mg/ml blasticidin S, to eliminate differentiated cells.

2.4. Differentiation of ES cells

Maintained undifferentiated ES cells were cultured for 3 days on gelatin-coated dishes in GMEM that was supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, and 2000 U/ml LIF. The cells were then trypsinized and cultured to form spheroids (embryoid bodies; EBs) from a single cell using a three-dimensional culture system in αMEM that was supplemented with 10% FBS, 2 mM L-glutamine without LIF in uncoated Petri dishes. When ES cells were differentiated into cardiomyocytes as positive controls, 0.15 g/ml Noggin (Noggin-Fc; R&D Systems) was added to the ES culture medium, αMEM, as described above, for 3 days in gelatin-coated dishes. The same concentration of Noggin (0.15 g/ml) was added to the three-dimensional culture system medium for ES cell differentiation. JNK inhibitor (420119) was purchased from CALBIOCHEM.

2.5. Preparation of Wnt2-conditioned medium

Wnt2-conditioned medium was freshly prepared from COS7 cell cultures with each cycle. The Wnt2 expression plasmid was kindly provided by Jan Kitajewski (Columbia University Medical Center, New York). Transfections were performed using Lipofectamine according to the manufacturer's recommendation. After transfection of the Wnt2 expression plasmid, the COS7 cells were incubated for 24 h. Protein contents were confirmed by Western blot analysis immediately.

2.6. Histologic and immunohistochemical analyses

EBs (Day 10) and mouse embryos were fixed in 4% paraformaldehyde for 45 min and embedded using Tissue-Teik OCT (Sakura Finetek). The samples were exposed to primary antibodies, which included anti-α-Actinin (EA-53, 1:800; Sigma), anti-GFP, anti-Troponin I (C-19, 1:500; Santa Cruz Biotechnology), and anti-atrial natriuretic peptide (ANP) (1:100; Chemicon). Bound antibodies were visualized using a secondary antibody conjugated with Alexa 488 or Alexa 546 (Cosmo Bio). Nuclei were stained with 4′,6-diamidine-2-phenylidole dihydrochloride (DAPI; Sigma-Aldrich) or propidium iodide (PI, Sigma), and TOTO3 (Molecular Probes). The percentage of α-Actinin-expressing cells was calculated for the Day 10 EBs.

2.7. Quantitative RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen), and RT-PCR was performed as described previously [24]. At least five replicates were carried out for each time-point. The *gapdh* gene was used as an internal control. For quantitative analysis of *nkx2.5*, *tbx5*, *brachyury T*, *mesp1*, *gata4*, *anp*, *mlc2a*, *myh6*, *mef2c* *wnt2*, *wnt3*, *wnt3a*, *wnt5a*, *wnt10b* and *wnt11* expression, the cDNA was used as template in a TaqMan real-time PCR assay using the ABI Prism 7700 sequence detection system (Applied Biosystems) with TaqMan probes, or with SYBR Premix ExTaq (Takara, Otsu, Japan), according to the manufacturer's instructions. All samples were run in triplicate. Data were normalized to *gapdh*. The primers and TaqMan probes (Applied Biosystems) for *tbx5* (Mm00803521_m1), *brachyury T* (Mm00436877_m1), *mesp1* (Mm00436877_m1), *gata4* (Mm00484689_m1), *anp* (Mm01255747_g1), *mlc2a* (Mm00491655_m1), *Myh6* (Mm00440354_m1), *mef2c* (Mm01340842_m1), *wnt2* (Mm00470018_m1), *wnt3* (Mm00437336_m1), *wnt3a* (Mm00437337_m1), *wnt5a* (Mm00437347_m1), *wnt10b* (Mm00442104_m1) and *wnt11* (Mm00437328_m1) were used. The PCR primers are listed in the Supplementary Table.

2.8. Western blotting

EBs were lysed in buffer that contained 20 mM Tris–HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA, 1.0% Triton X-100, 10% glycerol, 0.1% SDS, 1.0% deoxycholic acid, 50 mM NaF, 10 mmol/l $Na_3P_2O_7$, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Proteins were separated on 5–10% SDS-PAGE. Western blot analysis was performed as described previously [28]. Mouse monoclonal anti-α-Actinin (EA-53, Sigma), goat polyclonal anti-Troponin I (C-19, Santa Cruz Biotechnology), rabbit polyclonal anti-mef2c (C-21, Santa Cruz Biotechnology), goat polyclonal anti-gapdh (V-18; Santa Cruz Biotechnology) anti-JNK (9252, Cell Signaling Technology) and anti-phospho-JNK (9251, Cell Signaling Technology) were used as the primary antibodies, and peroxidase-conjugated horse anti-mouse IgG (Cell Signaling Technology), rabbit

anti-goat IgG (Rockland), and goat anti-rabbit IgG (Chemicon) were used as the secondary antibodies. The signals were visualized with SuperSignal West Pico (Pierce).

