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Augmented ST-Segment Elevation During Recovery From Exercise Predicts Cardiac Events in Patients With Brugada Syndrome

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Objectives The goal of this study was to evaluate the prevalence and the clinical significance of ST-segment elevation during recovery from exercise testing.

Background During recovery from exercise testing, ST-segment elevation is reported in some patients with Brugada syndrome (BrS).

Methods Treadmill exercise testing was conducted for 93 patients (91 men), 46 ± 14 years of age, with BrS (22 documented ventricular fibrillation, 35 syncope alone, and 36 asymptomatic); and for 102 healthy control subjects (97 men), 46 ± 17 years of age. Patients were routinely followed up. The clinical end point was defined as the occurrence of sudden cardiac death, ventricular fibrillation, or sustained ventricular tachyarrhythmia.

Results Augmentation of ST-segment elevation ≥0.05 mV in V_1 to V_3 leads compared with baseline was observed at early recovery (1 to 4 min at recovery) in 34 BrS patients (37% [group 1]), but was not observed in the remaining 59 BrS patients (63% [group 2]) or in the 102 control subjects. During 76 ± 38 months of follow-up, ventricular fibrillation occurred more frequently in group 1 (15 of 34, 44%) than in group 2 (10 of 59, 17%; p = 0.004). Multivariate Cox regression analysis showed that in addition to previous episodes of ventricular fibrillation (p = 0.005), augmentation of ST-segment elevation at early recovery was a significant and independent predictor for cardiac events (p = 0.007), especially among patients with history of syncope alone (6 of 12 [50%] in group 1 vs. 3 of 23 [13%] in group 2) and among asymptomatic patients (3 of 15 [20%] in group 1 vs. 0 of 21 [0%] in group 2).

Conclusions Augmentation of ST-segment elevation during recovery from exercise testing was specific in patients with BrS, and can be a predictor of poor prognosis, especially for patients with syncope alone and for asymptomatic patients. (J Am Coll Cardiol 2010;56:1576–84) © 2010 by the American College of Cardiology Foundation

Brugada syndrome (BrS) is recognized as a clinical syndrome that leads to sudden cardiac death (SCD) in middle-aged persons due to ventricular fibrillation (VF) (1). Brugada syndrome is defined by a distinct 12-lead electrocardiogram (ECG) pattern in precordial leads (V_1 to V_3) presenting coved-type ST-segment elevation. Both depolarization and repolarization hypotheses have been reported for the pathogenesis of phenotype in BrS (2–5). Although several indexes have been reported as predictive factors of VF occurrence (6), the recent largest series of BrS patients suggested that there were no reliable predictors of cardiac events except for prior symptoms and spontaneous type 1 ECG (7). However, risk stratification remains disputable, especially for BrS patients without documented VF episodes.

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Autonomic function has been suggested to relate to the occurrence of VF in BrS. It has also been shown that ST-segment elevation in patients with BrS was augmented

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by selective stimulation of muscarinic receptors but mitigated by beta-adrenergic stimulation (8). Heart rate during exercise testing is considered as 1 parameter to evaluate cardiac autonomic function (9). Sympathetic withdrawal and parasympathetic activation occur at early recovery after exercise (10), which are expected to augment ST-segment elevation directly by inhibition of calcium-channel current or by decreasing heart rate (5,11). Two cases of BrS were reported in which ST-segment was augmented during and after exercise (12). Amin et al. (13) recently assessed the ECG responses to exercise in BrS patients with and without *SCN5A* mutations and control subjects. They reported that exercise resulted in an increase of peak J-point amplitude in all groups, including control subjects, and more QRS widening in BrS patients with *SCN5A* mutation. The peak J-point amplitude measured by Amin et al. (13) is thought to represent the depolarization parameter as QRS duration, or at least the combined parameter of both depolarization and repolarization. Therefore, in the present study, we measured several points of ST-segment as a repolarization parameter rather than a depolarization parameter, and tried to investigate the relationship between augmented ST-segment elevation during recovery from exercise testing and prognosis of BrS patients. We also evaluated parasympathetic reactivation by using heart rate recovery (HRR), which is defined as heart rate decay in the first minute after exercise cessation, and its relation with ST-segment change.

Methods

Study population. The study population consisted of 93 consecutive Japanese patients with BrS (91 males; mean age 46 ± 14 years) admitted to the National Cerebral and Cardiovascular Center in Suita, Japan, between 1994 and 2006. Ventricular fibrillation was documented in 22 BrS patients, syncope alone in 35 patients, and the remaining 36 patients were asymptomatic. As control subjects, 102 age-, sex-, and QRS duration-matched healthy subjects were randomly selected from persons who underwent treadmill exercise testing between 2002 and 2007 (97 males; mean age 46 ± 17 years). They included 55 normal subjects with normal QRS duration (<100 ms), 21 with incomplete right bundle branch block (RBBB) (100 ms ≤QRS duration <120 ms), and 26 with complete RBBB (120 ms ≤QRS duration) but without structural heart disease or any ventricular arrhythmias.

Brugada syndrome was diagnosed when a coved ST-segment elevation (≥0.2 mV at J-point) was observed in >1 of the right precordial leads (V_1 to V_3) in the presence or absence of a sodium-channel–blocking agent, and in conjugation with 1 of the following: documented VF, polymorphic ventricular tachycardia, family history of SCD <45 years of age, family history of BrS, inducibility of VF with programmed electrical stimulation, syncope, or an nocturnal agonal respiration (6). Structural heart diseases were carefully excluded by history taking, physical examinations, chest roentgenogram, ECG, and echocardiogram.

Clinical, laboratory, electrocardiographic, and electrophysiologic study. The following clinical data were collected: family history of SCD (<45 years of age) or BrS, documented atrial fibrillation (AF), documented VF, syncope, age at the first cardiac event, and implantation of implantable cardioverter-defibrillator (ICD).

Abbreviations and Acronyms

AF = atrial fibrillation
BrS = Brugada syndrome
ECG = electrocardiogram
EPS = electrophysiologic study
HRR = heart rate recovery
ICD = implantable cardioverter-defibrillator
RBBB = right bundle branch block
RVOT = right ventricular outflow tract
SCD = sudden cardiac death
VF = ventricular fibrillation

A 12-lead ECG was recorded in all 93 BrS patients, and RR interval, PR interval (lead II), QRS duration (lead V_5), corrected QT interval (lead V_2), QRS axis, J-point amplitude (leads V_2), and amplitude of several points of ST-segment (leads V_1, V_2, V_3) were measured.

Signal-averaged ECG was recorded and analyzed in 91 patients by using a signal-averaged ECG system (1200EPX, Arrhythmia Research Technology, Milwaukee, Wisconsin). Three parameters were assessed using a computer algorithm: 1) total filtered QRS duration; 2) root mean square voltage of the terminal 40 ms of the filtered QRS complexes (V_{40}); and 3) duration of low-amplitude signals <40 μV of the filtered QRS complexes (T_{40}). Late potential was considered present when the 2 criteria (V_{40} <18 μV and T_{40} >38 ms) were fulfilled.

Electrophysiologic study (EPS) was performed in 79 BrS patients (21 documented VF patients, 30 syncope alone patients, and 28 asymptomatic patients). A maximum of 3 programmed ventricular extrastimuli were delivered from the right ventricular apex and RVOT, unless VF was induced. No patients received antiarrhythmic drugs before EPS. The atrio-His and His-ventricular intervals were measured during sinus rhythm. The EPS was conducted after all subjects gave written informed consent.

Genetic testing for the presence of an *SCN5A* mutation was also conducted.

Exercise testing. Treadmill exercise testing was conducted in all 93 patients with BrS and 102 control subjects. Neither BrS patients nor control subjects used antiarrhythmic agents. A symptom-limited or submaximal (up to 90% of the age-predicted maximum heart rate) graded treadmill exercise testing similar to modified Bruce protocol was used. All 93 BrS patients and 102 control subjects were in normal sinus rhythm, and none had atrioventricular block at the exercise testing. The standard 12-lead ECGs were recorded at rest, at the end of each exercise stage, at peak exercise, and at every minute during recovery. The amplitude of ST-segment from the isoelectric line at the right precordial leads (V_1 to V_3 leads) and QRS width at V_5 lead were manually measured. The ST-segment point was defined as the point

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where the vertical line from the end point of QRS at V_5 lead intersected the precordial leads. We also measured peak J-point amplitude in lead V_2 as a depolarization parameter, and amplitude of the point, which was 40 and 80 ms later than the peak J-points (ST40, ST80) in lead V_2 as a repolarization parameter. Measurements of ECG parameters were performed as the mean of 3 beats by single electrocardiologist who knew nothing about the patients. Significant augmentation of ST-segment elevation was defined as ST-segment amplitude increase ≥0.05 mV in at least 1 of V_1 to V_3 leads at early recovery (1 to 4 min at recovery) compared with the ST-segment amplitude at baseline (pre-exercise). We also recorded heart rate and blood pressure during exercise testing.

The HRR was defined as decay of heart rate from peak exercise to 1 min at recovery.

Follow-up. Follow-up was started after undergoing treadmill exercise testing. All patients with BrS were routinely followed up at the outpatient clinic of our hospital. The ICD implantation was performed in 63 BrS patients (20 documented VF patients, 25 syncope alone patients, and 18 asymptomatic patients). Antiarrhythmic drugs were prescribed for 7 patients; 2 patients who had episodes of VF but refused implantation of ICD (disopyramide 300 mg daily for 1 patient, and amiodarone 200 mg daily for another patient), 2 patients who had AF (quinidine 300 mg daily), and 3 patients who had previous history of both VF and AF and implanted ICD (quinidine 300 mg daily for 1 patient, amiodarone 200 mg daily for 2 patients).

Cardiac events were defined as SCD or aborted cardiac arrest, and VF or sustained ventricular tachyarrhythmia documented by ICD or ECG recordings.

Statistical analysis. Data were analyzed with Dr. SPSS II for Windows software package (SPSS Inc., Chicago, Illinois). Numeric values are expressed as mean ± SD. The chi-square test, Student *t* test, or 1-way analysis of variance was performed when appropriate to test for statistical differences. All p values <0.05 were considered statistically significant. Event rate curves were plotted according to the Kaplan-Meier method, and were analyzed with the log-rank test. Univariate and multivariate Cox regression were performed to assess whether 7 indexes can be significant and independent predictors of subsequent cardiac events. We used the forward step-wise approach with p to enter a value of 0.05 for multivariate analysis. Augmentation of ST-segment elevation at early recovery, family history of SCD or BrS, spontaneous coved-type ST-segment elevation, presence of *SCN5A* mutation, late potential, VF inducibility during EPS, and previous episodes of VF were included as indexes.

Results

There were no significant differences between 93 BrS patients and 102 control subjects with respect to age at exercise testing, sex, QRS duration (lead V_5), and QTc interval (lead V_2), as summarized in Table 1. The RR interval and PR interval (lead II) were significantly longer in BrS patients than in control subjects.

Table 1 Initial Characteristics of Patients and Control Subjects

	Brugada Patients (n = 93)	Control Subjects (n = 102)	p Value
Age at exercise testing, yrs	46 ± 14	46 ± 17	NS
Sex, male	91 (98%)	97 (95%)	NS
Electrocardiographic characteristics, ms			
RR	952 ± 151	903 ± 140	0.020
PR	178 ± 30	165 ± 24	0.001
QRS duration	98 ± 16	98 ± 20	NS
QTc	416 ± 44	406 ± 30	NS

Values are mean ± SD or n (%).
QTc = corrected QT interval.

Response of ST-segment elevation during treadmill exercise testing. Among 93 BrS patients, significant augmentation of ST-segment elevation mostly associated with coved pattern at early recovery phase was observed in 34 BrS patients (37% [group 1]), but not in the remaining 59 BrS patients (63% [group 2]). Conversely, ST-segment augmentation was never observed in any of the 102 control subjects (34 of 93 [37%] vs. 0 of 102 [0%], $p < 0.0001$). Typical responses of ST-segment amplitudes of 3 groups are shown in Figure 1. Composite data of serial changes of ST-segment amplitude in V_1 and V_2 leads during exercise testing are illustrated in Figure 2A. The serial changes of ST-segment amplitude in V_3 lead showed the same trend (not shown). In group 1, ST-segment amplitude decreased at peak exercise and started to reascend at early recovery, and culminated at 3 min of recovery (Figs. 1A and 2A). In contrast, ST-segment amplitude of group 2 patients and control subjects decreased at peak exercise, and gradually returned to the baseline amplitude rather than showing augmentation (Figs. 1B to 1D and 2A). Significant differences were identified between group 1 and group 2 patients in the ST-segment amplitude in leads V_1 and V_2 from peak exercise to 6 min of recovery, whereas no major differences were observed between group 2 patients and control subjects (Fig. 2A). Composite data of serial changes of peak J-point amplitude, ST40, and ST80 amplitudes are presented in Figure 2B. The peak J-point amplitude and ST40 amplitude during recovery showed the same trend as the ST-segment amplitude in Figure 2A. Significant differences were identified between group 1 and group 2 patients in the peak J-point and ST40 amplitudes from peak exercise to 6 min of recovery. The ST80 amplitude showed significant differences between group 1 and group 2 patients at 2, 3, and 4 min of recovery. At peak exercise, the peak J-point amplitude increased in 34 (37%) of 93 Brugada patients and in 26 (26%) of 102 control subjects, although the ST-segment

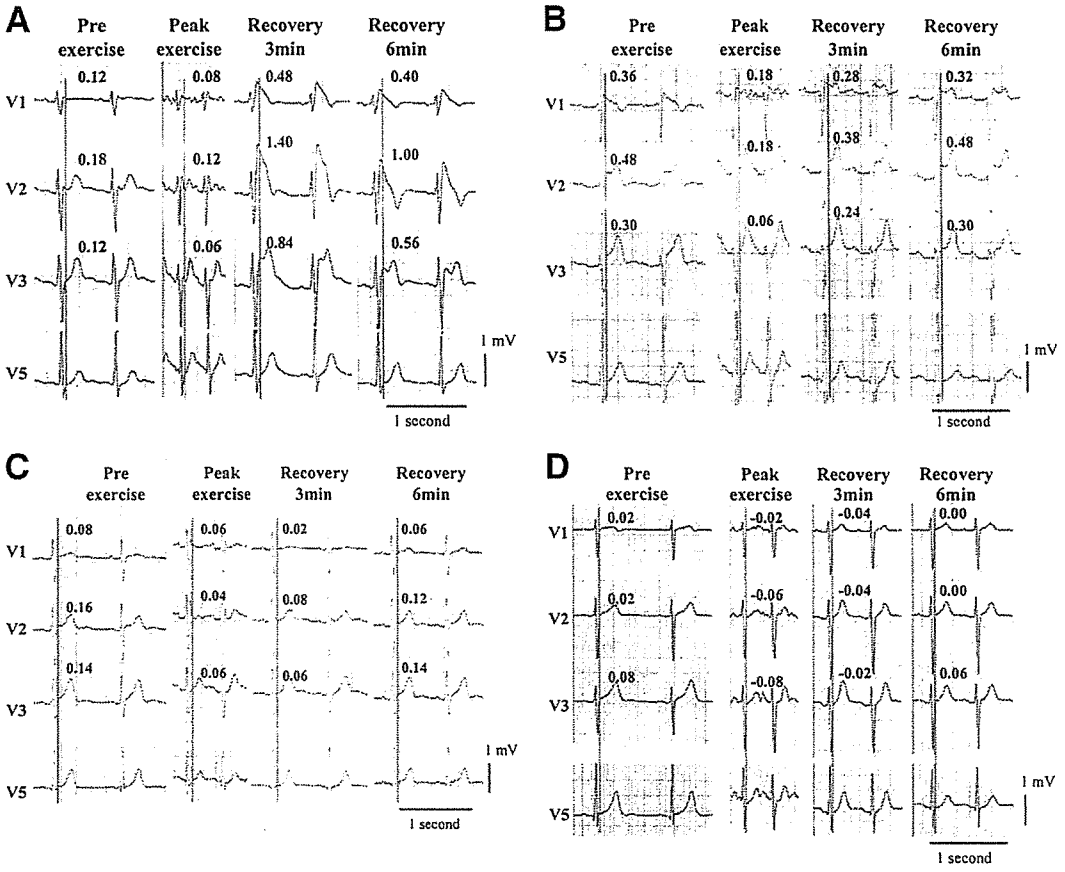

Figure 1 **Typical Responses of ST-Segment Amplitude in Leads V_1, V_2, V_3, and V_5 During Exercise Testing in Brugada Syndrome Patients**

(A) In the group 1 Brugada patient showing saddle-back type ST-segment (lead V_2) at baseline, ST-segment amplitude slightly decreased at peak exercise, but reascended at early recovery (3 min), resulting in typical coved-type ST-segment elevation. **(B, C)** In the group 2 Brugada patient and **(D)** in the control subject, ST-segment amplitude decreased at peak exercise and gradually recovered to the baseline at recovery. It is noteworthy that the peak J-point amplitude in lead V_2 was augmented despite not showing ST-segment augmentation in **A** and **C**. The ST-segment amplitudes are shown as numeric values expressed in millivolts (mV). The **red vertical line** indicates the line from the end point of the QRS interval at electrocardiography lead V_5.

amplitude and ST40 amplitude decreased in most patients of both groups.

Comparison of HRR is shown in Figure 3. The HRR of group 1 patients was significantly larger than that of group 2 patients (32 ± 15 vs. 23 ± 10, p = 0.0007) and control subjects (32 ± 15 vs. 26 ± 10, p = 0.021). The differences of HRR between group 2 patients and control subjects were also statistically significant (23 ± 10 vs. 26 ± 10, p = 0.026).