2.9. siRNA knockdown of Wnt2 and Wnt3a mRNA transcription

To achieve knockdown of *Wnt2* expression, ES cells were transfected with 1 nM to 100 nM of Wnt2 siRNA and Wnt3a siRNA (Silencer® Pre-designed siRNA; Ambion) or non-targeting siRNA as a negative control (Ambion) using Lipofectamine RNAiMAX (Invitrogen). The 2-ml transfection mixture, which contained 50 to 1000 pmol siRNA mix, 20 μl of transfection reagent, and α-MEM, was added directly to the culture medium (total volume, 12 ml). Two cognate sequences for *Wnt2* were utilized to eliminate potential design-specific RNAi inefficiency or non-specificity. The following sequences were used: "siWnt2-A", 5′-GCAAGGGCUUUAACUAAGU-3′; "siWnt2-B", 5′-GCCAACGAAAAAUGACCUC-3′; "siWnt3a", 5′-GGGUCUCAUACCUAAGGAC-3′.

2.10. Flow cytometry

EBs were dissociated to single cells by treating them with collagenase type II (Sigma) for 10–12 min at 37 °C, which was followed by treatment with Cell Dissociation Buffer (enzyme-free, Hanks-based, Invitrogen) for 5–8 min. Cells were stained with anti-Cardiac Troponin T (1C11, 1:400; Abcam), or anti-atrial natriuretic peptide (ANP) (FL-153, 1:400; Santa Cruz biotechnology) antibodies. Primary antibodies for intracellular antigens were detected with AlexaFluor 488- or AlexaFluor 633-conjugated anti-mouse or anti-rabbit IgG antibodies (Invitrogen). Analyses were carried out using a fluorescence-activated cell sorting, FACS Aria II flow cytometer (Beckton Dickinson).

2.11. Luciferase assay

HEK293 cells or EBs were plated in DMEM with 10% FBS, and transfected with Lipofectamine (Invitrogen) according to the manufacturer's instructions. Unless otherwise indicated, 100 ng of reporter and 100 ng of each Wnt expression plasmid were co-transfected. The total amount of DNA per well was kept constant by adding the corresponding amount of expression vector without a cDNA insert. CMV-*Renilla* luciferase was used as an internal control, to normalize for variations in transfection efficiency. All the proteins were expressed at very similar levels, as confirmed by Western blotting. The TCF reporter plasmid kit including TOPFLASH and FOPFLASH plasmids was purchased from Upstate Biotechnology. NFAT reporter plasmid and AP-1 reporter and AP-1 control plasmid were kindly provided from Dr. Gerald R. Crabtree and Dr. Arnd Kieser respectively. NFAT control plasmid is generated by deletion of NFAT binding site.

2.12. Statistical analysis

The data were processed using the StatView J-4.5 software. Values are reported as means ± SD. Comparisons among values for all groups were performed by one-way ANOVA. Scheffe's F test was used to determine the level of significance. The probability level accepted for significance was $P<0.05$.

3. Results

3.1. The expression levels of several Wnts are increased when ES cells are induced to differentiate into cardiomyocytes

To determine the specific functions of Wnt signaling in cardiomyocyte differentiation in vitro, we investigated the expression levels of several Wnts in differentiating ES cells, and compared the levels in control embryoid bodies (EBs) and cardiomyocyte-rich EBs. To induce cardiomyocytes from ES cells, we added Noggin, which is a bone morphogenetic protein (BMP) antagonist, to the ES cell culture medium (Fig. 1A) [29]. Noggin increased expression of certain genes in cardiomyocytes in the treated EBs, and cardiac-specific marker expression was confirmed by immunostaining and quantitative RT-PCR (Fig. 1B). Quantitative RT-PCR analysis revealed that, in the control EBs, *Wnt2* appeared on Day 10 of EB formation. The cardiomyocyte-rich EBs showed markedly higher *Wnt2* expression on Day 5 than the control EBs (Fig. 1C). Of the various Wnts, the levels of *Wnt3a*, *Wnt5a*, *Wnt10a* and *Wnt11* also increased when ES cells were induced to differentiate into cardiomyocytes. From these observations, we speculated that these Wnts might have positive effects on cardiomyocyte differentiation.

In vitro ES cell differentiation systems without using Noggin, quantitative RT-PCR analysis confirmed that, Brachyury T expression peaked on Days 3–4, Mesp1 expression peaked on Days 4–5, and Nkx2.5 expression gradually increased from Day 4 to Day 5. Wnt2 expression in the EBs began on Day 4 (Fig. 1D).