Although there were no sustained or nonsustained ventricular arrhythmias throughout exercise testing, single premature ventricular complexes were observed during exercise in 8 of the group 1 patients and in 11 of the group 2 patients, and at recovery in 10 of the group 1 patients and in 9 of the group 2 patients. There were no significant differences between groups 1 and 2 in incidences of premature ventricular complexes.

Clinical, laboratory, electrocardiographic, and electrophysiologic characteristics. Comparison of the clinical, laboratory, electrocardiographic, and electrophysiologic characteristics between groups 1 and 2 patients are shown in Table 2. There were no significant differences in these characteristics between groups 1 and 2 except for the presence of *SCN5A* mutation and late potential (*SCN5A* mutation, 17% vs. 5%, p = 0.048; late potential, 82% vs. 53%, p = 0.004).

Follow-up. The mean follow-up period for the 93 BrS patients was 75.7 ± 38.4 months. During follow-up, 25 of all 93 BrS patients (27%) had cardiac events, and the incidence of cardiac events was significantly higher in group 1 than in group 2 patients (44% vs. 17%, p = 0.004). The period from exercise testing to cardiac events ranged from 1 to 78 months (median 12 months). One patient in group 2, who refused implantation of ICD and was taking disopyr-

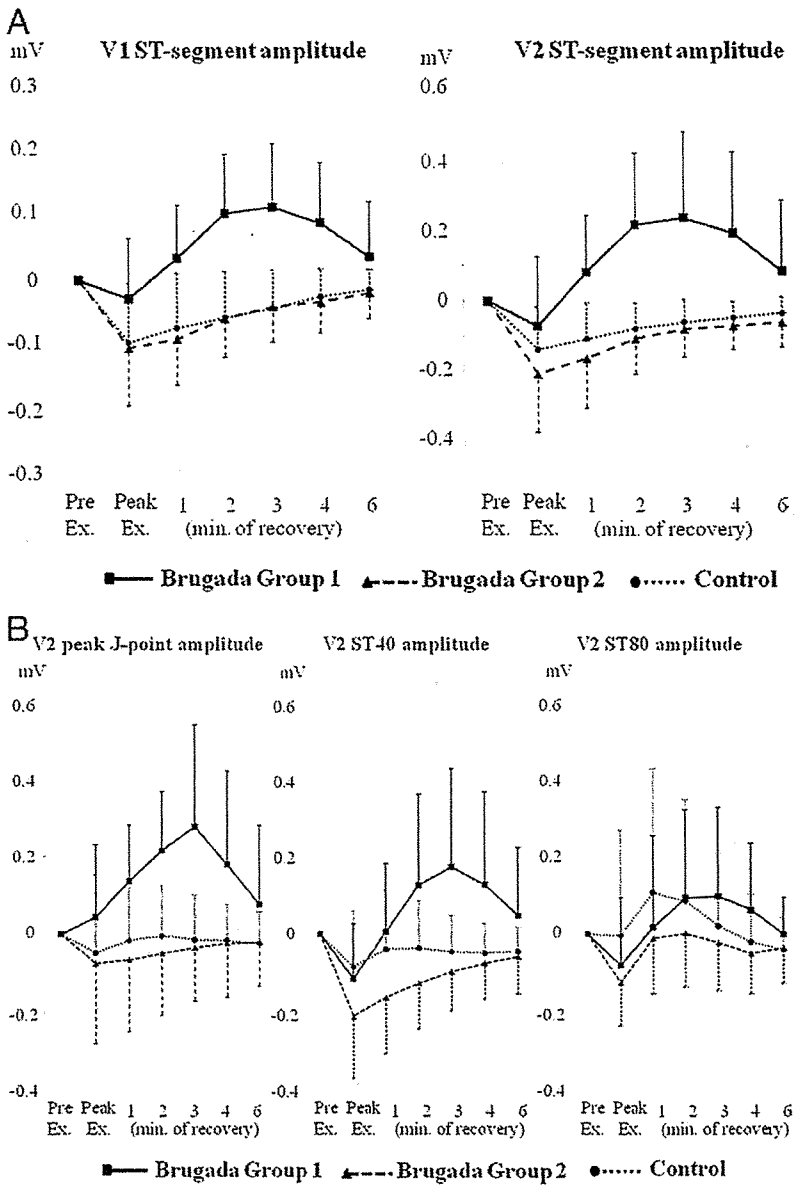

Figure 2 Composite Data of Serial Changes of ST-Segment Amplitude

(A) Composite data of serial changes of ST-segment amplitude in lead V_1 **(left)** and lead V_2 **(right)** during exercise (Ex.) testing in group 1 Brugada syndrome patients **(squares)** and group 2 Brugada syndrome patients **(triangles)**, and in control subjects **(circles)**. **(B)** Peak J-point amplitude **(left)**, ST40 amplitude **(middle)**, and ST80 amplitude **(right)** in lead V_2. The ST-segment amplitude decreased at peak exercise and started to reascend at early recovery, and culminated at 3 min of recovery in group 1 Brugada patients. In the group 2 Brugada patients and control subjects, the ST-segment amplitude decreased at peak exercise and gradually recovered to the baseline level during recovery. The peak J-point amplitude and ST40 amplitude during recovery showed the same trend as the ST-segment amplitude. Since ST80 amplitude was influenced by T wave, especially at rapid heart rate, the trends of the 3 groups were somewhat different from ST-segment amplitude or ST40 amplitude. The ST-segment amplitudes are shown as values compared to pre-exercise ST-segment amplitudes. $p < 0.05$.

amide 300 mg daily, died of VF. Three of 7 patients with medication had cardiac events, including 1 death.

Predictors of outcome. Kaplan-Meier analysis demonstrated significant differences in the time to the first cardiac event depending on the presence of ST-segment augmentation during recovery from exercise (Fig. 4A). Group 1 patients had a significantly higher cardiac event rate than group 2 patients (log-rank, $p = 0.0029$). Previous history of VF (Fig. 4B) and positive *SCN5A* mutation (Fig. 4C) also had significant values for occurrence of subsequent cardiac events ($p = 0.0013$ and $p = 0.028$, respectively); however, spontaneous coved-type ST-segment elevation did not predict cardiac events ($p =$

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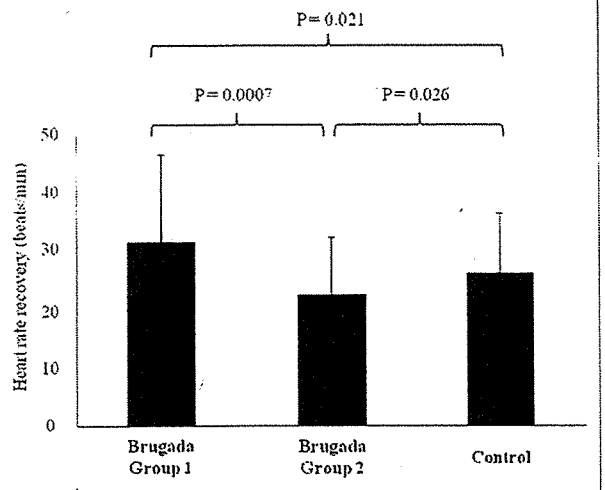

Figure 3 Comparison of HRR After Exercise Cessation

Comparison of heart rate recovery (HRR) 1 min after exercise cessation among Brugada syndrome patients of groups 1 and 2 and control subjects. The HRR in group 1 patients was significantly larger than that in group 2 (32 ± 15 beats/min vs. 23 ± 10 beats/min, p = 0.0007) and in control subjects (32 ± 15 beats/min vs. 26 ± 10 beats/min, p = 0.021). The differences of HRR between group 2 patients and control subjects were also significant (23 ± 10 beats/min vs. 26 ± 10 beats/min, p = 0.026).

0.068) (Fig. 4D). The results of Cox regression analysis are shown in Table 3. In univariate analysis, indexes predictive of cardiac events were previous episodes of VF (p = 0.003), ST-segment augmentation at early recovery (group 1; p = 0.005), and presence of *SCN5A* mutation (p = 0.037). In multivariate Cox regression analysis, previous episodes of VF and ST-segment augmentation at early recovery were significant and independent predictors of subsequent cardiac events (p = 0.005 and p = 0.007, respectively).

The incidence of cardiac events during follow-up in the subgroups according to symptoms before exercise testing is shown in Table 4. In the subgroup of 35 BrS patients with syncope alone, group 1 had a significantly higher cardiac event rate than group 2 (log-rank, 6 of 12 [50%] vs. 3 of 23 [13%], p = 0.016). Of note, among 36 asymptomatic patients, only 3 patients (9%) in group 1 experienced cardiac events. The log-rank test also demonstrated higher cardiac event risk in group 1 compared with group 2 (3 of 15 [20%] vs. 0 of 21 [0%], p = 0.039).

Discussion

The major findings of the present study were the following: 1) 37% of BrS patients showed ST-segment augmentation at early recovery during exercise testing; 2) ST-segment augmentation at early recovery was specific in BrS patients, and was significantly associated with a higher cardiac event rate, notably for patients with previous episode of syncope or for asymptomatic patients; and 3) BrS patients with ST-segment augmentation at early recovery showed significantly larger HRR. This is the first systematic report on the relationship between ST-segment augmentation during recovery from exercise and prognosis for BrS patients.

Augmentation of ST-segment elevation and possible mechanism. It is well known that autonomic function influences an extent of ST-segment elevation in BrS (8). The ST-segment elevation is mitigated by administration of β-adrenergic agonists and is enhanced by parasympathetic agonists such as acetylcholine in experimental and clinical investigations (5,14–16). Parasympathetic reactivation is thought to occur at early recovery after treadmill exercise testing, especially in the first minute after cessation of exercise (10,17). In the present study, we measured the ST-segment amplitude as a repolarization parameter rather than a depolarization parameter, and evaluated HRR to investigate the correlation between ST-segment augmentation and parasympathetic activity (9,18). The BrS patients who had ST-segment augmentation had significantly larger HRR compared with patients who did not, suggesting that the ST-segment augmentation was closely related to higher parasympathetic activity. However, it is still unclear whether ST-segment augmentation observed in the 34 BrS patients was simply due to more increased parasympathetic activity or to more increased susceptibility (hypersensitivity) to the parasympathetic reactivation.

Conversely, the *SCN5A* mutation was more frequently identified in group 1. Scornik et al. (19) reported that *SCN5A* mutation can accentuate parasympathetic activity toward the heart directly. It was also reported that specific mutations in the *SCN5A* gene may lead to augmentation of J-point amplitude or ST-segment amplitude during beta-adrenergic stimulation (20,21). Veldkamp et al. (20) demonstrated that a specific *SCN5A* mutation, 1795insD, augments slow inactivation, and delays recovery of sodium channel availability, thus reducing the sodium current and resulting in augmented peak J-point amplitude at rapid heart rate. Increased body temperature induced by exercise can be a risk of life-threatening arrhythmias in patients with BrS (22). A specific *SCN5A* missense mutation, T1620M, was reported to cause a faster decay of the sodium channel but slower recovery from inactivation, resulting in increased ST-segment elevation in precordial leads at higher temperatures during exercise. Although Amin et al. (13) reported that exercise induced augmentation of peak J-point amplitude, a depolarization parameter or at least combined parameter of both depolarization and repolarization, in all subjects tested, the incidence of increase in the peak J-point amplitude at peak exercise was lower (37%) in our Brugada patients. This is probably in part because only 9 (10%) of our 93 BrS patients had the *SCN5A* mutation. We could not identify significant differences in HRR, QRS duration, peak J-point amplitude (lead V_2), and ST-segment amplitude (leads V_1, V_2, V_3) at peak exercise between patients with and without *SCN5A* mutation (not shown), and that may be also due to the small number of BrS patients with *SCN5A* mutation.

Risk stratification in BrS. Implantation of an ICD is a first line of therapy for secondary prevention in patients with BrS who exhibited previous history of VF. The American College

Table 2 Clinical, Laboratory, Electrocardiographic, and Electrophysiologic Characteristics and Long-Term Follow-Up of Groups 1 and 2 Brugada Syndrome Patients

Characteristic	Group 1 (n = 34)	Group 2 (n = 59)	p Value
Clinical characteristics			
Age at exercise testing, yrs	42 ± 11	48 ± 15	NS
Men	34 (100%)	57 (97%)	NS
Family history of SCD at age <45 yrs or Brugada syndrome	7 (21%)	16 (27%)	NS
Documented AF	7 (21%)	12 (20%)	NS
Documented VF before exercise testing	7 (21%)	15 (25%)	NS
Syncope alone before exercise testing	12 (35%)	23 (39%)	NS
Asymptomatic before exercise testing	15 (44%)	21 (36%)	NS
Age at first cardiac event, yrs	42 ± 13	45 ± 15	NS
ICD implantation	25 (74%)	38 (64%)	NS
Laboratory characteristics			
SCN5A mutation	6 (17%)	3 (5%)	0.048
Electrocardiographic characteristics			
RR, ms	951 ± 170	953 ± 140	NS
PR, ms	184 ± 28	175 ± 31	NS
QRS, ms	98 ± 14	98 ± 17	NS
QTc, ms	418 ± 46	415 ± 43	NS
ST-segment amplitude (mV) at baseline			
V_1	0.14 ± 0.09	0.16 ± 0.12	NS
V_2	0.41 ± 0.22	0.38 ± 0.26	NS
V_3	0.22 ± 0.13	0.19 ± 0.14	NS
Spontaneous coved-type ST-segment elevation in right precordial leads	30 (88%)	43 (73%)	NS
Signal-averaged electrocardiogram			
TfQRS, ms	122 ± 15	118 ± 17	NS
Late potential	28/34 (82%)	30/57 (53%)	0.004
Premature ventricular complexes during exercise	8 (24%)	11 (19%)	NS
Premature ventricular complexes at recovery	10 (29%)	9 (15%)	NS
Electrophysiologic characteristics			
AH interval, ms	107 ± 24	98 ± 27	NS
HV interval, ms	45 ± 8	44 ± 11	NS
Induction of VF	26/31 (84%)	33/47 (70%)	NS
Follow-up			
Cardiac events	15 (44%)	10 (17%)	0.004
Follow-up period, months	74.1 ± 42.2	76.5 ± 36.4	NS

AF = atrial fibrillation; ICD = implantable cardioverter-defibrillator; SCD = sudden cardiac death; TfQRS = total filtered QRS duration; VF = ventricular fibrillation; other abbreviations as in Table 1.

of Cardiology/American Heart Association/Heart Rhythm Society guidelines refer to BrS patients who have had syncope as having Class IIa indication for ICD therapy (23). However, there is still much room for argument with respect to treatments for patients who have had only syncope, and for asymptomatic patients (24–28). Although inducibility of VF during EPS (25,26), family history of SCD (24), spontaneous type 1 ECG (25,27), and late potential (28) have been proposed as predictors of cardiac events, the availability of these indexes remains controversial (7,29).

In the present study, a previous episode of VF (or aborted cardiac arrest) was the strongest predictor of subsequent cardiac events, as in previous studies (7,30,31). Moreover, ST-segment augmentation at early recovery during exercise testing was a significant and independent predictor of subsequent cardiac events in the present study. The results suggested that parasympathetic activity plays an important role in both ST-segment augmentation and subsequent cardiac events. As previously noted, it remains unclear that the cause of ST-segment augmentation in our 34 patients was a result of more increased parasympathetic activity or of more increased susceptibility of the patients to the increased parasympathetic reactivation.

Study limitations. First, BrS patients were confined to those who were hospitalized in our hospital for close investigation. That indicates these patients can be biased toward relatively high risk. Second, the present study is based on data from a small population of 93 patients; hence, it was not sufficient to evaluate the prognosis, and there also was a small number of events. Although we adopted a step-wise approach, the limited number of events can lessen the precision of the consequences for multivariate Cox regression analysis.

Conclusions

The presence of *SCN5A* mutation was a significant predictor of subsequent cardiac events by univariate Cox regression analysis. However, multivariate Cox regression analysis showed it was not a significant predictor of prognosis.