3.2. Wnt2 is strongly expressed in the murine embryonic early developing heart

To clarify whether these candidate Wnts are expressed in vivo, we investigated the expression patterns of these Wnts in the developing mouse heart. Whole-mount in situ hybridization revealed initial expression of *Wnt2* in the cardiac crescent of the E7.5 mouse embryo (Supplementary Fig. 1A). *Wnt2* was also clearly expressed at the late crescent stage of the E8.0 mouse, in the linear heart tube of the E8.5 mouse and in both the atria and ventricles of E9.5 and E10.0 mice. *Wnt2* was strongly expressed in the murine embryonic early developing heart, and this expression pattern was quite similar to that of *Nkx2.5* in early murine embryos at E7.5 to E8.0 (Supplementary Fig. 1A). Sectional analysis of the E8.5 mouse embryo revealed that Wnt2 was expressed in the sinus venosus and primitive heart, and was strongly expressed in the primitive atria compared with the primitive ventricles (Supplementary Fig. 1B). These data are consistent with previous reports showing that Wnt2 is expressed in early cardiac mesoderm in the cardiac crescent at E7.5 [30] and developing inflow tract mesoderm atria at E9.5 and E10.5 [31,32]. The heart specific expression of Wnt2 in mouse embryo strongly suggested this protein might be critically involved in heart development. Therefore, we focused on the role of Wnt2 on cardiomyocyte differentiation.

3.3. Knockdown of intrinsic Wnt2 significantly reduces cardiomyocyte differentiation from murine ES cells in a stage specific manner

From the findings of Wnt2 expression in the primitive heart and ES cell-derived developing cardiac progenitors, we speculated its critical positive role in cardiomyocyte differentiation. We next investigated whether the knocking down of intrinsic Wnt2 could reduce cardiomyocytes differentiation from ES cells by Wnt2 siRNA at various developmental phases from Day −1 to Day 7. At first, we examined specificity and continuousness of Wnt2 siRNA effect and found that Wnt2 siRNA specifically inhibit Wnt2 expression and sustained its effect for approximately 5 days (Supplementary Figs. 2A, B, C, D). Day 0 was just after the discontinuation of LIF and plating into Petri dishes, and the number of spontaneously beating EBs was counted on Day 10. The beating incidence was markedly reduced by Wnt2 siRNA treatment, and the most efficient timing to reduce cardiomyocyte differentiation was Day 3 (Fig. 2A). Quantitative RT-PCR analysis revealed that the levels of the cardiac-specific transcription factors Nkx2.5 and Tbx5 were reduced when ES cells were treated with Wnt2 siRNA on Days 1 to 4 (Figs. 2B and C), which was concordant with the beating incidence data.

Furthermore, we demonstrated a dose-dependent relationship between the siRNA concentrations and the beating incidence reduction caused by knocking down Wnt2 on Day 3 (Fig. 2D). In addition,

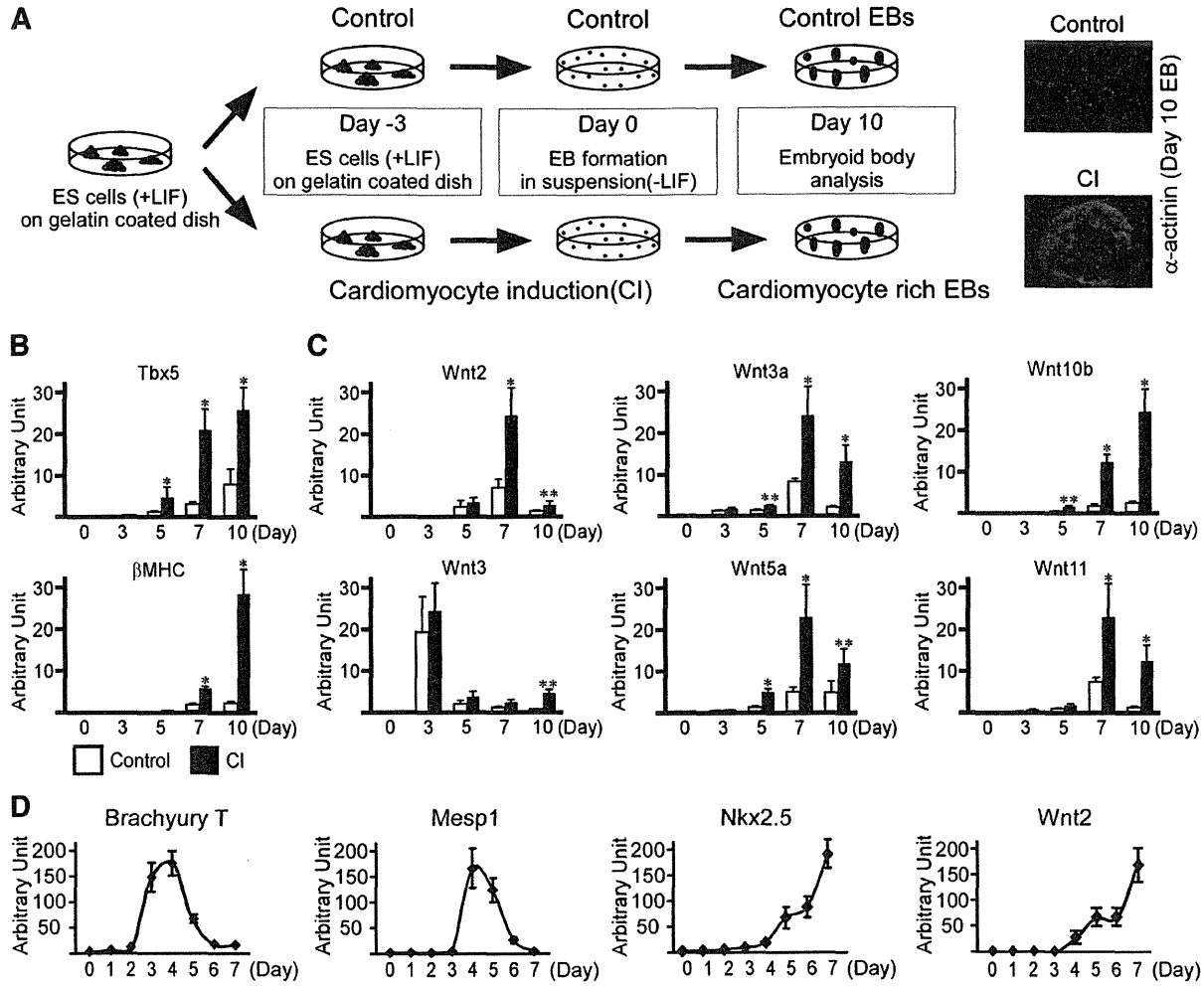

Fig. 1. *Wnt2* expression is increased when cardiomyocytes are induced from ES cells. (A) A representative schema of the protocols for ES cell culturing, EB formation, and cardiomyocyte induction (CI) from ES using Noggin. Right panels indicate that sections of differentiated Day 10 EBs are immunostained with the anti-α-Actinin antibody. The Noggin-treated EBs stain positive for α-Actinin. (B) Quantitative RT-PCR analysis of *Tbx5* and *βMHC* gene expressions. Noggin treatment strongly induces the expression of these heart-specific genes. (C) Quantitative RT-PCR analysis of the expression levels of Wnt genes during the differentiation of ES cells into differentiated control EBs and Noggin-treated EBs. The levels of *Wnt2*, *Wnt3a*, *Wnt5a* *Wnt10b* and *Wnt11* expression are markedly increased in the Noggin-treated cardiomyocytes. (D) Quantitative RT-PCR analysis shows the expression levels of early mesodermal marker (Brachyury-T), early cardiac marker (Mesp1 and Nkx2.5) and Wnt2 transcripts during the differentiation of ES cells into cardiac myocyte. *P<0.01, **P<0.05 versus control.

the levels of the cardiac-specific genes Nkx2.5 and Tbx5 were reduced in a dose-dependent manner by knocking down Wnt2 on Day 3 (Figs. 2E, F). Quantitative RT-PCR revealed that knockdown of Wnt2 on Day 3 EBs markedly reduced the expression of the cardiac-specific marker genes, Gata4, MEF2C, α-myosin heavy chain (αMHC), β-myosin heavy chain (βMHC), and myosin light chain 2v (MLC2v), and atrial natriuretic peptide (ANP) in the 10 day EBs compared to those of the control (Fig. 2G). To confirm that Wnt2 siRNA treatment decreased cardiomyocyte content, we performed Western blotting analysis for the cardiac marker proteins α-Actinin and Troponin I. The levels of these cardiac markers were decreased in the Wnt2 siRNA-treated EBs compared to the control siRNA-treated EBs (Fig. 2H). These results indicated that intrinsic expression of Wnt2 was critically involved in differentiation of cardiomyocytes, and that this effect was specific to the early cardiac developmental phase.

3.4. Administration of Wnt2 increases cardiomyocyte differentiation in murine ES cells in a stage specific manner

We then investigated whether administration of extrinsic Wnt2 could enhance cardiomyocyte differentiation from ES cells. First, we prepared Wnt2-contained conditioned medium from the Wnt2-transfected COS7 cells. We added it to the ES cell culture medium on Days 0 to 7 using the protocol illustrated in Fig. 3A. We did not use Noggin in this protocol, and either Wnt2-contained or control conditioned medium was administered at various time points. Adding conditioned medium was equalized with control conditioned medium. ES cells were cultured in the same medium. Quantitative RT-PCR, performed on Day 10 EBs, demonstrated that Wnt2 administration significantly increased the expression of Nkx2.5 and Tbx5 (Fig. 3B). The optimal timing was determined as Day 3, which was concordant with the results of the knockdown experiment. Dose–response experiment revealed that maximal effect was obtained by addition of 20 μl of Wnt2-conditioned medium (Fig. 3C). These results indicated that the effect of Wnt2 on cardiomyocyte differentiation required a narrow time window and an optimum concentration.