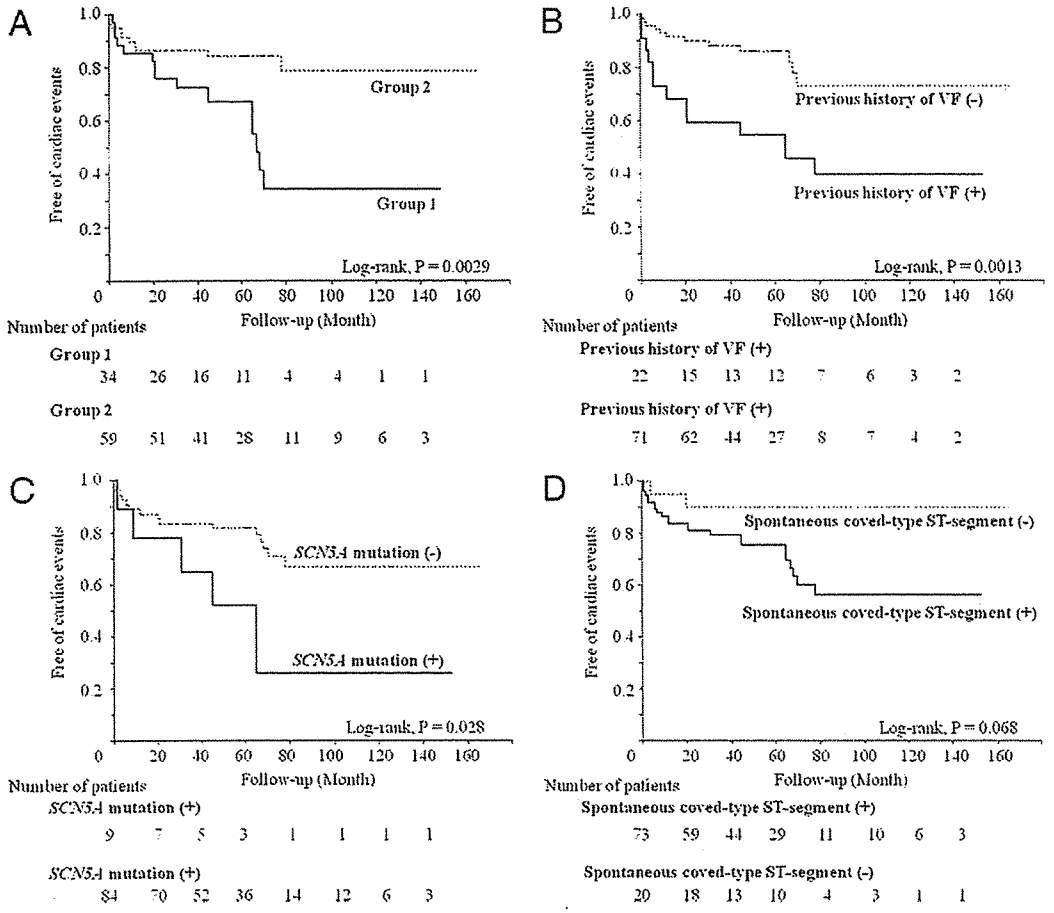

Figure 4 Kaplan-Meier Analysis of Cardiac Events During Follow-Up

Kaplan-Meier analysis of **(A)** cardiac events during follow-up, depending on patterns in response to ST-segment elevation during exercise test (groups 1 and 2), **(B)** incidence of previous episode of ventricular fibrillation (VF), **(C)** *SCN5A* mutation, and **(D)** spontaneous coved-type ST-segment elevation. Group 1 Brugada patients had a significantly higher cardiac event rate than did group 2 Brugada patients (log-rank, p = 0.0029). Brugada patients with previous episodes of VF or with *SCN5A* mutation had significantly greater values for occurrence of subsequent cardiac events than did patients without VF episodes or *SCN5A* mutation (p = 0.0013, p = 0.028, respectively), whereas spontaneous coved-type ST-segment elevation in Brugada patients did not predict cardiac events compared with patients not having such ST-segment elevation (p = 0.068).

Further study with a larger number of BrS patients will be required to evaluate the significance of the index as a predictor of subsequent cardiac events.

As for BrS patients with only syncope, subsequent cardiac events occurred in 50% (6 of 12) patients who exhibited ST-segment augmentation at early recovery. Asymptomatic

Table 3 Predictive Capabilities of Cardiac Events

		Univariate Analysis		Multivariate Analysis	
	Positive, n (%)	HR (95% CI)	p Value	HR (95% CI)	p Value
Previous episodes of VF	22 (24%)	3.40 (1.54–7.53)	0.003	3.25 (1.43–7.37)	0.005
Augmentation of ST-segment elevation at early recovery phase	34 (37%)	3.17 (1.42–7.09)	0.005	3.17 (1.37–7.33)	0.007
SCN5A mutation	9 (10%)	2.86 (1.07–7.66)	0.037		
Spontaneous coved-type ST-segment	72 (77%)	3.51 (0.83–14.9)	0.089		
Late potential	58/91 (64%)	2.25 (0.84–5.99)	0.11		
VF inducible in EPS	59/78 (76%)	0.73 (0.30–1.75)	0.48		
Family history of SCD or BrS	23 (25%)	1.19 (0.47–3.02)	0.72		

BrS = Brugada syndrome; CI = confidence interval; EPS = electrophysiologic study; HR = hazard ratio; other abbreviations as in Table 2.

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Table 4 Incidence of Cardiac Events According to Symptoms Before Exercise Testing

Type	n	Treadmill Exercise Test	n	VF Occurrence	p Value (vs. Group 1)
Documented VF	22	Group 1	7	6 (86%)	
		Group 2	15	7 (47%)	0.14
Syncope alone	35	Group 1	12	6 (50%)	
		Group 2	23	3 (13%)	0.016
Asymptomatic	36	Group 1	15	3 (20%)	
		Group 2	21	0 (0%)	0.039

The p value was calculated according to the log-rank test.
VF = ventricular fibrillation.

patients who had ST-segment augmentation at early recovery had a higher incidence of cardiac events than patients who did not. These data suggested the potential utility of exercise testing to predict cardiac events for patients with BrS who have had previous episodes of only syncope but not VF or who have had no symptoms.

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Key Words: Brugada syndrome ■ exercise testing ■ ST-segment elevation.

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Reduction in incidence and fatality of out-of-hospital cardiac arrest in females of the reproductive age

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Aims The aim of this study was to determine relative risk (RR) of incidence and fatality of out-of-hospital cardiac arrest (OHCA) by gender and oestrogen status.

Methods and results In a prospective, population-based observational study from 1998 through 2007, incidence and neurologically intact 1-month survival after OHCA were compared by gender after grouping: 0–12 years, 13–49 years, and ≥50 years according to menarche and menopause age. Among 26 940 cardiac arrests, there were 11 179 females and 15 701 males. Age-adjusted RR of females for OHCA incidence compared with males was 0.72 [95% confidence interval (CI), 0.58–0.91] in age 0–12 years, 0.39 (95% CI, 0.37–0.43) in age 13–49 years, and 0.54 (95% CI, 0.52–0.55) in age ≥50 years. Females aged 13–49 years had a significantly higher good neurological outcome than males [adjusted odds ratio (OR), 2.00 (95% CI 1.21–3.32)]. This sex difference was larger than that in the other age groups [adjusted OR, 0.82 (95% CI, 0.06–12.02) in age 0–12 years and 1.23 (95% CI, 0.98–1.54) in age ≥50 years].

Conclusion Reproductive females had a lower incidence and a better outcome of OHCA than females of other ages and males, which might be explained by cardioprotective effects of endogenous oestrogen on OHCA.

Keywords Out-of-hospital cardiac arrest • Sudden death • Cardiopulmonary resuscitation • Epidemiology • Gender • Women

Introduction

Sudden cardiac arrest (SCA) is the leading cause of death in the industrialized world,[1] and approximately 50 000 events occur every year in Japan.[2] Females are generally less likely to die of cardiovascular disease than males,[3,4] and there are age-related differences in incidence and outcomes of SCA in males and females.[5–10] But the aetiology of gender-related differences in cardiac arrest remains to be determined.

Animal models of cardiac arrest suggest that acute doses of oestrogen are associated with a good prognosis after cardiac arrest.[11,12] Although some protective effects of oestrogen on the cardiovascular system have been suggested,[13–17] no clinical studies have assessed the gender differences in incidence and outcomes of SCA by focusing on oestrogen exposure of females.

The Utstein Osaka Project is a large prospective population-based cohort study of out-of-hospital cardiac arrests (OHCAs) in Osaka, Japan, which covered about 8.8 million residents and

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was launched in 1998.[5,18,19] During the initial 9 years and 8 months, there were 29 192 emergency medical service (EMS)-resuscitated OHCAs of presumed cardiac aetiology. Our hypothesis is that endogenous oestrogen has protective effects on OHCA incidence and outcome in this population.

Methods

Study design, setting, and population

This observational study enrolled all patients who suffered OHCA of presumed cardiac aetiology before EMS arrival, were treated by EMS, and were transported to medical institutions in Osaka Prefecture from 1 May 1998 through 31 December 2007. Osaka is the second largest prefecture in Japan with a population of 8 805 081 inhabitants in an area of 1892 km^2. The census population included 4 293 763 males and 4 495 591 females, and the proportion of people aged 65 years and over is 14.9% in 2000.[20] Cardiac arrest was defined as the cessation of cardiac mechanical activity as confirmed by the absence of signs of circulation.[21] The arrest was presumed to be of cardiac aetiology unless it was caused by trauma, drowning, drug overdose, asphyxia, exsanguinations, or any other non-cardiac causes. These diagnoses were determined by the physician in charge in collaboration with the EMS rescuers.

The research protocol was approved by the institutional review board of Osaka University with the assent of the EMS authorities of the local governments in Osaka Prefecture.

Emergency medical service systems in Osaka

In Osaka Prefecture, there are 35 fire stations with emergency dispatch centres. The EMS system is operated by the local fire stations. The free telephone emergency number 119 is used to call for ambulance from anywhere in Japan. Emergency services are provided 24 h each day by them, which are single tiered in 33 stations and two tiered in two stations. The latter uses medics followed by physicians. The most highly trained pre-hospital emergency care providers are the emergency life-saving technicians (ELSTs). When called, an ambulance is dispatched from the nearest fire station. Usually, each ambulance has a crew of three emergency providers including at least one ELST. They were allowed to insert an intravenous line and an adjunct airway, and to use a semi-automated external defibrillator for OHCA patients during the study period. Specially trained ELSTs were permitted to insert tracheal tubes after July 2004 and administer intravenous epinephrine after April 2006. The use of automated external defibrillators by citizens was legally approved in July 2004. Do-not-resuscitate orders or living wills are not generally accepted in Japan. Emergency medical service providers are not permitted to terminate resuscitation in the field. Therefore, all patients with OHCA who were treated by EMS personnel were transported to hospital and registered in this study. Details of the EMS system in Osaka were described previously.[18]

Data collection and quality control

Data were prospectively collected using a form that included all core data recommended in the Utstein-style reporting guidelines for cardiac arrests.[21] Initial rhythm was recorded and diagnosed by the EMS personnel with semi-automated defibrillators on the scene, and confirmed by the physician who was responsible for the on-line medical direction. The time of EMS call receipt and vehicle arrival at the scene was recorded automatically at the dispatch centre. The time of collapse and initiation of bystander CPR was obtained by EMS interview with the bystander before leaving the scene. The time of defibrillation was recorded in the semi-automated defibrillator.

The data form was filled out by the EMS personnel in cooperation with the physicians in charge of the patient, transferred to the Information Center for Emergency Medical Services of Osaka, and then checked by the investigators. If the data sheet was incomplete, the relevant EMS personnel were contacted and questioned, and the data sheet was completed.

All survivors were followed up for up to 1 month after the event by the EMS personnel and investigators with the cooperation of the Osaka Medical Association and medical institutes in this area. Neurological outcome was determined by a follow-up telephone interview 1 month after successful resuscitation, using the cerebral performance category (CPC) scale: category 1, good cerebral performance; category 2, moderate cerebral disability; category 3, severe cerebral disability; category 4, coma or vegetative state; and category 5, death.[21]

Key group definition and main outcome measures

To assess our hypothesis that endogenous oestrogen would influence OHCA incidence and their outcomes, we divided the patients into the following three groups: 0–12 years, 13–49 years, and 50 years and over according to the published data on mean age of menarche (12.2 ± 0.9 years) and menopause (49.5 ± 3.5 years) among Japanese females.[22,23] According to this criterion, females aged 13–49 years would be considered to be exposed to endogenous oestrogen. The primary outcome measure was annual incidence and neurologically intact 1-month survival. Age-adjusted annual incidence of EMS-treated OHCA of presumed cardiac aetiology by gender were calculated by the direct method using 2000 census data and 1985 Japanese model population.[20,24] Neurologically intact outcome was defined as CPC category 1 or 2.[21] Secondary outcome measures included return of spontaneous circulation (ROSC), admission to hospital, and 1-month survival.

Statistical analysis

Patient characteristics were compared between groups using unpaired *t*-test for numerical variables, and χ^2 test or Fisher's exact test for categorical variables. The relative risk (RR) and its 95% confidence interval (CI) of females against males for the incidence were calculated with the Mantel–Haenszel statistic stratifying by 5-year age stratum. Multiple logistic regression analysis assessed the factors associated with better neurological outcome, and odds ratios (ORs) and their 95% CIs were calculated. As potential confounders, factors that were biologically essential and considered to be associated with clinical outcomes were taken in the multivariable analyses. These variables included age, gender, bystander witnessed status, location of arrest, activity of daily living before arrests, bystander CPR status, first recorded rhythm, time interval from call to the initiation of CPR by EMS personnel, and year of arrest. The interaction between gender and age group on outcomes was also calculated. All of the tests were two-tailed and a *P*-value of <0.05 was considered statistically significant. All statistical analyses were performed using SPSS statistical package ver16.0J (SPSS, Inc., Chicago, IL, USA).

Results

Figure 1 shows an overview of the study patients based on the Utstein template. A total of 53 526 OHCAs were documented during these 9 years and 8 months. Resuscitation was attempted in 50 119, and 29 192 of them were presumed of cardiac aetiology.

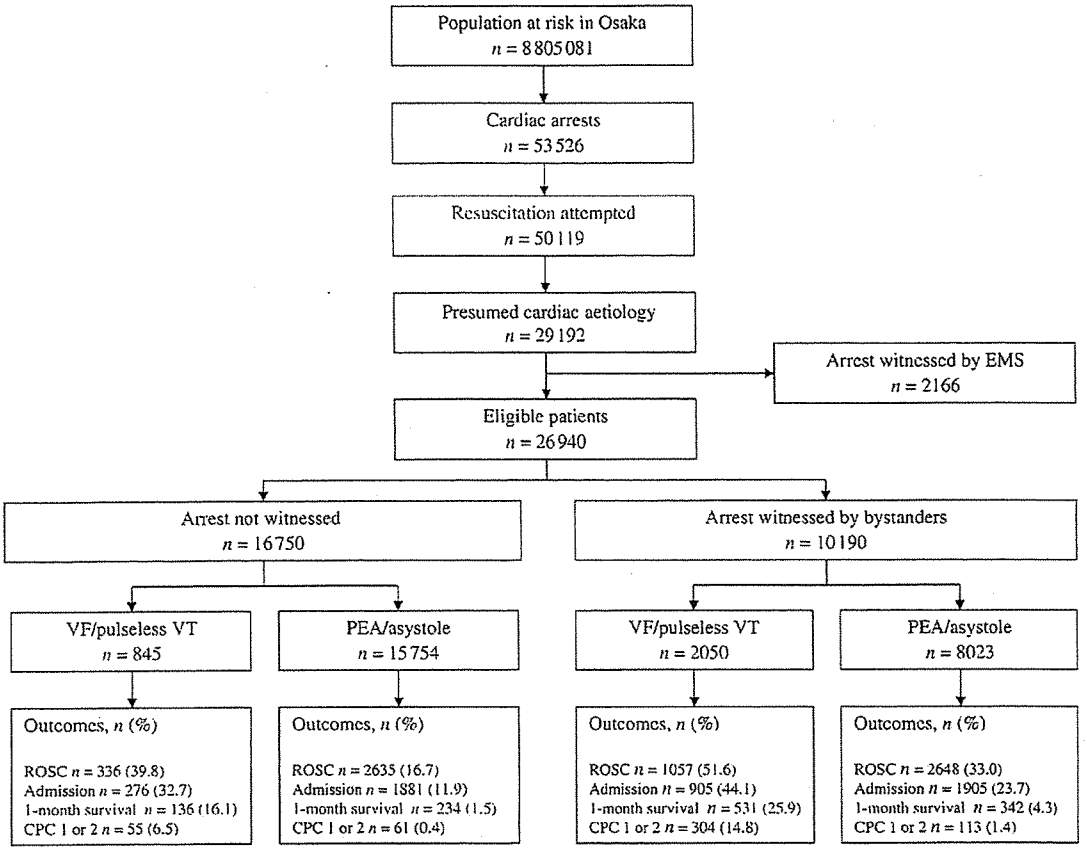

Figure 1 Overview of emergency medical service-treated cardiac arrests with an abridged Utstein template from 1 May 1998 through 31 December 2007. EMS, emergency medical service; VF, ventricular fibrillation; VT, ventricular tachycardia; PEA, pulseless electrical activity; ROSC, return of spontaneous circulation; CPC, cerebral performance category.

Excluding 2166 victims who were witnessed by EMS, 26 940 were eligible for our analyses. Among them, 15 701 (58.3%) were males, 11 179 (41.6%) were females, and the remaining 60 (0.2%) were missed for gender. Of these victims, 10 190 were witnessed by bystanders, and 16 750 were not. Among witnessed cases, 2050 had ventricular fibrillation (VF) or pulseless ventricular tachycardia (VT), and 8023 had pulseless electrical activity (PEA) or asystole. Among non-witnessed cases, 845 had VF or pulseless VT and 15 754 PEA or asystole. We could not obtain neurologically intact 1-month survival data for 13 (0.04%) among 26 940 eligible victims. The proportions of neurologically intact 1-month survival among those with VF or pulseless VT and among those with PEA or asystole were 14.8 and 1.4%, respectively, when witnessed, whereas 6.5 and 0.4%, respectively, when not witnessed.

The characteristics of patients who experienced OHCA of presumed cardiac aetiology are shown in *Table 1*. Females were significantly older than males (mean age, 75.5 ± 16.4 vs. 67.9 ± 16.5 years; $P < 0.001$). The proportions of females in the 0–12 years group, the 13–49 years group, and the 50 years and over group were 127/311 (40.8%), 612/2120 (28.9%), and 10 440/24 449 (42.7%), respectively ($P < 0.001$). Females with cardiac arrest were more likely to have their arrests at home than males, and less likely to be witnessed and have VF as initial rhythm, although these differences are statistically insignificant in the age group of 0–12 years. The mean time intervals from call to CPR were similar between genders in each age group.

The incidence of OHCA increased with advancing age group in a non-linear manner in both genders (*Table 2*). Females had a lower incidence rate than males in every age group. The age-adjusted population-based incidence of OHCA of presumed cardiac aetiology was 32.5 per 100 000 person-years in males and 13.2 in females, and that of witnessed OHCA in males and females was 15.6 and 5.3, respectively. Age-adjusted RR of females for the incidence of OHCA in the group of age 13–49 years was 0.39 (95% CI; 0.37–0.43; $P < 0.001$), which was smaller than that in the group of age 0–12 years (0.72; 95% CI 0.58–0.91, $P = 0.005$) and 50 years and over (0.54; 95% CI 0.52–0.55, $P < 0.001$). In cases of bystander-witnessed OHCA of presumed cardiac aetiology, age-adjusted RRs of females for the incidence in the group of age 0–12 years, 13–49 years, and 50 years and over were 0.67 (95% CI 0.41–1.11, $P = 0.092$), 0.32 (95% CI 0.27–0.37, $P < 0.001$), and 0.45 (95% CI 0.43–0.47, $P < 0.001$), respectively.

Table 1 Characteristics of the study participants by age and gender

Characteristic	Age 0–12 years			Age 13–49 years			Age 50 years and over		
	Female (n = 127)	Male (n = 184)	P	Female (n = 612)	Male (n = 1508)	P	Female (n = 10 440)	Male (n = 14 009)	P
Age (years)	1.7 (3.1)	2.0 (3.2)	0.419	37.5 (9.3)	38.1 (9.3)	0.217	78.7 (11.1)	72.0 (11.9)	<0.001
Arrest witnessed by bystanders	19.7 (25)	21.2 (39)	0.777	36.9 (226)	46.8 (705)	<0.001	33.6 (3505)	40.5 (5668)	<0.001
Location of arrest									
Home	92.1 (116)	88.6 (163)		77.0 (470)	59.6 (894)		75.1 (7823)	70.4 (9836)	
Public space	4.0 (5)	4.9 (9)		11.3 (69)	19.9 (299)		5.8 (609)	14.4 (2009)	
Work place			0.732	2.0 (12)	9.4 (141)	<0.001	0.5 (56)	3.7 (512)	<0.001
Health care facility[a]	0.0 (0)	1.1 (2)		2.1 (13)	0.5 (7)		14.5 (1516)	4.8 (673)	
Other	4.0 (5)	5.4 (10)		7.5 (46)	10.7 (160)		4.0 (417)	6.7 (936)	
Activity of daily living before arrests									
Good	73.6 (92)	77.8 (140)		81.2 (492)	84.4 (1257)		63.0 (6524)	72.1 (10 024)	
Disability	20.0 (25)	17.8 (32)	0.647	12.2 (74)	6.8 (101)	<0.001	33.6 (3481)	20.7 (2879)	<0.001
Unknown	6.4 (8)	4.4 (8)		6.6 (40)	8.8 (131)		3.5 (358)	7.2 (1006)	
Type of bystander CPR									
No CPR	51.6 (65)	52.8 (92)		66.7 (405)	67.9 (1017)		65.7 (6826)	72.0 (10 049)	
Chest compression-only CPR	9.5 (12)	6.1 (11)	0.535	12.5 (76)	14.2 (212)	0.244	15.1 (1570)	13.6 (1890)	<0.001
Conventional CPR	38.9 (49)	41.1 (74)		20.8 (126)	17.9 (268)		19.2 (2001)	14.4 (2009)	
Type of initial rhythm									
VF or pulseless VT	2.4 (3)	3.9 (7)		12.5 (76)	23.0 (344)		5.7 (588)	13.4 (1873)	
PEA	11.4 (14)	14.5 (26)	0.467	12.5 (76)	10.1 (151)	<0.001	17.5 (1817)	17.7 (2471)	<0.001
Asystole	85.4 (105)	81.6 (146)		74.8 (456)	66.6 (997)		76.4 (7930)	68.4 (9533)	
Other	0.8 (1)	0.0 (0)		0.3 (2)	0.3 (5)		0.5 (50)	0.4 (50)	
Call to CPR time by EMS personnel (min)	7.5 (2.4)	7.4 (2.6)	0.921	7.9 (2.9)	8.0 (3.1)	0.780	7.8 (3.0)	7.8 (3.1)	0.961

Data are mean (SD) and % (n).
CPR, cardiopulmonary resuscitation; EMS, emergency medical system; VF, ventricular fibrillation; VT, ventricular tachycardia; PEA, pulseless electrical activity.
[a]Health care facility includes chronic facilities and medical clinics.

Table 2 Annual incidence of out-of-hospital cardiac arrest by age and gender

	0–12 years	P	13–49 years	P	50 years and over	P
Female						
Number of victims/population at risk	127/526 398		612/2 193 247		10 440/1 775 946	
Age-adjusted incidence rate per 100 000 population	2.4		3.8		50.1	
Male						
Number of victims/population at risk	184/551 328		1508/2 200 452		14 009/1 541 983	
Age-adjusted incidence rate per 100 000 population	3.3		9.7		98.5	
Adjusted RR of the incidence for female vs. male (95% CI)[a]	0.72 (0.58–0.91)	0.005	0.39 (0.37–0.43)	<0.001	0.54 (0.52–0.55)	<0.001

RR, relative risk; CI, confidence interval.
[a]RRs are adjusted for age, and estimated by the Mantel–Haenszel statistic.

Table 3 Outcomes after out-of-hospital cardiac arrest by age and gender

Outcomes	Age 0–12 years (female = 127, male = 184)	P	Age 13–49 years (female = 612, male = 1508)	P	Age 50 years and over (female = 10 440, male = 14 009)	P	Interaction P[c]
ROSC							
Female	22.0 (28)		26.3 (161)		23.6 (2465)		<0.001
Male	19.6 (36)		20.2 (305)		26.8 (3751)		
Crude OR[a] (95% CI)	1.16 (0.67–2.03)	0.595	1.40 (1.13–1.75)	0.003	0.85 (0.80–0.90)	<0.001	
Adjusted OR[b] (95% CI)	1.35 (0.72–2.54)	0.404	1.90 (1.49–2.44)	<0.001	1.02 (0.95–1.09)	0.385	
Admission							
Female	16.5 (21)		21.6 (132)		17.3 (1810)		<0.001
Male	16.3 (30)		15.9 (240)		19.9 (2788)		
Crude OR[a] (95% CI)	1.02 (0.55–1.87)	0.957	1.45 (1.15–1.84)	0.002	0.84 (0.79–0.90)	<0.001	
Adjusted OR[b] (95% CI)	1.06 (0.53–2.13)	0.990	2.09 (1.60–2.72)	<0.001	1.05 (0.98–1.13)	0.102	
Survival at 1 month							
Female	2.4 (3)		7.4 (45)		3.4 (360)		0.037
Male	4.9 (9)		7.8 (118)		5.2 (733)		
Crude OR[a] (95% CI)	0.47 (0.13–1.77)	0.265	0.94 (0.65–1.34)	0.719	0.65 (0.57–0.74)	<0.001	
Adjusted OR[b] (95% CI)	0.49 (0.11–2.08)	0.344	1.57 (1.05–2.36)	0.026	1.19 (1.03–1.37)	0.019	
Neurologically intact 1-month survival							
Female	1.6 (2)		4.9 (30)		1.3 (139)		0.020
Male	1.6 (3)		4.4 (67)		2.2 (312)		
Crude OR[a] (95% CI)	0.97 (0.16–5.18)	0.969	1.11 (0.71–1.72)	0.641	0.59 (0.48–0.73)	<0.001	
Adjusted OR[b] (95% CI)	0.82 (0.06–12.02)	0.857	2.00 (1.21–3.32)	0.005	1.23 (0.98–1.54)	0.073	

ROSC, return of spontaneous circulation; OR, odds ratio; CI, confidence interval.
[a]OR is for female vs. male. Data shows % (*n*).
[b]ORs are adjusted for age, bystander witnessed status, location of arrest, activity of daily living before arrests, bystander CPR status, first recorded rhythm, time interval from call to the initiation of CPR by EMS personnel, and year of arrest.
[c]Interaction *P*-values are calculated for evaluating the interaction between gender and age group in outcomes.

Table 3 shows the age-stratified outcomes after OHCA of presumed cardiac aetiology by gender. Among OHCAs of presumed cardiac aetiology, females aged 13–49 years had a significantly higher neurologically intact 1-month survival than males (4.9 vs. 4.4%; adjusted OR, 2.00; 95% CI 1.21–3.32, $P = 0.005$). As for ROSC (26.3 vs. 20.2%; adjusted OR, 1.90; 95% CI 1.49–2.44, $P < 0.001$), admission (21.6 vs. 15.9%; adjusted OR, 2.09; 95% CI 1.60–2.72, $P < 0.001$) and 1-month survival (7.4 vs. 7.8%; adjusted OR, 1.57; 95% CI 1.05–2.36, $P = 0.026$) as secondary outcomes, females aged 13–49 years also showed significantly greater frequencies than males. In the group aged 0–12 years in which only a few patients survived, frequency of neurologically intact 1-month survival of females was similar to that of males (1.6 vs. 1.6%; adjusted OR, 0.82; 95% CI 0.06–12.02, $P = 0.86$). In the

group aged 50 years and over, on univariable analysis, females had poorer outcomes than males. However, on multivariable analysis, females had a greater neurologically intact 1-month survival (1.3 vs. 2.2%; adjusted OR, 1.23; 95% CI 0.98–1.54, $P = 0.073$) and 1-month survival (3.4 vs. 5.2%; adjusted OR, 1.19; 95% CI 1.03–1.37, $P = 0.019$), though the differences were small.

When limiting witnessed OHCA with presumed cardiac aetiology, findings were almost identical as those in the whole OHCA. Neurologically intact 1-month survival in witnessed OHCA of females and males was 8.0 vs. 7.7%, respectively (adjusted OR, 0.83; 95% CI 0.07–8.60, $P = 0.86$) in the group aged 0–12 years, 9.7 vs. 8.2%, respectively (adjusted OR, 2.08; 95% CI 1.16–3.73, $P = 0.006$) in the group aged 13–49 years, and 2.9 vs. 4.2%, respectively (adjusted OR, 1.28; 95% CI 0.98–1.67, $P = 0.080$) in the group aged 50 years and over.

Discussion

This study showed that females of reproductive age had a lower incidence of OHCA and better outcomes than males in a large population. Although many studies have examined gender- and age-related differences in OHCA occurrence and outcome,[5–10] the impact of oestrogen status on gender difference was not fully evaluated in the preceding studies.

This study suggests that endogenous oestrogen is protective against the occurrence of OHCA. The mechanism of how oestrogen works protectively against coronary heart disease including SCA is still unclear. It is reported that oestrogen reduces levels of lipoprotein (a), inhibits oxidation of low-density lipoprotein, and improves vascular function.[16,17] In animal models, oestrogen has cardioprotective effects by binding the oestrogen receptor on vascular cells and producing nitric oxide, which is required for the maintenance and repair of vascular endothelium and dilation of vascular smooth muscle.[25,26] In autopsies of female SCA victims, vulnerable plaques varied by age, and the association between hypercholesterolaemia and coronary plaque rapture was found only in post-menopausal age.[15,27] Our data are consistent with these previous studies and strengthen the evidence that endogenous oestrogen has a protective effect against the occurrence of coronary heart disease.

In addition to the protective effect on the occurrence of OHCA, better outcomes after OHCA in females of the reproductive age were demonstrated. Some experimental studies showed that oestrogen might have cardioprotective effects for cardiac arrest.[11,12] Another report suggested that oestrogen had a possible neuroprotective effect.[28] In an animal experiment, a single low dose of E2, one of the major oestradiols in humans, has neuroprotective effects on CPR after cardiac arrest.[29] These findings suggest the protective effects of endogenous oestrogen against not only the occurrence but also survival after cardiac arrests.

If oestrogen exposure reduces the risk for incidence and fatality of SCA, hormone replacement therapy (HRT) might be effective for reducing the occurrence of OHCA and improving survival after OHCA. Observational studies suggested that post-menopausal HRT reduced CVD and sudden death.[17,30] However, some randomized controlled trials indicated that HRT rather increased overall coronary heart disease risk,[17,31] and HRT is not recommended for the prevention of coronary heart disease.[32] This discrepancy between observational studies and randomized trials is under debate, but it could be partially explained by factors such as the initiation time, dose, and delivery route of HRT, as well as genetics, statin use, and socioeconomic status of the recipients.[17,33,34] Further study on the effect of oestrogen as a new therapeutic approach for OHCA would be needed.

This study also showed a lower incidence of OHCA and better outcomes in females compared with males regardless of the age group. There might be other factors than oestrogen which were associated with lower OHCA occurrence and fatality in females. Cardiovascular disease is a major cause of SCA, and representative risk factors for CVD, such as smoking, hypertension, and diabetes mellitus, partially explain these results because CVD risk factors were less likely to occur in females than in males.[35–38] Some underlying diseases of SCA, such as Brugada syndrome and hypertrophic cardiomyopathy, which are less likely to occur in females, might affect the frequency imbalance of OHCA between genders.[39,40] A heavy alcohol consumption and physical exercise could also confound the lower incidence of SCA in females[41–43] because fewer females drink excess alcohol or exercise vigorously.[38] Unfortunately, no information about a history of smoking, drinking, and physical exercise was available in this study. Androgens and testosterone might also be taken into consideration. Although one study suggested that high androgen levels were associated with an increased risk of CVDs,[44] the effects of male sex hormones on CVDs are left unresolved.[45]

Limitations

This study has some limitations. First, we stratified study patients at ages of 12 and 50 years based on the mean age of menarche and menopause in Japanese females.[22,23] We did not obtain any information on menarche and menopausal status and history of HRT from each female suffering from OHCA. Therefore, we might underestimate the effects of active hormonal status owing to misclassification. However, the prevalence of HRT in Japanese females is $\leq$10%, and its influences would be small.[46] Secondly, the presence of CVD and the role of CVD risk factors were not assessed in this study. Thirdly, information about the care after admission was lacking. In-hospital diagnosis (e.g. coronary angiographies) and treatment (e.g. hypothermia) might affect survival after OHCA. Fourthly, data integrity, validity, and ascertainment biases are potential limitations. The data collected by EMS providers included relatively few data points that were easy to attain in an accurate manner at the scene, based on the clear and concise Utstein-style guidelines for reporting cardiac arrest.[21] The uniform data collection, consistent definitions, time synchronization process, and large sample size in the population-based cohort study were intended to minimize these potential sources of bias.

More detailed information about age of menarche and menopause, HRT use, and other potential factors associated with OHCA, including CVD risk factors and in-hospital diagnosis and treatment, and measurement of oestrogen concentration in females suffering from OHCA or interventional trial of HRT, would be needed for better ascertainment of the protective effects of oestrogen on OHCA.

Conclusion

Females in the period between menarche and menopause had a lower incidence and better outcomes of OHCA, which might be explained by cardioprotective effects of endogenous oestrogen on OHCA occurrence and outcome.

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The Cellular Prion Protein Identifies Bipotential Cardiomyogenic Progenitors

Kyoko Hidaka, Manabu Shirai, Jong-Kook Lee, Takanari Wakayama, Itsuo Kodama, Michael D. Schneider and Takayuki Morisaki

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The Cellular Prion Protein Identifies Bipotential Cardiomyogenic Progenitors

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Rationale: The paucity of specific surface markers for cardiomyocytes and their progenitors has impeded the development of embryonic or pluripotent stem cell–based transplantation therapy. Identification of relevant surface markers may also enhance our understanding of the mechanisms underlying differentiation.

Objective: Here, we show that cellular prion protein (PrP) serves as an effective surface marker for isolating nascent cardiomyocytes as well as cardiomyogenic progenitors.

Methods and Results: Embryonic stem (or embryo-derived) cells were analyzed using flow cytometry to detect surface expression of PrP and intracellular myosin heavy chain (Myhc) proteins. Sorted cells were then analyzed for their differentiation potential.

Conclusions: PrP^+ cells from beating embryoid bodies (EBs) frequently included nascent $Myhc^+$ cardiomyocytes. Cultured PrP^+ cells further differentiated, giving rise to cardiac troponin I^+ definitive cardiomyocytes with either an atrial or a ventricular identity. These cells were electrophysiologically functional and able to survive in vivo after transplantation. Combining PrP with a second marker, platelet-derived growth factor receptor (PDGFR)α, enabled us to identify an earlier cardiomyogenic population from prebeating EBs, the $PrP^+PDGFR\alpha^+$ (PRa) cells. The $Myhc^-$ PRa cells expressed cardiac transcription factors, such as Nkx2.5, T-box transcription factor 5, and Isl1 (islet LIM homeobox 1), although they were not completely committed. In mouse embryos, PRa cells in cardiac crescent at the 1 to 2 somite stage were $Myhc^+$, whereas they were $Myhc^-$ at headfold stages. PRa cells clonally expanded in methlycellulose cultures. Furthermore, single $Myhc^-$ PRa cell–derived colonies contained both cardiac and smooth muscle cells. Thus, PrP demarcates a population of bipotential cardiomyogenic progenitor cells that can differentiate into cardiac or smooth muscle cells. (*Circ Res.* 2010;106:111-119.)