Western blotting of the EBs on Day 10, which were treated with Wnt2 on Day 3, revealed that the cardiac marker genes were expressed at higher levels in EBs treated with Wnt2-conditioned medium than in those with control-conditioned medium (Fig. 3D). We then performed immunostaining for cardiomyocyte markers, Troponin I, α-Actinin, and ANP. The Wnt2 apparently increased positive

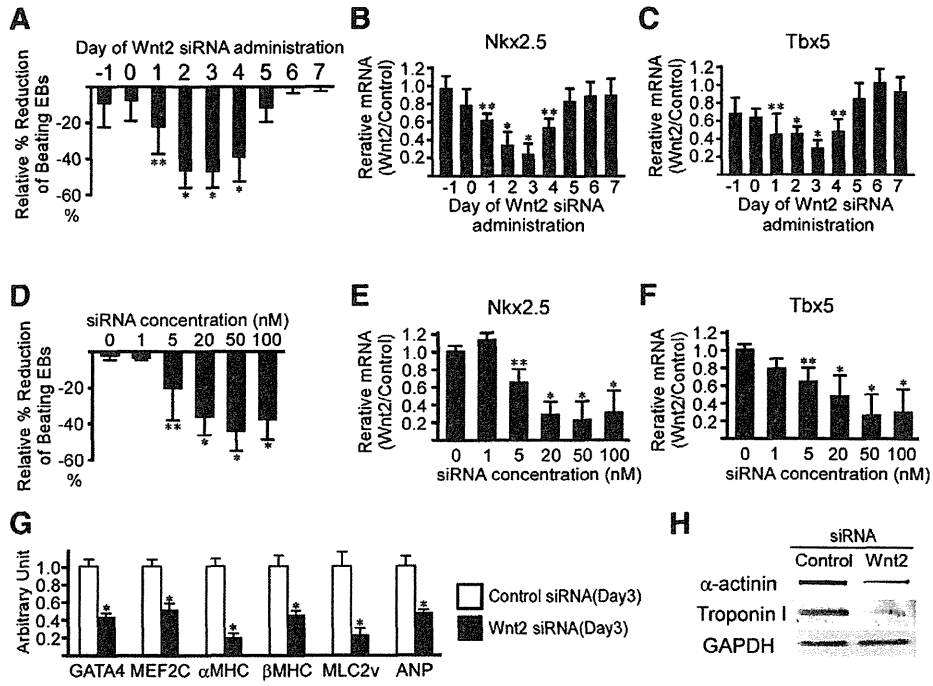

Fig. 2. Knockdown of intrinsic *Wnt2* using siRNA reduces cardiomyocyte generation from ES cells. (A–C) Knockdown of *Wnt2* at various developmental phases of ES cell differentiation. (A) Knocking down *Wnt2* inhibits the formation of beating EBs during Days 1 to 4. (B, C) Quantitative RT-PCR analysis of the Day 10 EBs treated with Wnt2 siRNA from Day −1 to Day 7, as compared to the negative control siRNA-treated EBs. Knocking down *Wnt2* on Day 1 to Day 4 reduces the levels of the cardiac-specific transcription factors *Nkx2.5* and *Tbx5*. (D) The number of beating EBs on Day 10 is reduced in a dose-dependent manner by knocking down *Wnt2* on Day 2. (E, F) Quantitative RT-PCR analysis of the Day 10 EBs treated with Wnt2 siRNA on Day 3, as compared to the negative control siRNA-treated EBs. Knocking down *Wnt2* on Day 3 reduces in a dose-dependent manner the levels of the cardiac-specific transcription factors *Nkx2.5* and *Tbx5*. (G, H) Knocking down *Wnt2* on Day 3 reduces the expression levels of the cardiac-specific genes and proteins in the Day 10 EBs, as determined by RT-PCR (G) and Western blotting (H). *P<0.01, **P<0.05 versus control.

areas for these markers compared with the control EBs (Fig. 3E). We quantitated the number of cardiomyocytes in differentiated EBs at Day 10 by immunostaining and FACS analyses. These experiments showed that Wnt2 significantly increased their number (Fig. 3F and Supplementary Fig. 3). These findings indicated that Wnt2 promoted cardiomyocyte differentiation from ES cells, and this effect was in a time-specific and concentration-specific manner.

3.5. Wnt2 administration on Day 3 affects ES cell differentiation into cardiac myocytes after mesodermal induction

To elucidate the step at which intrinsic Wnt2 signaling is essential for cardiogenesis, we investigated the mesodermal and cardiac marker gene expression patterns by quantitative RT-PCR analysis. At the optimal timing for Wnt2 administration (Day 3), Wnt2 did not affect significantly the *Brachyury T* and *Mesp1* expression patterns (Fig. 4A). In contrast, the cardiac markers, *Gata4*, *Nkx2.5* and *Tbx5* expression levels were increased by Wnt2 administration (Fig. 4B). The levels of *MLC2a*, *ANP* and *Myh6* expression were also increased (Fig. 4C). Moreover, when we knocked down intrinsic *Wnt2* in ES cells on Day 3 under the condition in which Noggin was not used for cardiac induction, the *Brachyury T* and *Mesp1* levels were not affected but *Tbx5* expression was decreased significantly (Fig. 4D). Brachyury T is needed for mesodermal formation, and Mesp1 is expressed at the onset of gastrulation and is essential for development of endocardium and myocardium [33,34]. Therefore, these data show that Wnt2 does not affect the expression of Brachyury T and Mesp1, suggesting that Wnt2 does not affect the primitive development of the mesodermal progenitor cells. However, Wnt2 critically promotes cardiac specification after mesodermal induction and increases the eventual cardiac musculature. This is supported by the finding that both *Brachyury T* and *Mesp1* started to be expressed before the expression of *Wnt2*. The genes that were affected by Wnt2 administration or *Wnt2* knockdown, i.e., *Gata4*, *Nkx2.5*, *Tbx5*, *ANP*, *MLC2a*, and *Myh6*, were not expressed before Day 4, at which time-point intrinsic *Wnt2* expression had begun. These findings imply that neither additional Wnt2 nor intrinsic *Wnt2* knockdown affects *Brachyury T* and *Mesp1* but could affect cardiac genes. In order to examine cooperative effect of Wnt2 and canonical Wnt, Wnt3a, we examined mesodermal and cardiac induction with Wn2 siRNA and Wnt3a siRNA. Single Wnt2 siRNA addition didn't show any effect on Brachyury T expression but Wnt3a siRNA on Day 0 decreased Brachyury T expression in the presence of Wnt2 siRNA. And Wnt2 siRNA and Wnt3a siRNA addition cooperatively and independently decreased cardiac differentiation (Fig. 4E). Taken together, our results show that Wnt2 is not crucial for primitive development of the mesodermal progenitor cells, but that Wnt2 acts positively in cardiac development just after definition of the cardiac mesoderm.