Key Words: cardiogenic precursor ■ differentiation ■ embryonic stem cells ■ surface marker regeneration

Heart, the first functional organ to develop in vertebrate embryos, contains cardiac, smooth muscle, and endothelial cells.[1–3] Cardiac transcription factors, such as Nkx2.5 (NK2 transcription factor related, locus 5) and T-box transcription factor (Tbx)5, are first expressed in cardiogenic mesoderm, which is located in the anterior lateral plate mesoderm.[4] Subsequently, genes encoding cardiac-specific structural proteins are expressed in the cardiac crescent.[4] The cardiac crescent then fuses at the ventral midline to form the linear heart tube, which develops into the chambered heart after looping. In the cardiac crescent, endothelial (or endocardial) markers, such as Flk1 (fetal liver kinase-1), do not markedly overlap with cardiomyogenic markers, suggesting that these 2 lineages have already segregated at this stage. Recent studies using embryonic stem (ES) cells have suggested the presence of multipotential cardiovascular stem cells that can differentiate into cardiac, smooth muscle, and endothelial cells[5,6]; these cells have been identified as $Flk1^+$ and/or islet LIM homeobox 1 $(Isl1)^+$.[7] Intermediate cardiomyogenic progenitor cells may be bipotential, differentiating into both cardiac and smooth muscle cells.[8] Because of the paucity of known specific surface markers, however, these intermediate progenitors have been difficult to characterize. Several studies using ES cells or embryonal carcinoma cells have suggested that temporally regulated Wnt signaling is important for the specification and differentiation of cardiomyocytes.[9–12] Progeny derived from ES cells, however, are a mixture of different cell types, and additional analysis is needed to define the immediate progenitors of cardiomyocytes more precisely.

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Non-standard Abbreviations and Acronyms	
ANP	atrial natriuretic peptide
cTn	cardiac troponin
E	embryonic day
EB	embryoid body
ES	embryonic stem
Flk1	fetal liver kinase-1
GFP	green fluorescent protein
Isl1	islet LIM homeobox 1
MEC	methylcellulose
Myhc	myosin heavy chain
Mylc	myosin light chain
Nkx2.5	NK2 transcription factor related, locus 5
PDGFR	platelet-derived growth factor receptor
PrP	prion protein
SMA	smooth muscle actin
SMMyhc	smooth muscle myosin heavy chain
Tbx	T-box transcription factor

In addition to their utility in developmental studies, ES cells or induced pluripotent stem cells are a potential cellular source for cell transplantation therapy to treat damaged hearts. Several groups have shown that transplanted ES cell–derived cells can repair damaged heart tissue.[13,14] In most of the previous studies, however, the transplanted cells were not purified cardiomyocytes, and including undifferentiated ES cells was often tumorigenic. To improve the efficacy of ES cell–derived cardiomyocytes, cardiomyocytes or committed progenitors should be isolated. Moreover, because functional engraftment requires a large number of cells, scalable and reproducible preparation methods should be developed. To date, few surface markers that can be used to isolate cardiomyocytes have been reported.[15] This has hindered efforts to isolate pure cardiomyocytes.

During course of studies to identify cardiogenesis-associated genes in ES cells,[16,17] we found that that *Prnp*, which encodes cellular prion protein (PrP), was expressed in cardiomyocyte-rich embryoid bodies (EBs). In this study, we show that PrP can be used to separate the cardiomyogenic and noncardiomyogenic cellular fractions. Furthermore, we identified a bipotential, $PrP^+PDGFR\alpha^+$ cardiomyogenic population that was able to differentiate into either cardiac or smooth muscle cells depending on Wnt signaling and the culture conditions.

Methods

ht7 cells (derived from the CGR8 cell line; a kind gift from Dr H. Niwa, RIKEN Center for Developmental Biology, Kobe, Japan) and the derivative hcgp7 cell line (Nkx2.5 EGFP [enhanced green fluorescent protein] knock-in) were maintained and differentiated as described previously.[1] Briefly, we created hanging drops (500 cells/drop) of differentiation medium containing 10% fetal calf serum (FCS) and Glasgow Minimum Essential Medium (GMEM). On day 2 of differentiation, medium was added to the plates and the cells were cultured as floating embryoid bodies (EBs). To differentiate EB3 ES cells (derived from the E14tg2a cell line; a kind gift from Dr Niwa), Minimum Essential Medium Eagle Alpha Modification (αMEM) was used as the basal medium instead of GMEM. Typically, ht7 and hcgp7 cells began to beat spontaneously on day 7, whereas EB3 cells began beating on day 6. The percentages of beating EBs were approximately 100% (ht7 and hcgp7 cells) and 80% (EB3 cells).

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results

PrP Is Expressed in ES Cell–Derived Cardiomyocytes

To confirm that *Prnp* was expressed in EBs during cardiogenesis, we performed RT-PCR analysis. *Prnp* expression was upregulated by 7- to 8-fold before spontaneous beating began (Online Figure I, A). In beating EBs, the PrP^+ area partially overlapped with the area containing sarcomeric tropomyosin protein–expressing cardiomyocytes (Online Figure I, B). Although the physiological functions of PrP are not yet known,[18] PrP has been detected on the surface membranes of various cell types.[19] To examine the specificity of PrP expression at the cellular level, we used flow cytometry to examine an ES cell line in which *EGFP* was knocked into the *Nkx2.5* locus ($Nkx2.5^{GFP/+}$ ES cells)[20]; all of the green fluorescent protein $(GFP)^+$ cardiomyocytes expressed PrP on their surfaces on day 7 (Figure 1A). Although GFP has been used to isolate cardiomyocytes from beating EBs, we

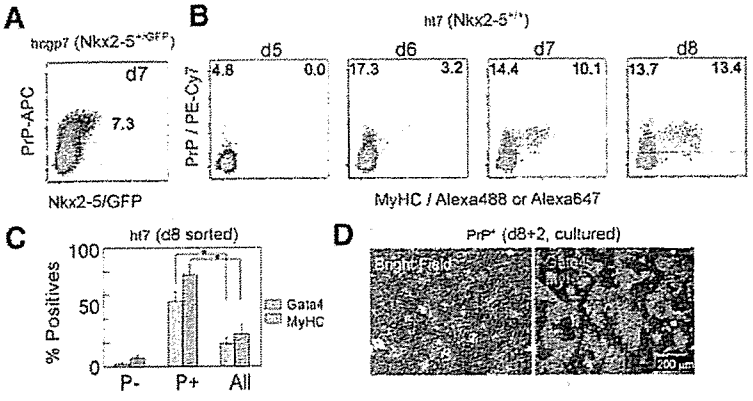

Figure 1. PrP is expressed in ES cell-derived cardiomyocytes. A, Flow cytometric analysis of PrP cell surface expression in $Nkx2.5^{GFP/+}$ ES cells (hcgp7). On day 7 of differentiation, GFP^+ cardiomyocytes were found in the PrP^+ fraction. B, Flow cytometric analysis of cell surface PrP and intracellular sarcomeric Myhc in the parental cell line of hcgp7 cells (ht7). After staining the cells with an anti-PrP antibody, the cells were fixed, permeabilized, and stained with an anti-Myhc antibody. The earliest $Myhc^+$ cells appeared on day 6 and were all PrP^+. C and D, Cardiomyocyte samples were enriched from beating EBs using the anti-PrP antibody. Cells were analyzed for Myhc and GATA-binding protein 4 (Gata4) expression immediately after cell sorting (C) or after they had been cultured on gelatin-coated plates for 2 days (D). Error bars represent the SEMs (n=3 independent samples for each group). $^*P<0.05$ from an unpaired Student *t* test.

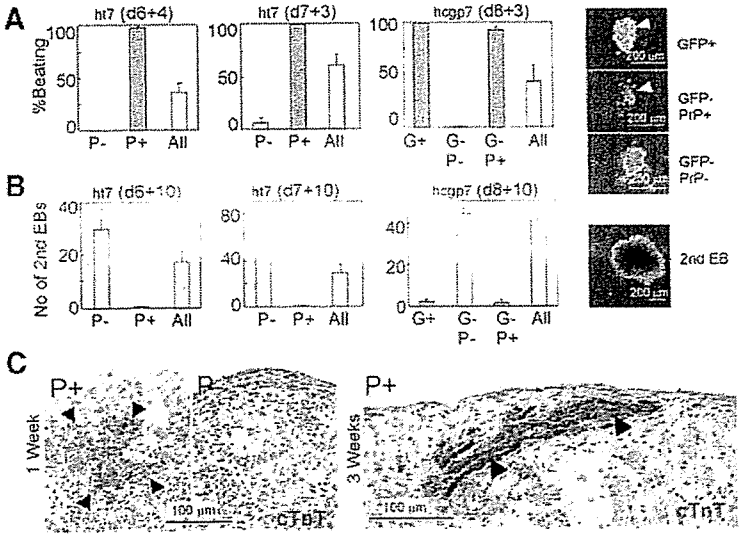

Figure 2. PrP specifically marks a cardiomyogenic population derived from ES cells. A, The cardiomyogenic potential of the PrP$^+$ fraction of post- and prebeating EBs. Cells were sorted on day 6, 7, or 8 (beating began on day 7), reaggregated, and cultured in Matrigel for 3 to 4 days. Beating foci (denoted with yellow arrowheads) were found in all reaggregates of PrP$^+$ cells. Error bars represent the standard error of the mean (n=3). B, Sorting using PrP depleted the undifferentiated cell population. Reaggregates of PrP$^+$ or PrP$^-$ cells were grown in Matrigel for 10 days. Clearly visible colonies (secondary EBs) were scored. Error bars represent the SEMs (n=3). C, Transplantation and survival of PrP$^+$ cells in vivo. Reaggregates of PrP$^+$ cells were transplanted under the kidney capsule. Arrowheads indicate cTnT$^+$ cells.

found that GFP expression was not strong enough to allow us to capture all of the Nkx2.5$^+$ cells (see next section). Therefore, we simultaneously detected the surface expression of PrP and intracellular sarcomeric proteins. On day 6 or 7, all of the sarcomeric myosin heavy chain (Myhc)$^+$ cells that were derived from the Nkx2.5$^{+/+}$ ES cell (Figure 1B) or Nkx2.5$^{GFP/+}$ ES cell population (not shown) were PrP$^+$, suggesting that the nascent cardiomyocytes were PrP$^+$. Expression of PrP on Myhc$^+$ cells gradually started to decrease on day 8. Immunofluorescence in cytospin samples (Figure 1C) and cultured cells (Figure 1D) further confirmed that PrP can be used to enrich the Myhc$^+$ and GATA-binding protein 4 (Gata4)$^+$ cell populations.

PrP Specifically Identifies a Cardiomyogenic Cell Population Derived From ES Cells

To determine whether PrP can be used to enrich not only differentiated cardiomyocytes but also cardiac progenitors, we used reaggregation assays to assess the cardiomyogenic activities of cells sorted from pre- or postbeating EBs. PrP$^+$ cells sorted at day 6 (prebeating), day 7 (onset of beating), or day 8 (beating) generated beating reaggregates, whereas PrP$^-$ cells did not (Figure 2A). In contrast, the PrP$^-$ fraction gave rise to secondary EBs in Matrigel cultures, suggesting the presence of undifferentiated cells[21] that were not produced from the PrP$^+$ fraction (Figure 2B). We also found that GFP$^-$PrP$^+$ reaggregates derived from Nkx2.5$^{GFP/+}$ ES cells had beating foci. Consistent with this result, the GFP$^-$PrP$^+$ cells expressed low levels of mRNA encoding Nkx2.5 and other cardiac transcription factors (Figure 3A). Thus, enriching the cardiomyogenic fraction using the cardiogenic marker PrP yielded better results than when enrichment was performed using Nkx2.5$^{GFP/+}$ knock-in ES cells. Importantly, even without using genetically modified ES cells, we were able to purify the cardiogenic fraction before beating began.

To test whether PrP can be used to separate transplantable cells, we transplanted reaggregates from day 6 EBs under the kidney capsules of nude mice. Immunohistochemical analysis demonstrated that cardiac troponin (cTn)T$^+$ cells were present in the PrP$^+$ cell grafts, but not in the PrP$^-$ grafts (Figure 2C). Tumor formation was frequently observed in unsorted cell grafts (data not shown) but not in PrP$^+$ grafts (41 transplanted aggregates). These results suggested that the PrP$^+$ cell population was able to generate cardiomyocytes and survive in vivo for at least 3 weeks.

PrP$^+$ Cells Differentiate From Primitive to Definitive Cardiomyocytes

Monolayer cultures of PrP$^+$ cells sorted from day 7 or day 8 EBs started to beat within 24 hours. After 5 days of culture, approximately one third of the cells spontaneously beat (data not shown). Single-cell recordings of membrane potentials from 16 of 18 quiescent cells revealed action-potential profiles similar to those of working (atrial or ventricular) cardiomyocytes (Figure 3A). The rest of the cells displayed sinus nodal cell-like spontaneous beating activity. Pharmacological studies using multielectrode arrays indicated that the cultured PrP$^+$ cells were responsive to isoproterenol and carbamylcholine, suggesting the presence of functional β adrenergic receptors and sinoatrial cardiomyocytes, respectively (Figure 3B). Immunofluorescence indicated that PrP$^+$ cells differentiated into cTnI$^+$ (a definitive marker of cardiomyocytes) cells. These were either myosin light chain 2v (Mylc2v)$^+$ or Mylc2v$^-$ (Figure 3C). A few atrial natriuretic peptide (ANP)$^+$ cells were also detected.

To examine the cell types quantitatively, we performed flow cytometry with atria and ventricles from mouse embryos as reference samples (Online Figure I, E). Among the atrial and ventricular cells from embryonic day (E)13.5 embryonic heart, 25% and 2% to 4% were ANP$^+$, respectively. Myosin light chain 2a (Mylc2a) was detected in both the atrial and ventricular cells, although higher levels were observed in atrial cells. Thus, neither ANP nor Mylc2a can be used to distinguish atrial cells from ventricular cells. In contrast, all embryonic ventricular cells were Mylc2v$^+$, whereas all atrial cells were Mylc2v$^-$. Approximately 50% of cultured PrP$^+$ cells were Mylc2v$^+$, although the expression level varied between cells (Figure 3D). Consistent with the immunofluo-

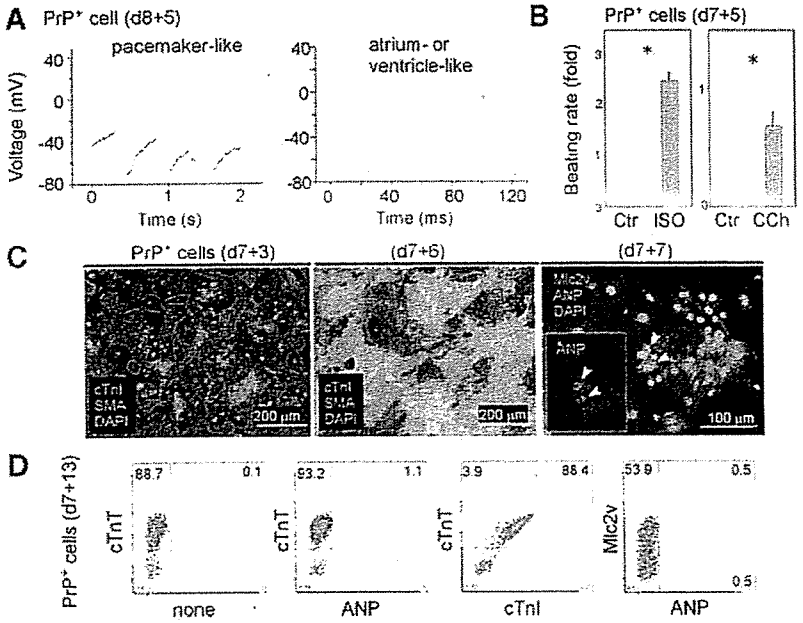

Figure 3. PrP$^+$ cells differentiate into cTnI$^+$ definitive cardiomyocytes with an atrial or a ventricular identity. A, Traces of the membrane potential recorded from a single PrP$^+$ cell. PrP$^+$ cells sorted on day 8 were cultured for 5 days. Approximately, one third of the cells spontaneously beat (data not shown). Sixteen of 18 quiescent cells exhibited a working myocardium-type (atrial or ventricle) action potential, whereas the rest exhibited a pacemaker-type action potential. Action potentials were elicited at 1 Hz. B, Pharmacological analysis of PrP$^+$ cells. PrP$^+$ cell aggregates were analyzed using multielectrode arrays and isoproterenol (ISO) (1 μmol/L) or carbamylcholine (CCh) (10 μmol/L). All aggregates were sensitive to CCh, suggesting that they contained atrial cells. Error bars represent the SE (n=9 independent samples for each group). *P<0.05 from a paired t test. C, Immunofluorescence analysis for cTnI (a definitive cardiomyocyte marker), Mylc2v (a ventricle marker), and ANP (a chamber myocardium marker). PrP$^+$ cells sorted on day 7 were cultured on gelatin-coated dishes for 1 week. The number of cTnI$^+$ cells increased during the culture. Mylc2v$^+$ cells were frequently observed, whereas the percentage of ANP$^+$ cells was low (1% to 2%). Arrows indicate cells that are ANP$^+$MLC2v$^+$, a profile characteristic of left ventricular cardiomyocytes. D, Flow cytometric analysis of PrP$^+$ cell cultures. Sorting on day 7 revealed that the cardiomyocytes were cTnT$^+$ but not cTnI$^+$ (data note shown). After culturing the cells for 13 days, 90% to 95% expressed both cTnT and cTnI, 50% to 60% expressed Mylc2v, and 1% expressed ANP. Control experiments using embryonic heart and PrP$^-$ fractions are shown in Online Figure III.

rescence results, only 1% to 2% of the cells were ANP$^+$. The PrP$^+$ cell fraction initially contained cells expressing Myhc and cThT but not cTnI (data not shown).[20,22] Interestingly, after 10 to 13 days of culture, 90% of the cells differentiated into cTnI$^+$ cardiomyocytes. Collectively, these results suggested that PrP$^+$ cells differentiated from cTnI$^-$ to cTnI$^+$ cardiomyocytes with an atrial or a ventricular identity.

The PrP$^+$PDGFRα$^+$ Population Contains Myhc$^-$ Cardiomyogenic Progenitors

Although PrP expression was first detected at day 5, we were unable to enrich the cardiomyogenic population using this marker until day 6. To determine whether combination with other surface markers would allow better enrichment of the progenitor population at day 5, we examined Flk1, c-kit, and platelet-derived growth factor receptor (PDGFR)α as secondary surface markers. Flk1 is a marker of cardiovascular progenitors: common progenitors for cardiac, smooth muscle, and endocardial cells.[5] PrP$^+$ cells expressed a very low level of Flk1 on day 5 and even less on day 6 (Online Figure IV, A), suggesting that PrP$^+$ cells may have already diverged from the vascular lineage. c-kit, which is reported to be a cardiovascular stem cell marker in adult and embryonic heart,[8] was not expressed on PrP$^+$ cells. PDGFRα is widely expressed in mesoderm, including the cardiac lineage, although its expression is gradually downregulated in heart tube (elsewhere[23] and Online Figure VI). PrP expression was first observed on day 5 on PDGFRα$^+$ and PDGFRα$^-$ cells (Figure 4A). Molecular analysis indicated that the PrP$^+$PDGFRα$^+$ cell fraction from day 5 EBs expressed cardiac marker genes, including *Nkx2.5*, *Tbx5*, and *Isl1*, whereas the PrP$^+$PDGFRα$^-$ cell fraction did not (Figure 4B and 4C). Consistent with these data, only the PrP$^+$PDGFRα$^+$ cell fraction gave rise to beating reaggregates (Online Figure IV, D). These results suggested that double-positive PrP$^+$PDGFRα$^+$ cells, hereafter referred to as PRa cells, are the earliest known cardiomyogenic population.

PRa Cells Differentiate Into Cardiac Cells and Smooth Muscle Cells

To test the cardiomyogenic potential of PRa cells, we used PRa cells sorted from day 5.5 EBs, at which point Myhc was not expressed (data not shown), and directly differentiated the cells as 3D reaggregates (3D cultures) or as sparsely cultured 2D monolayers (2D cultures). The reaggregates developed into beating cardiomyocytes, whereas the monolayer cultures did not (Online Figure V, D). Reaggregate cultures expressed such cardiac-specific genes as *Myl7* (encoding Mylc2a), *Myh6* (encoding myosin heavy chain α), and *Tnni3* (encoding cTnI) (Figure 5A). In contrast, smooth muscle genes, including *Tagln* (encoding SM22α), *Acta2* (encoding smooth muscle actin, SMA), and *Myh11* (encoding smooth muscle myosin heavy chain [SMMyhc]), were expressed in the monolayer cultures. Interestingly, adding Wnt3a (wingless-related MMTV integration site 3A) to the reaggregates inhibited cardiac differentiation (Figure 3C). Wnt, however, did not increase the levels of smooth muscle markers, suggesting that it did not drive the progenitors to a smooth muscle fate. Immunostaining experiments revealed that aggregates expressed the cardiac muscle proteins Myhc and cTnI, whereas the

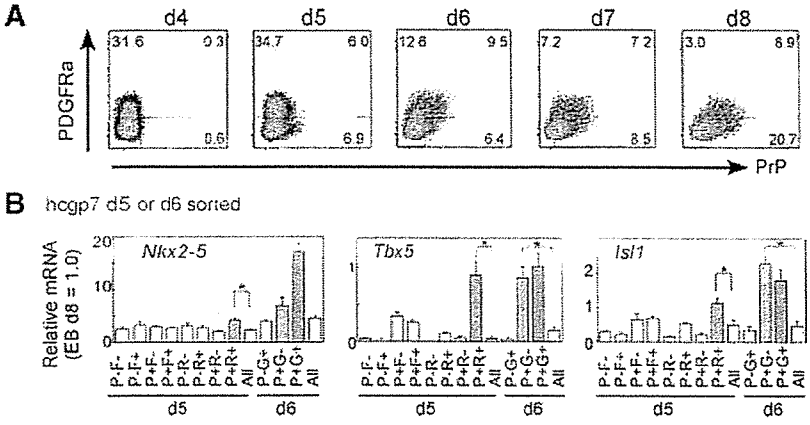

Figure 4. The PrP$^+$PDGFRα^+ (PRa) population contains bipotential cardiomyogenic progenitors that can differentiate into cardiomyocytes or smooth muscle cells. A, Flow cytometric analysis of EBs using PrP- and PDGFRα-specific antibodies. PrP$^+$ cells detected on day 5 were PDGFRα^+ or PGDFRα^-. The expression profiles of PDGFRα and PrP overlapped on day 6 and then gradually receded as differentiation proceeded. B, RT-PCR analysis of hcgp7-derived cells sorted using PrP (P), PDGFRα (R), Flk1 (F), and GFP (G). PrP$^+$ cells on day 5 were PDGFRα^+ or PDGFRα^- and Flk1$^{Low/-}$ (see Online Figure II). Compared with the unsorted fraction, Nkx2.5, Tbx5, and Isl1 were expressed at significantly higher levels in the P$^+$R$^+$ fraction (pink bars) and P$^+$G$^-$ fraction (blue) on day 5, as well as in the P$^+$G$^+$ fraction (green) on day 6. Error bars represent the SEM (n=3 independent samples for each group). *P<0.05 from an unpaired Student t test. Other marker genes are shown in Online Figure II.

monolayer cells expressed such smooth muscle proteins as SMA, SM22α, and calponin (Figure 5B). SMMyhc, a very specific marker of smooth muscle cells,[8] was also detected in the monolayer cells. These results suggested that the PRa cell fraction contained 2 different lineages and/or bipotential progenitors. To examine the latter possibility, we next cultured single cells isolated from days 5 to 5.5 EBs in methylcellulose (MEC). Typically, ≈100 colonies

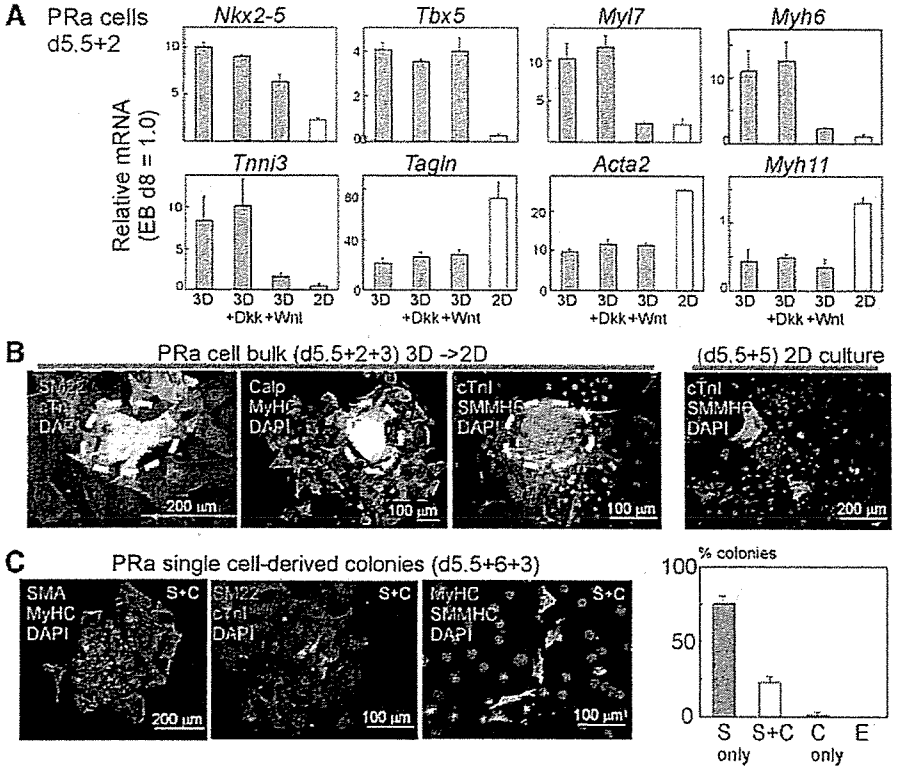

Figure 5. Developmental potential of PRa cells. A, Differentiation of reaggregated cultures of PRa cells. PRa cells sorted on day 5.5, at which point the cells were still negative for Myhc (Online Fig V), were allowed to differentiate as reaggregates (3D) or sparse monolayers (2D) with or without Wnt or the Wnt inhibitor Dkk1 (dickkopf homolog 1). After 2 days of culture, cardiac (*Myl7*, *Myh6*, *Tnni3*) and smooth muscle (*Tagln*, *Acta2*, *Myh11*) markers were analyzed in RT-PCRs. Error bars represent the SEMs (n=3 independent samples for each group). B, Immunostaining of 3D or 2D cultures of PRa cells for cardiac and smooth muscle marker proteins. PRa cells sorted on day 5.5 were aggregated in an ultralow binding plate for 2 days and then transferred to a gelatin plate for 3 days (3D→2D) or were cultured on only a gelatin plate for 5 days (2D). Beating aggregates (marked with the dotted lines) strongly expressed the cardiac proteins Myhc and cTnI. Monolayer cells expressed a number of smooth muscle cell markers, including SMA, SM22, calponin, and SMMyhc. C, Expansion and differentiation of single PRa cells. PRa cells sorted on day 5.5 were expanded in MEC at clonal density for 6 days and colonies derived from single cells were transferred to gelatin-coated multiwell plates for immunostaining. Colonies composed of SMA$^+$Myhc$^-$ smooth muscle cells only (S only), SMA$^+$Myhc$^-$ smooth muscle cells and SMA$^+$Myhc$^+$ cardiac muscle cells (S+C), Myhc$^+$SMA$^+$ cardiac muscle cells only (C only), or platelet/endothelial cell adhesion molecule-positive endothelial cells (E) were counted. Error bars represent the SEMs (n=5 independent samples for each group).

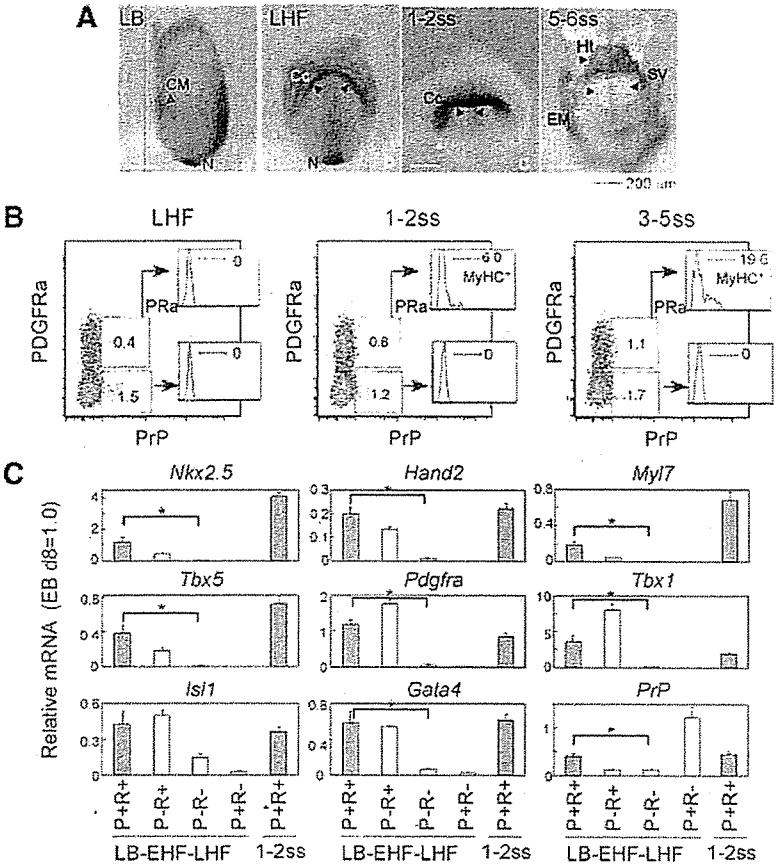

Figure 6. PrP is expressed before Myhc in mouse embryonic cardiac mesoderm. A, Whole-mount in situ hybridization analysis for *Prnp* mRNA (a through c) and immunohistochemical analysis for PrP protein (d). Note that *Prnp* mRNA was expressed in the cardiac mesoderm (CM) beginning at the late-bud stage (LB) and expression was maintained in the cardiac crescent (Cc) and heart tube (Ht) at the late headfold stage (LHF), 1 to 2 somite stage (1 to 2ss), and 3 to 4 somite stage (not shown). PrP was also expressed in the node (N). PrP protein was detected in the cardiac crescent at the late headfold stage (see Online Fig. VI). At the 5- to 6-somite stage, expression was also observed in the sinus venosus (Sv). Nonspecific staining was detected in extraembryonic membrane (EM). B, Flow cytometric analysis of surface and intracellular proteins. After staining the cell surfaces with anti-PrP and anti-PDGFRα antibodies, cells were fixed and permeabilized. Myhc$^+$ cells were first detected in 1- to 2-somite stage embryos and were exclusively observed in the PRa fraction. C, RT-PCR analysis of sorted cells from pools of late-bud to late headfold embryos (LB-EHF-LHF) or 1 to 2 somite embryos (1 to 2 ss). Note that the PRa cell fraction from presomite stages, before Myhc was expressed, was specifically enriched with cardiac markers. Error bars represent the SEMs (n=3 independent samples for each group).

were created from 5000 single-cell isolates (Online Figure III, C). Colony formation was inhibited by the addition of the Wnt inhibitor Dkk1 (dickkopf homolog 1) and promoted by Wnt3a or a glycogen synthase kinase-3β inhibitor, suggesting that the proliferation of PRa cells was dependent on canonical Wnt signaling. Individual colony analysis indicated that approximately 25% of Myhc$^-$ PRa cell–derived colonies expressed both smooth and cardiac muscle proteins (Figure 5C), whereas the rest of the colonies expressed only smooth muscle proteins. Endothelial cell differentiation, assessed based on platelet/endothelial cell adhesion molecule expression, was not observed. These results strongly suggested that the Myhc$^-$ PRa cells contained bipotential (cardiac and smooth muscle) progenitor cells.

Expression of PrP in Mouse Embryos

We next examined the spatial and temporal profiles of PRa cells in embryos. Transgenic mouse studies using β-galactosidase suggested that *Prnp* is expressed in embryonic heart at E8.5.[24] Endogenous expression of PrP mRNA and protein during embryonic early stages (E7 to E7.5) has not been adequately elucidated, however.[25,26] To examine the expression of PrP in early mouse embryos, we performed whole-mount in situ hybridizations and immunohistochemical analysis. During early developmental stages, we observed specific expression of *Prnp* mRNA in the cardiac mesoderm during late-bud stage (Figure 6A). We also detected PrP protein in the cardiac crescent at headfold stages and in the heart tube at somite stages (Online Figure VI). On the other hand, PDGFRα expression was observed widely in the mesoderm, including cardiac mesoderm, of late-bud to 1 to 2 somite stage embryos (Online Figure VI). PDGFRα expression gradually decreased in cardiomyocytes after they reorganized into the heart tube.

We next analyzed PrP expression in mouse embryos using flow cytometry. As expected from the immunohistochemical analysis, PrP$^+$ cells were either PRa cells or PrP$^+$PDGFRα$^-$ cells from the E7 pool (primarily consisting of presomite stage mouse embryos) and the E8 pool (primarily consisting of somite stage mouse embryos) (Online Figure VII, A). As observed in ES cell–derived cells, little overlap between PrP and Flk1 expression was detected at these stages. Sorting using PrP clearly enriched the cardiac marker–expressing cells from the E8 pool (Online Figure VII, B), suggesting that PrP was a specific marker of cardiomyocytes at these stages. To determine whether the PRa population contained both Myhc$^+$ cardiomyocytes and Myhc$^-$ progenitor cells, flow cytometric analysis was performed with embryos at different developmental stages. Myhc$^+$ cells were detected at 1 to 2 somite stages but not at presomite stages. The nascent Myhc$^+$ cells were exclusively identified in the PRa cell

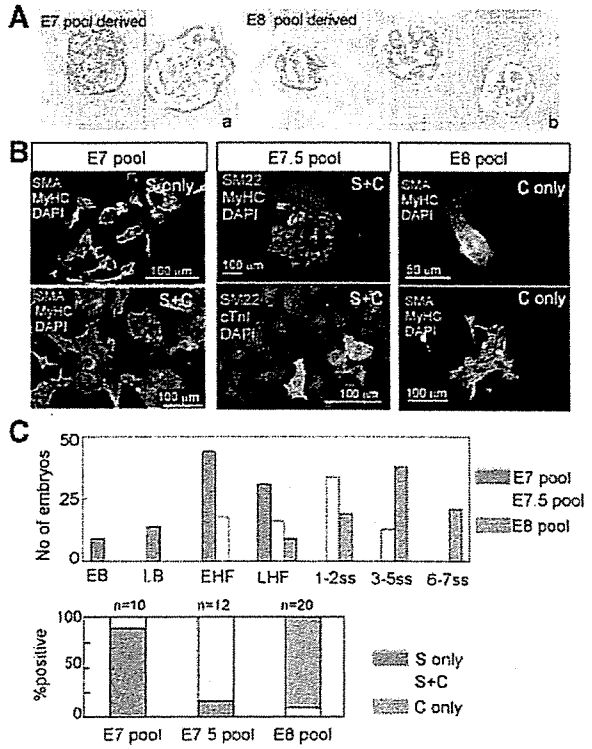

Figure 7. Analysis of single PRa cell–derived colonies from mouse embryos. A, Schematic of the experiment. B, Representative colonies from MEC cultures of E7 pool (a) and E8 pool (b) embryos. Individual colonies from MEC cultures derived from E7 pool, E7.5 pool, or E8 pool embryos were transferred to 96-well plates for immunofluorescence analysis. C, Percentages of the colony types based on immunofluorescence analysis. Embryonic stages in each pool used (top) and colony types derived from each pool (bottom) are shown. The numbers of colonies composed of SMA^+ cells (S only), SMA^+Myhc^+ cells and SMA^+ cells (S+C), or SMA^+Myhc^+ cells (C only) were counted. Eighteen of 20 colonies from the E8 pool contained only cardiomyocytes (C only) and exhibited spontaneous beating.

fraction (Figure 6B). PRa cells sorted from presomite stage embryos (a pool of late-bud to headfold stage embryos) expressed mRNA encoding Nkx2.5, Isl1, Tbx5, and other cardiac transcription factors (Figure 6C). $PrP^+PDGFR\alpha^-$ cells, probably from such extracardiac regions as node, did not express cardiac genes. These results suggested that the embryonic PRa population, similar to ES cell–derived PRa cells, contained $Myhc^+$ nascent cardiomyocytes as well as $Myhc^-$ cardiomyogenic progenitors.