3.6. The canonical Wnt pathway is not activated during early embryonic cardiac development

We found that *Wnt2* was clearly expressed in the early developing heart. To investigate whether the canonical Wnt pathway is activated in the region of *Wnt2* expression during embryonic heart development, we precisely analyzed reporter-transgenic ins-TOPEGFP mice, which harbor a β-catenin-dependent reporter gene that drives the expression of EGFP. In this construct, the expression of EGFP is controlled by six-multimerized LEF/TCF consensus-binding sequences and a minimal thymidine kinase promoter, which is stimulated by β-catenin nuclear translocation. In addition, this transgenic construct is flanked by a core element of the chicken β-globin HS4 insulator, to minimize positional effects imposed by chromosome structure [23,35]. The insulator is a DNA element that protects genes from inappropriate signals in the surrounding environment [23,36]. By investigating

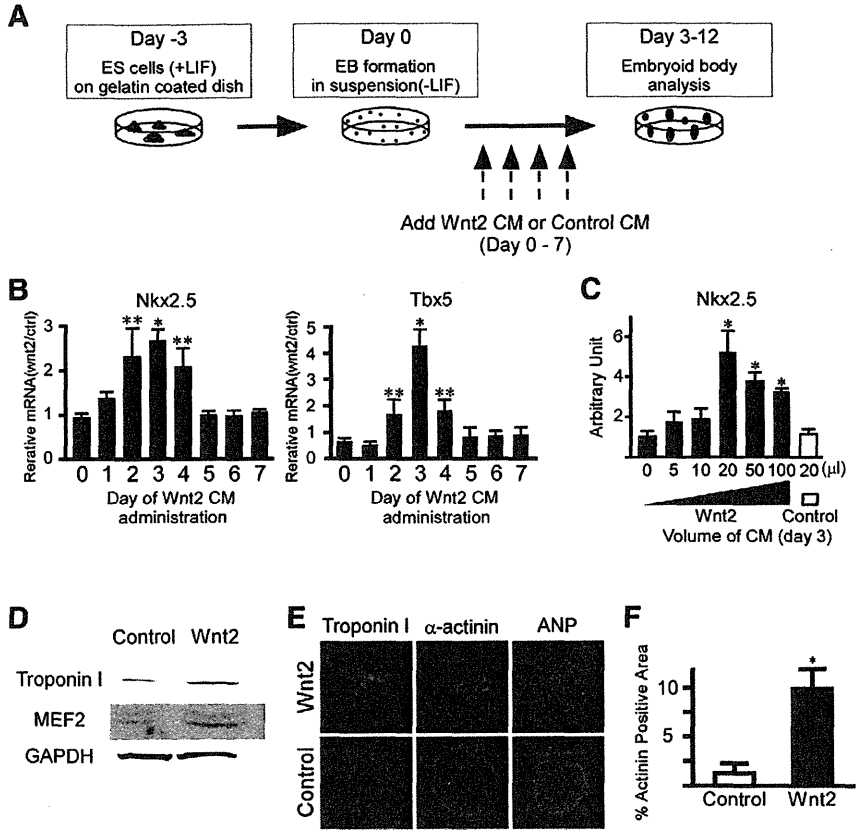

Fig. 3. The addition of Wnt2 protein increases cardiomyocyte in ES cells. (A) A representative schema of the protocol used for ES cell culture and EB formation. Wnt2 protein-conditioned medium and control-conditioned medium were added at various phases. (B) Wnt2 protein was added at the indicated phases of ES cell differentiation. The cardiac transcription factors, Nkx2.5 and Tbx5 are markedly increased, as determined by quantitative RT-PCR, only when Wnt2 was added on Day 2 to Day 4. (C) Dose–efficiency relationship of Wnt2 protein administration for the cardiac-specific genes in the Day 10 EBs, as analyzed by quantitative RT-PCR. (D) The addition of Wnt2 protein on Day 3 increases the expression of the cardiac-specific genes, Troponin I and MEF2 in the Day 10 EBs, as analyzed by Western blotting. (E) Sections of the differentiated Day 10 EBs were immunostained for Troponin I, α-Actinin, and ANP. (F) The addition of Wnt2 protein on Day 3 increases percentages of anti-Actinin positive area in the sections of the differentiated Day 10 EBs. *P<0.01, **P<0.05 versus control.

the ins-TOPEGFP embryos in the period from E5.5 to E9.0, we found that this transgenic mouse expressed strong EGFP signals and that the EGFP expression patterns were similar to those of the reporter signals previously shown in other Wnt reporter transgenic mice [23,37–39].