Commitment Status of PRa Cells Derived From Mouse Embryos

We then cultured PRa cells from mouse embryos. Similar to cells from EBs, PRa cells from embryos formed colonies in MEC cultures (Figure 7A). Immunostaining analysis revealed that one of ten colonies derived from an E7 pool contained $Myhc^+$ cells (Figure 7B–C). The other colonies expressed the smooth muscle proteins SMA, SM22, and SMMyhc. PRa cells from the E7.5 pool frequently generated $Mhyc^+$ cardiomyocyte–containing colonies (10 of 12). These samples also contained smooth muscle colonies, suggesting from the presence of bipotential progenitors. PRa cells from the E8 pool generated small beating cardiac colonies. Such beating colonies exclusively contained cardiomyocytes, and not smooth muscle cells, suggesting that the isolated cells were committed to the cardiomyogenic lineage. These results suggested that PRa cells isolated from the cardiac mesoderm could differentiate into either cardiac or smooth muscle cells. Thus, PRa cells from mouse embryos and EBs may share common differentiation and proliferation potentials.

Discussion

In this study, we have demonstrated that the surface marker PrP can be used to enrich cardiomyocytes derived from ES cells. Several groups have examined methods to enrich cardiomyocytes using gravity or introduction of a selectable marker. Surface markers that allow cardiomyocytes to be selected directly, however, have not been extensively elucidated. Importantly, up to 90% of cells derived from PrP^+ cell cultures expressed cTnI (a marker for definitive cardiomyocytes). The other cells expressed SMA, suggesting that the cultures contained at least 2 different lineages. To improve the purity of the population, other markers or directional differentiation methods should be combined with PrP expression. We also showed that the cultured PrP^+ cells differentiated into both atrial and ventricle cardiomyocytes. Hcn4 protein, a specific marker for sinus node, was barely detectable. Because spontaneous pacemaker-like activity was detected in immature atrial or ventricular cardiomyocytes, PrP^+ cells may not have differentiated into mature sinus node cells, at least under our culture conditions. Cells expressing ANP, a marker of chamber myocardium,[27] were not found in significant numbers in the PrP^+ cell cultures, suggesting that the PrP^+ cells had not differentiated into chamber myocardium cells. Consistent with this idea, flow cytometric analysis of the expression patterns of Mylc2v and ANP produced results that were more similar to those observed for E9.5 heart than for E13.5 heart.

At earlier stages, PrP did not define a cardiomyogenic population. The $PrP^+PFGFR\alpha^+$ fraction (PRa cells) contained nascent cardiomyocytes and their progenitors. It would be interesting to understand the relationship between PRa cells and previously described cardiovascular progenitor/stem cells. Because PRa cells did not markedly express Flk1, they may have segregated from the endothelial lineage. Consistent with this idea, we did not observe endothelial cell differentiation from PRa cell–derived cells even in the OP9 culture system, which efficiently supported endothelial cell differentiation (Online Figure V). The proliferation and differentiation of PRa cells are regulated positively and negatively by Wnt signals, respectively. The presence of $Isl1^+$ cells in the PRa cell population (Online Figure IV, E) indicates that PRa cells represent an intermediate progenitor, located between $Isl1^+$ multipotential cells and committed cardiomyocytes. Other groups have described bipotential progenitors, such as $c\text{-}kit^+Nkx2.5^+$ cells and $Tbx18^+$ epicardial cells.[28,29] Because PRa cells did not display surface expression of c-kit,

they are likely not related to c-kit$^+$Nkx2.5$^+$ cells. Interestingly, we observed Tbx18 expression in PRa cell–derived 2D cultures but not in 3D cultures (data not shown). Recent studies showed that the Nkx2.5$^+$ lineage also diverges into Tbx18$^+$ epicardial cells.[30] Thus, it would be intriguing to know if PRa cells can differentiate into epicardial cells.

Embryonic localization in the cardiac crescent and their limited differentiation potential strongly suggest that PRa cells include progenitors from the primary heart field. Consistent with this idea, PRa cells sorted from presomite-stage embryos expressed Tbx5. On the other hand, PRa cells also include progenitors from the secondary heart field, because they also expressed Tbx1 and Isl1. Flow cytometric analysis further revealed that PRa cells were present in the outflow tract segment, which is derived from the secondary lineage (Online Figure VII, C). As in the early somite stages, outflow-derived PRa cells included Myhc$^+$ cells. Thus, PRa can serve as a pan-marker for cardiomyogenic progenitors independent of the heart field lineage. Interestingly, compared with embryo-derived PRa cells, EB-derived PRa cells expressed lower levels of Tbx1. This suggests that, under our culture conditions, primary heart lineage progenitors preferentially developed. However, it should be determined whether or not EB-derived PRa cells differentiated into outflow cardiomyocytes.

Based on molecular studies and in vitro cultures of isolated cells, we propose that PRa cells include a cardiomyogenic population, which can differentiate into cardiac or smooth muscle cells (Online Figure VIII). Considering their limited differentiation potential, PRa cells may be downstream of Flk1$^+$ cells, separate from the endothelial lineage. Immediate early progenitors may be in the Flk1$^+$PDGFRα^+ fraction, because this transient cell faction (observed on day 5) specifically expressed *Mesp1* (data not shown). Multipotent or pluripotent stem cells, including induced pluripotent stem cells, are expected to be a powerful tool for transplantation therapy and drug screening. Testing the ability of ES cell–derived cardiomyocytes to rescue in vivo heart function has been hampered by a lack of markers that allow scalable purification of the population without recourse to the genetic manipulations required to insert lineage- or stage-specific selectable markers. The mouse cell surface marker PrP defines a cardiogenic population in differentiating ES cells, enabling efficient isolation and enrichment of ES cell–derived cardiomyocytes.

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Disclosures

None.

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Supplement Material

Materials and Methods

Flow cytometry.
Embryos and 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 allophycocyanin (APC)-, phycoerythrin (PE)-, or biotin-conjugated anti-PrP (SPI-Bio, mouse monoclonal, clone SAF83), anti-Flk1 (BD Bioscience, rat monoclonal, clone Avas 12α1), or anti-PDGFRα (eBioscience, rat monoclonal, clone APA5) antibodies. Biotin-conjugated antibodies were further stained with PE-Cy7-conjugated streptavidin. To detect surface and intracellular antigens simultaneously, live cells were first stained for surface antigens, and then fixed and permeabilized using Foxp3 staining buffer (eBioscience). Primary antibodies for intracellular antigens were detected with AlexaFluor 488- or AlexaFluor 647-conjugated anti-mouse or anti-rabbit IgG antibodies (Invitrogen). Analyses were carried out using a FACSCanto flow cytometer (Beckton Dickinson, San Jose, CA). Cell sorting was performed using a FACSAria cell sorter (Beckton Dickinson). Flow cytometric data were analyzed using FlowJo software (Treestar, San Carlos, CA).

Cultures of sorted cells.
To examine the cardiac differentiation of PrP^+ cells, sorted cells were plated at 5×10^4 cells/cm^2 in gelatin-coated plates containing GMEM medium supplemented with 10% FCS. To examine the cardiomyogenic potential of PRa cells, sorted cells were plated at 1×10^3 cells/well in low-cluster 96-well plates (Ultra Low Attachment, U-bottom, Costar 7007) to promote the formation of reaggregates or in gelatin-coated 96-well plates to promote the formation of monolayers. For long-term cultures (> Day 10), reaggregates were embedded and cultured in Matrigel (BD Bioscience). When indicated, Wnt3a (R&D Systems, 100 ng/ml) or Dkk1 (R&D Systems, 150 ng/ml) was added to the reaggregates. To form single cell–derived colonies, sorted cells were plated at a clonal density (500 cells/cm^2) in methylcellulose (MEC, R&D Systems) as described previously,[2] although DT4 conditioned medium was not added to the samples. Instead of 150 ng/ml Dkk1, 100 ng/ml Wnt3a, or 2.5 μM (2′Z,3′E)-6-bromoindirubin-3′-oxime (BIO, GSK3β inhibitor IX, Calbiochem) was added to the samples. Six days after plating, the colonies that formed in the MEC were individually transferred onto gelatin-coated 96-well plates and further cultured for immunofluorescence analysis.

RT-PCR.
RNA was extracted from different samples using Trizol Reagent (Invitrogen). cDNA was generated with SuperScript III (Invitrogen). Real-time PCRs were performed with SYBR Green ER qPCR SuperMix (Invitrogen) and the products were analyzed using an ABI PRISM 7900 sequence detection system (Applied Biosystems). Levels of GAPDH mRNA were determined using rodent GAPDH control reagents (Applied Biosystems) and used to normalize the cDNA levels of other genes. Levels of expression relative to uterus (for *Myh11*), Day-4 EBs (*Mesp1* and *T*), or Day-8 EBs (for the rest of the genes) are shown. The primers are listed in Online Table I. RT-PCR data with error bars in the figures are from at least three independent experiments, and are not a result of replicates of the PCRs.

Immunohistochemistry and *in situ* hybridization.
Immunohistochemistry and immunofluorescence analyses were carried out with the antibodies listed in Online Table II. To detect PrP in fixed samples, clone SAF32 was used instead of SAF83. Embryos or cultured cells were fixed in 4% paraformaldehyde at 4°C. Unlabeled primary antibodies were detected with secondary antibodies conjugated to AlexaFluor 488, AlexaFluor 546, or horseradish peroxidase (Nichirei). APC-conjugated anti-PECAM or Cy3-conjugated anti-SMA antibodies were also used. Whole-mount mouse immunohistochemistry was performed according to protocols from Dr. Andras Nagy's laboratory (http://www.mshri.on.ca/nagy/). Whole-mount *in situ* hybridization was performed as described previously.[3]

Patch clamp.
Membrane potentials were recorded in P-positive cells on a cover slip using the patch clamp technique in

the whole-cell configuration and an amplifier (Axopatch-1D, Axon Instruments, Burlingame, CA). The bath temperature was maintained at 37°C and cells were perfused with normal Tyrode's solution (143 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.25 mM $MgCl_2$, 0.25 mM NaH_2PO_4, 5 mM HEPES, and 5 mM glucose at pH 7.4). The pipette solution contained 80 mM KCl, 60 mM KOH, 40 mM aspartate, 5 mM HEPES, 10 mM EGTA, 5 mM MgATP, 5 mM sodium creatine phosphate, and 0.65 mM $CaCl_2$ (pH 7.3). Action potentials were elicited at 1.0 Hz. Data were analyzed using pClamp software (Clampfit, Axon Instruments).

Transplantation of sorted cells.
Reaggregates of PRa cells were labeled with DiI, mixed with Matrigel and injected under the kidney capsules of nude mice using a glass capillary tube. One week following transplantation, DiI-labeled cells were detected. Three weeks following transplantation, the kidneys were fixed overnight in 4% paraformaldehyde at 4°C and embedded in Optimal Cutting Temperature compound. After cryosectioning (7 μm), cardiomyocytes were detected with anti-cardiac troponin T antibodies.

Figure legends
Online Fig. I. A, RT-PCR analysis of EBs. Expression of PrP was observed 2 days before spontaneous beating began. Arrows indicate the onset of spontaneous beating. Representative results from two or three independent experiments are shown. **B**, Immunodetection of PrP protein in ES cell-derived cells. Note that most of the area that was positive for tropomyosin (green) also contained PrP (red).

Online Fig. II. A, Experiments using EB3 ES cells, which show different differentiation kinetics than those of ht7 cells (see Online Fig. IA). (a) Cytospin analysis of $Myhc^+$ cells after sorting on Day 7 (1 day after beating began). The PrP^+ cell fraction contained $Myhc^+$ cells, whereas the PrP^- cell fraction did not. (b) The PrP^+ cell fraction sorted on Day 5 (1 day before beating began) gave rise to beating reaggregates, whereas the PrP^- fraction did not. (c) Additional culturing of the PrP^- cell fraction produced secondary EB-like cells, suggesting the presence of undifferentiated cells. Values are the means ± SE from three independent experiments. **B**, RT-PCR analysis of hcgp7 cells ($Nkx2.5^{GFP/+}$ ES cell). Cells were sorted on Day 8 using PrP (P) and GFP (G). The GFP^-PrP^+ (G-P+) cell fraction expressed cardiac markers. Values are means ± SE from five independent experiments.

Online Fig. III. A, Flow cytometry analysis of intracellular proteins in embryonic heart–derived cells. Whole hearts dissected from E9.5 embryos, and atria and ventricles dissected from E13.5 embryos were dissociated using collagenase and dissociation buffer (Invitrogen). Cells were fixed, permeabilized, and stained with anti-cTnT, anti-cTnI, anti-Mylc2v, anti-Mylc2a, and anti-ANP antibodies. Note that ventricular cardiomyocytes were $Mylc2v^+$ and atrial cells were $Mylc2v^-$, suggesting that Mylc2v can be used to distinguish ventricular cells from atrial cells. ANP, a chamber myocardium marker, is preferentially expressed in atrial cardiomyocytes; the percentage of positive cells was similar to that observed in the immunofluorescence analysis of cultured cells (approximately 20%; data not shown). Mylc2a was expressed in both atria and ventricles, although higher expression levels were observed in atria. **B**, Flow cytometry analysis of PrP^- and PrP^+ cell cultures. Cells were sorted on Day 7 and cultured on gelatin-coated plates for 10 days. Note that PrP^- cells did not markedly differentiate into $cTnT^+$ cardiomyocytes. In PrP^+ cell cultures, $cTnT^-$ cells were SMA^+, suggesting that the noncardiomyocytes in the cultures were smooth muscle cells.

Online Fig. IV. A, Flow cytometry analysis of cell fractions sorted using PrP (P), PDGFRα (R), and Flk1 (F). **B**, RT-PCR analysis of cell fractions. Relative mRNA levels are shown (Day-4 EB = 1.0 for T and Mesp1; Day-8 EB = 1.0 for the rest of the genes). Expression levels of Nkx2.5, Tbx5, Isl1 (see Fig 3), Hand2, and Gata4 were enriched in the $PrP^+PDGFR\alpha^+$ (PRa) fraction (pink bars) on Day 5, whereas cardiac contractile protein markers were not expressed. Kdr (also known as Flk1), T (primitive streak marker), Mesp1 (mesoderm marker), Gata1 (hematopoietic cell marker), Tbx1 (second heart field marker), and Nfatc1 (endocardial cell marker) were not expressed in the PRa cell fraction. The P^-R^+ fraction may contain progenitors that are earlier in the developmental process than PRa cells, because Mesp1 expression was detected in this fraction. Error bars represent the standard errors of the mean (n = 3). **C**, The percentage of beating reaggregates of sorted cells. Cells were sorted on Day 5 and plated

in a 96-well ultra-low binding plate. Wells containing beating aggregates were counted. The PRa cell fraction frequently gave rise to beating aggregates. Error bars represent the standard errors of the mean (n = 5).

Online Fig. V. A, Schematic diagram of the culture methods for PRa cells. Sorted PRa cells were plated on OP9 stromal cells or in methylcellulose (MEC) at a clonal density to obtain colonies derived from single cells. Colonies grown in MEC were picked on Day 6 and plated on gelatin-coated plates. To differentiate cells directly, cells were cultured on gelatin-coated plates (2D cultures) or after reaggregation in ultra-low binding multiwell plates (3D cultures). **B**, Analysis of the ability of the OP9 or MEC culture system to support the undifferentiated state. PRa or P^-R^+ cell fractions were sorted and cultured on OP9 cells (b, c, d) or in MEC (a, e, f, g, h) for 8-9 days. After cell dissociation, cell surface and intracellular markers were analyzed using flow cytometry. We found that OP9 cells expressed PDGFRα (data not shown) and could be distinguished from ES cell–derived cells (pink open circles in b and c; the $PDGFR\alpha^+$ fraction was gated out in d). Whereas OP9 cells induced the differentiation of both endothelial ($CD31^+$, b) and smooth muscle cells (SMA^+, d), MEC did not (a, c, e), suggesting that MEC supported the undifferentiated state. When MEC colonies were cultured on gelatin-coated plates for 3 days (MEC → 2D), they started to differentiate into smooth muscle cells and cardiomyocytes (h). In contrast to P^-R^+ cells, the PRa cell fraction did not give rise to endothelial cells (d, g), suggesting that the PRa cell fraction did not contain multipotent cardiovascular progenitors. **C**, Colony formation from single PRa cells in MEC cultures depended on Wnt signaling. The colonies derived from Day-5.5 PRa cells were counted. **D**, Differentiation of Day-5.5 PRa cells after 2D or 3D cultures. Bars represent standard errors of the mean from three independent experiments.