In the E7.5 embryo, EGFP expression was not found in the cardiac crescent area, while EGFP signals were clearly detected in the primitive streak (Figs. 5Aa' and b'). In the cross-section of the E7.5 embryo, no EGFP signal was detected in the cardiac crescent, whereas strong signals were detected in the primitive streak (Fig. 5Bi–k). Whole-mount in situ hybridization revealed that *Wnt3a* was expressed in the posterior side of the E7.5 embryo (data not shown), and that this transgene was activated as expected, and suggested that β-catenin signal activation in the posterior region during this phase may have originated from Wnt3a. This result corroborates the notion that Wnt3a acts as a positive regulator of mesodermal induction in primitive streak through Wnt/β-catenin signaling [40–42]. As gastrulation proceeded, Wnt/β-catenin signaling persisted at the posterior side of the embryo, in the primitive streak and node, but not in the cardiac developing area at E8.0 (Figs. 5Ac' and d'). Furthermore, as somite differentiation proceeded, EGFP expression gradually increased in the dorsal domain of the dermomyotome and then faded in the more-anterior somites. The transgenic mouse embryo at E8.5 displayed positive signals in both the midbrain and paraxial mesoderm (Fig. 5Ag'). But EGFP signal was not clearly detected in the hearts at E8.5 and E9.0 (Figs. 5Ag', h' and Bl–s).

3.7. Wnt2 activates JNK/AP-1 pathway during cardiac differentiation from ES cells

To explore the further mechanisms by which Wnt2 promotes cardiomyocyte differentiation from ES cells through canonical Wnt pathway or non-canonical Wnt pathway, we used luciferase analysis in which luciferase gene is driven by β-catenin dependent transcription reporter, namely the TOPFLASH reporter. Wnt signaling is transduced in HEK293 cells [43]. HEK293 cells were transfected with TOPFLASH reporter and Wnt2 or Wnt3 expression plasmids. Wnt3 significantly activates TOPFLASH, while no significant activity increase was observed when a FOPFLASH reporter, which lacks appropriate TCF binding sites (Fig. 6A, Supplementary Fig. 4A). We also examined the non canonical Wnt pathways, NFAT signaling pathway and AP-1 pathway. In HEK293 cells, Wnt2 but not Wnt3 significantly activated both NFAT and AP-1 pathway (Figs. 6B, C). In order to clarify whether these signal transduction pathways are commonly conserved in differentiating ES cells, we performed the same experiments in differentiating EBs. Wnt3 significantly activates TOPFLASH, while Wnt2 scarcely increased TOPFLASH reporter activity (Fig. 6D, Supplementary Fig. 4D). Wnt2 couldn't activate NFAT reporter activity either but significantly activated AP-1 reporter activity (Figs. 6E, F), while Wnt3 didn't activate both NFAT and AP-1 reporter activity (Figs. 6E, F). And both Wnt2 and Wnt3 didn't affect the activity of NFAT control luciferase without NFAT binding site and AP-1 control luciferase without AP-1 binding

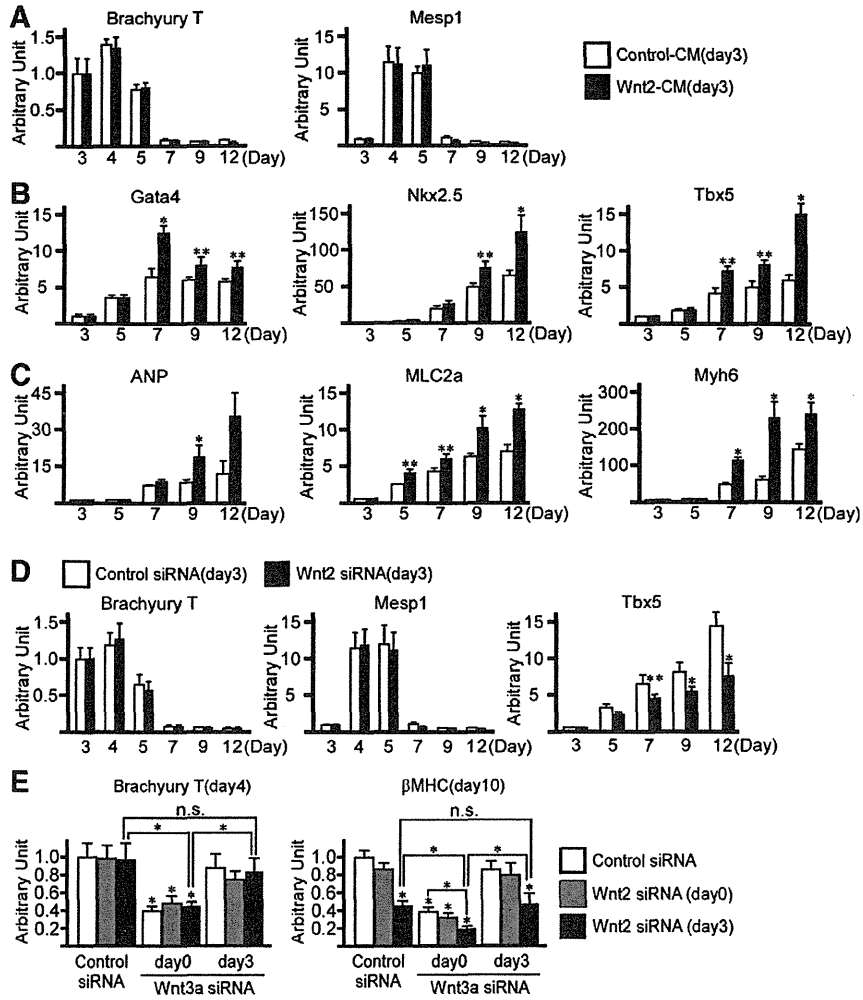

Fig. 4. Effects of Wnt2 and Wnt3a treatment on the expression levels of primitive mesodermal, cardiomyocyte, and other mesodermal markers. Quantitative RT-PCR analysis of the expression levels of several genes after Wnt2 protein addition to ES cells on Day 3. (A) The levels of primitive mesodermal markers, *Brachyury T* and *Mesp1*, are unchanged, (B) while those of the cardiac transcription factors *Gata4*, *Nkx2.5*, and *Tbx5* are increased. (C) The levels of the cardiac-specific proteins *ANP*, *MLC2a*, and *Myh6* are increased. (D) By administration of Wnt2 siRNA, the levels of primitive mesodermal markers, *Brachyury T* and *Mesp1*, are unchanged, while cardiac transcription factors *Tbx5* are decreased. (E) Quantitative RT-PCR analysis of the expression levels of *Brachyury T* (day4) and *βMHC* (Day 10) with or without Wnt2 and Wnt3a siRNA addition on Day 0 or Day 3. Wnt3a siRNA addition on Day 0 decreases the expression levels of *Brachyury T* and *βMHC*, and the effect is independent of Wnt2 siRNA. *$P<0.01$, **$P<0.05$ versus control.

site (Supplementary Figs. 4B, C, E and F). In developing EBs, Wnt2 addition significantly increased JNK phosphorylation (Fig. 6G). Wnt2 dependent AP-1 activity is eliminated by JNK inhibitor addition (Fig. 6H). Furthermore, JNK inhibitor diminished Wnt2 dependent cardiac differentiation and a dose-dependent relationship between the JNK-inhibitor concentrations and the beating incidence reduction of EBs in the presence of Wnt2 were observed (Fig. 6I). Taken together, our results show that JNK/AP-1 pathway is crucial for Wnt2 dependent cardiac differentiation from ES cells.

4. Discussion

Canonical Wnt/β-catenin signals have been investigated in detail and shown to have stage-specific and biphasic effects on cardiomyogenesis [44]. Wnts comprise a large family, and individual Wnts have potent effects on organ development, homeostasis, and pathogenesis [45]. Many Wnts regulate temporally cardiac development in various ways. In order to clarify when, how and what types of Wnts are involved in cardiogenesis from ES cells, we examined the Wnt expression patterns in a cardiomyocyte differentiation system using murine ES cells. We found that *Wnt2* was strongly up-regulated in the cardiomyocyte differentiation group, as compared to the control. Of the Wnts tested, *Wnt2* was expressed most strongly in the cardiac crescent. Therefore, we analyzed Wnt2 using the cardiomyocyte differentiation system with ES cells. We confirmed that *Wnt2* gene expression pattern in ES cells mirror the in vivo embryonic gene expression pattern. We applied these findings to the ES cell culture differentiation system. We showed that Wnt2 is a critical positive regulator of cardiomyocyte differentiation by demonstrating that knocking down intrinsic *Wnt2* notably decreased generation of ES cell-derived cardiomyocytes and that exogenous Wnt2 administration remarkably increased them. Our results also demonstrated that neither additional Wnt2 nor intrinsic *Wnt2* knockdown affects *Brachyury T* and *Mesp1* but could affect cardiac genes. In summary, although Wnt2 is not crucial for the development of mesodermal progenitor cells, Wnt2 has strong positive effects on cardiac differentiation following the determination of the cardiac mesoderm.

Wnt2 knockdown in developing EBs significantly reduced cardiac myocyte differentiation from ES cells but did not lead to the complete disappearance of cardiomyocytes. This probably reflects redundancy, compensation or incomplete knockdown. Although the knockdown system using siRNA has the limitation that knockdown is incomplete, it is also advantageous in that the knockdown timing can be altered. A