Online Fig. VI. A, Immunohistochemical analysis of PrP protein. PrP was detected in cardiac crescent (Cc, black arrowhead) and node (N, yellow arrowhead) at the late headfold stage (LHF). Expression of PrP persisted in heart tube (Ht) from E8.5 to E10.5. PrP expression was detected in ganglia at E9.5 (yellow arrowheads). Background staining was observed in extraembryonic mesoderm (EM). **B**, Immunohistochemical analysis of PDGFRα. PDGFRα expression was observed in mesodermal tissues, including cardiac mesoderm (CM) at the early headfold stage (EHF) and in cardiac crescent (Cc) at E8.0. At E8.25, PDGFRα was expressed in the sinus venosus (SV, black arrowheads), whereas expression levels were downregulated in the heart tube (white arrowhead). **C**, Immunofluorescence analysis of PrP and PDGFRα. Overlapping expression was observed in cardiac crescent (Cc) at the late headfold stage (LHF) and in heart tube (Ht) at the 6-7 somite stage (6-7ss). **D**, *In situ* hybridization analysis of cardiac transcription factor mRNA at the early headfold stage. *Nkx2.5*, *Tbx5*, and *Isl1* mRNA was detected cardiac mesoderm (CM).

Online Fig. VII. A, Flow cytometry analysis of surface proteins on mouse embryo–derived cells. Mouse embryos were dissected and the extraembryonic region was removed. E8 pools (containing embryos from LHF to 7ss) were stained with antibodies specific for PrP, PDGFRα, and Flk1. **B**, RT-PCR analysis of sorted cells from E8 pool embryos. Note that the PrP^+ fraction was specifically enriched for cardiac markers. Representative results from three independent experiments are shown. **C**, Flow cytometry analysis of PrP, PDGFRα, and Myhc in the outflow tract (OFT) segment derived from E9.5 embryos. The OFT was dissected, dissociated, and analyzed using flow cytometry. Overlapping expression of PrP and PDGFRα was observed. Intracellular staining revealed that PRa cells included $Myhc^+$ cells.

Online Fig. VIII. Potential and committed cardiac progenitor cells based on ES cell studies. The earliest cardiomyogenic population was the PRa cells. PRa cells include $Myhc^+$ nascent cardiomyocytes as well as $Myhc^-$ bipotential progenitors that can differentiate into cardiac or smooth muscle cells. PrP can also be used to isolate nascent cardiomyocytes that differentiate into atrial or ventricular cardiomyocytes.

Online Table I. Primers used for real-time RT-PCRs

	Gene Symbol	Sense primers (5'→3')	Antisense primers (5'→3')
Early mesoderm	*T*	TCCCGAGACCCAGTTCATAG	GGTCGTTTCTTTCTTTGGCA
	Mesp1	GCGACATGCTGGCTCTTCTA	TGGTATCACTGCCGCCTCTTCC
	Kdr (Flk1)	GGCGGTGGTGACAGTATCTT	CTCGGTGATGTACACGATGC
	Pdgfra	CAACCACACTCAGACGGATG	CTCCCGTTATTGTGCAAGGT
Cardiac transcription factors	*Isl1*	TCATCCGAGTGTGGTTTCAA	CCATCATGTCTCTCCGGACT
	Tbx1	CGACAAGCTGAAACTGACCA	ACTGTCTTTTCGAGGGTCCA
	Tbx5	GGAGCCTGATTCCAAAGACA	TTCAGCCACAGTTCACGTTC
	Hand2	TCAAGGCGGAGATCAAGAAG	TGGTTTTCTTGTCGTTGCTG
	Hand1	GCCTACTTGATGGACGTGCT	GCGCCCTTTAATCCTCTTCT
	Nkx2-5	ACCCAGCCAAAGACCCTC	GACAGGTACCGCTGTTGCTT
	Gata4	TCTCACTATGGGCACAGCAG	GCGATGTCTGAGTGACAGGA
	Mef2c	ACTGGGAAACCCCAATCTTC	ATCAGACCGCCTGTGTTACC
	Tbx2	GGTCATCTGCTAGCCTCAGT	AAAGTGGGCATTGGGATT
	Tbx3	CCTTCCACCTCCAACAACAC	GCATGCTGTTCAAATTGAGG
	Tbx18	ACGAAATAGGCACCGAGATG	CCTGCCACCATCCACTTAGA
Endocardial cells	*Nfatc1*	TCATCCTGTCCAACACCAAA	TCACCCTGGTGTTCTTCCTC
Hematopoietic cells	*Gata1*	AGCATCAGCACTGGCCTACT	AGGCCCAGCTAGCATAAGGT
Cardiac muscle	*Prnp*	CTGAAGCATTCTGCCTTCCT	GCCGACATCAGTCCACATAG
	Myl2 (Mylc2v)	AAAGAGGCTCCAGGTCCAAT	CCTCTCTGCTTGTGTGGTCA
	Myl7 (Mylc2a)	TCAGCTGCATTGACCAGAAC	AAGACGGTGAAGTTGATGGG
	Tnni3 (cTnI)	CTGCCAACTACCGAGCCTAT	CTCGTTCCATCTCCTGCTTC
	Myh6	GAGATTTCTCCAACCCAG	TCTGACTTTCGGAGGTACT
	Hcn4	CTGGGGTCAACAAATTCTCC	ATCAGCAACAGCATCGTCAG
	Nppa	AGTGGACTAGGCTGCAACAGCTTC	ACACACCACAAGGGCTTAGGATC
Smooth muscle	*Myh11* (SMMyhc)	CAAATGAAGCCTCGTTTCCT	ATGAGGCCACAGAGAGCAAT
	Tagln (SM22a)	GATGGAACAGGTGGCTCAAT	TTTTGGTCACAGCCAAACTG
	Acta2 (SMA)	AGCCAGTCGCTGTCAGGAA	CTTACAGAGCCCAGAGCCAT

Online Table II. Antibodies used in this study

		Protein Name	Abbreviation used in this study	Antibody source (clone name)	Label
Surface staining of live cells		Prion protein	PrP	SPI-BIO, mouse monoclonal (SAF83)	Biotin APC
		PDGF Receptor α	PDGFRα	eBioscience, rat monoclonal, (APA5)	Biotin, PE, APC
		Flk1	Flk1	BD Bioscience, rat monoclonal, (Avas12α1)	PE
		c-kit	c-kit	eBioscience, rat monoclonal,	PE
		PECAM	CD31	eBioscience, rat monoclonal,	APC
Intracellular staining of fixed cells	Transcription factors	Gata4	Gata4	Santa Cruz, rabbit polyclonal	
		Islet-1	Isl1	Hybridoma Bank, mouse monoclonal (clone 39.4.D5)	
	Cardiac proteins	Prion protein	PrP	SPI-BIO, mouse monoclonal (SAF32)	
		Myosin heavy chain	Myhc	Hybridoma Bank (clone MF20)	
		Tropomyosin	Tm	Sigma, mouse monoclonal (clone CH1)	
		Cardiac troponin I	cTnI	DSMZ, mouse monoclonal (clone TI-1)	
		Cardiac troponin T	cTnT	Santa Cruz, goat polyclonal	
		Cardiac troponin T	cTnT	Abcam, mouse monoclonal (clone 13-11)	
		Atrial natriuretic peptide	ANP	Protos Biotech (rabbit polyclonal)	
		Myosin light chain 2v	Mylc2v	Alexis, mouse monoclonal (clone F109.3E1)	
		Myosin light chain 2a	Mylc2a	Sigma, rabbit polyclonal	
		Hyperpolarization activated cyclic nucleotide-gated potassium channel 4	HCN4	Chemicon, rabbit polyclonal	
	Smooth muscle proteins	Smooth muscle α actin	SMA	Sigma, mouse monoclonal (1A4)	
		SM22α	SM22	Abcam, goat polyclonal	
		Calponin	Calp	Abcam, rabbit monoclonal	

		Smooth muscle myosin heavy chain	SMMyhc	Biomedical Technologies, rabbit polyclonal	

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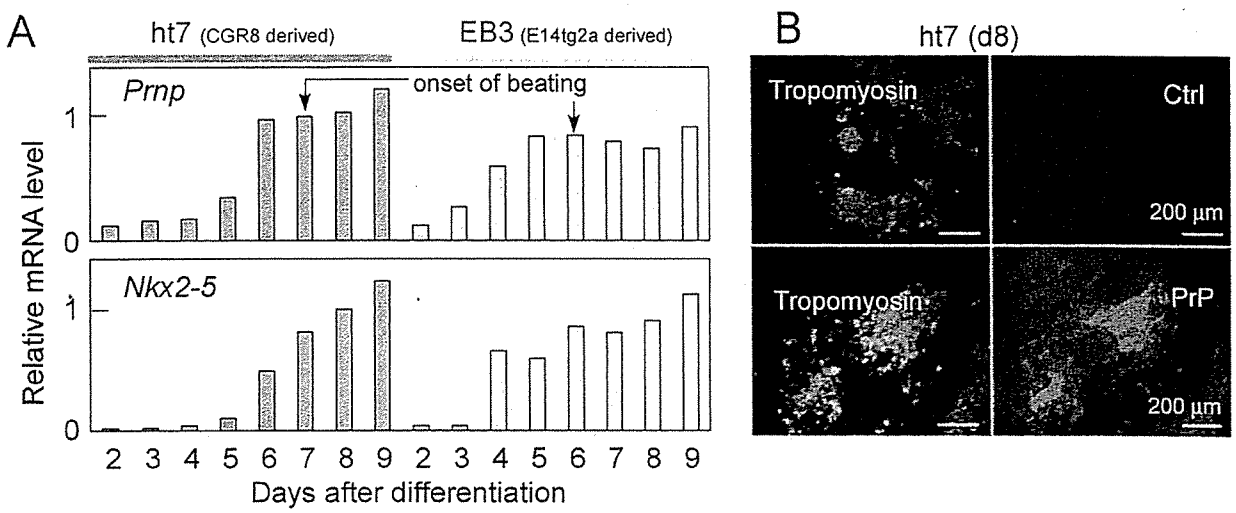

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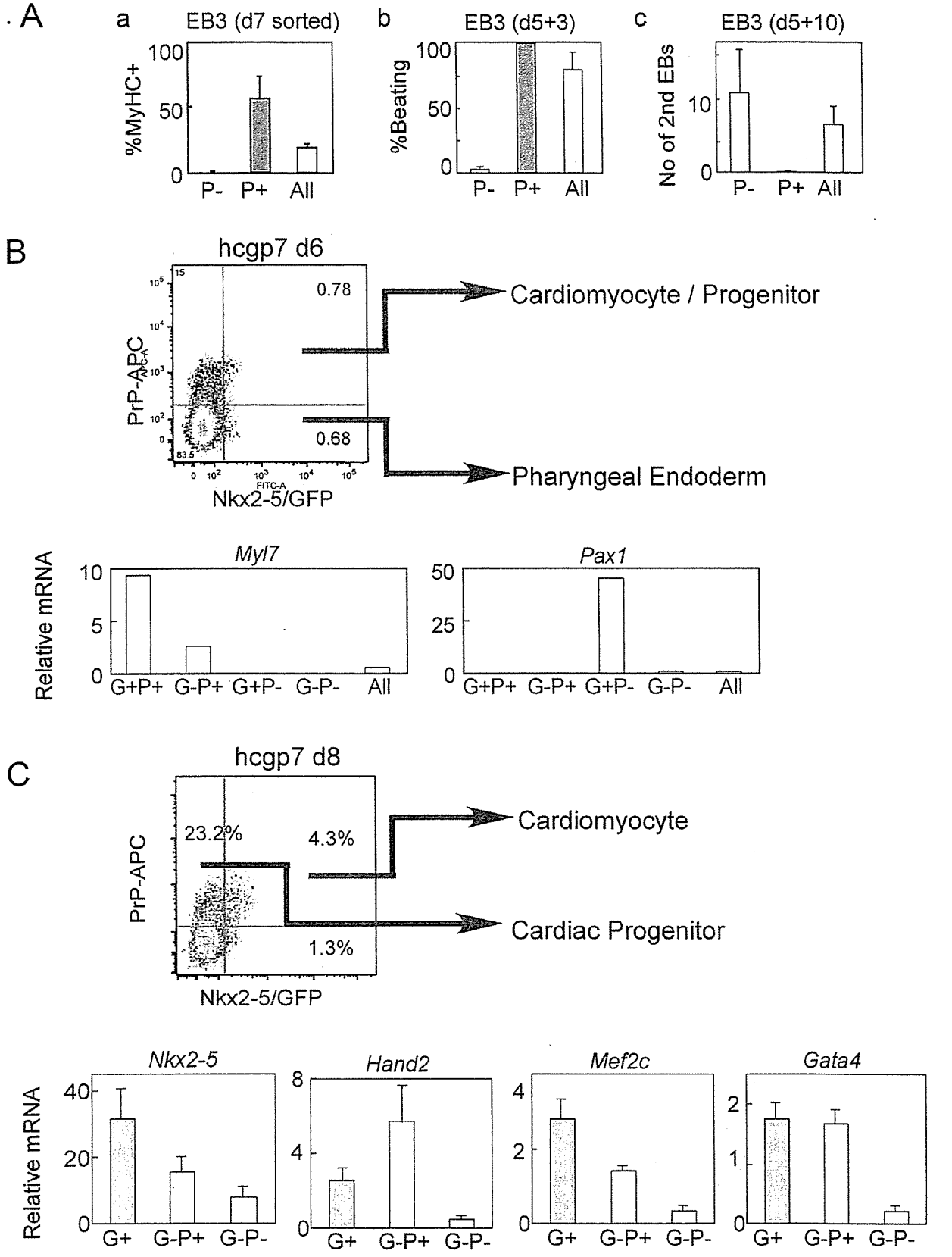

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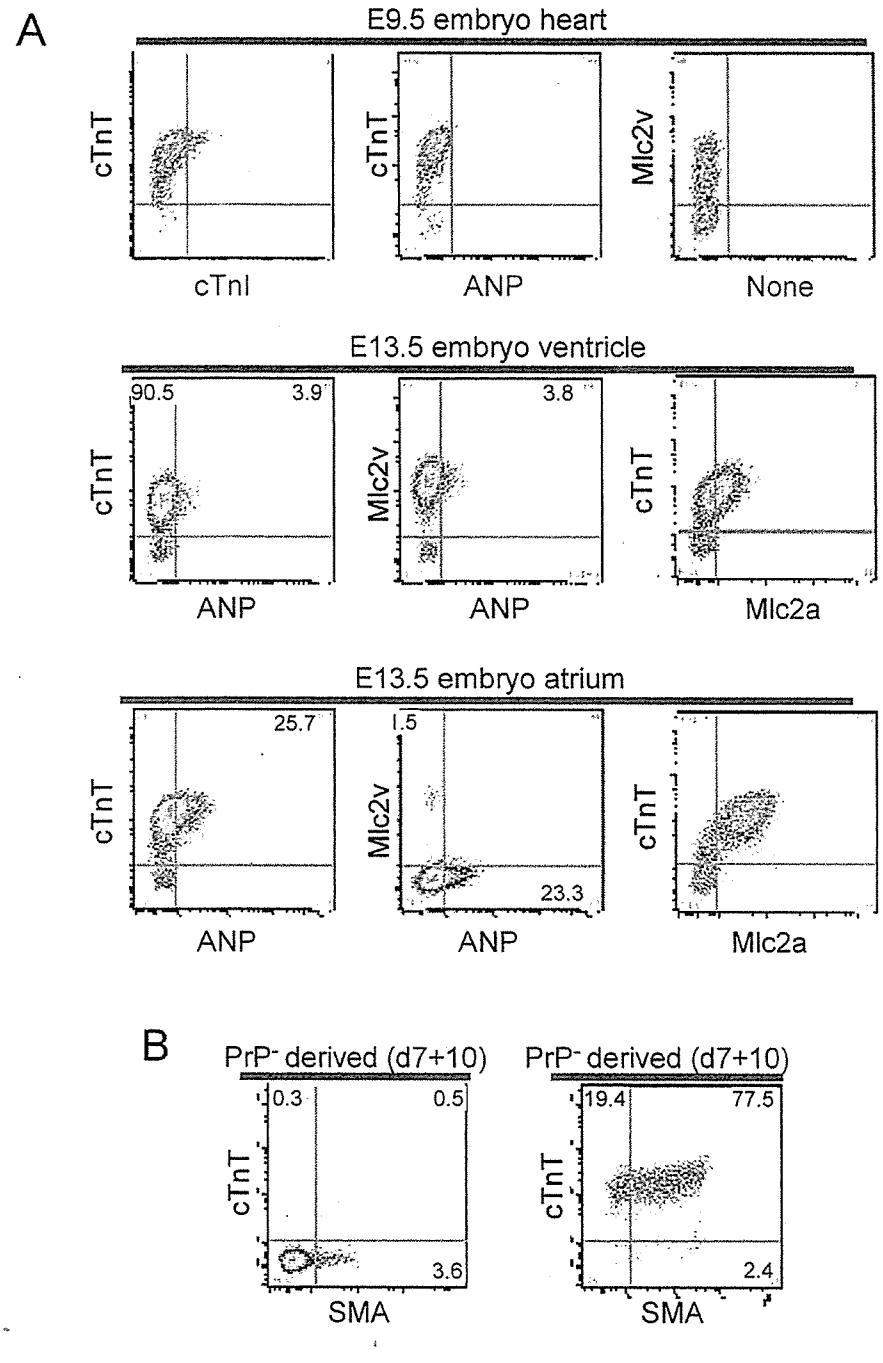

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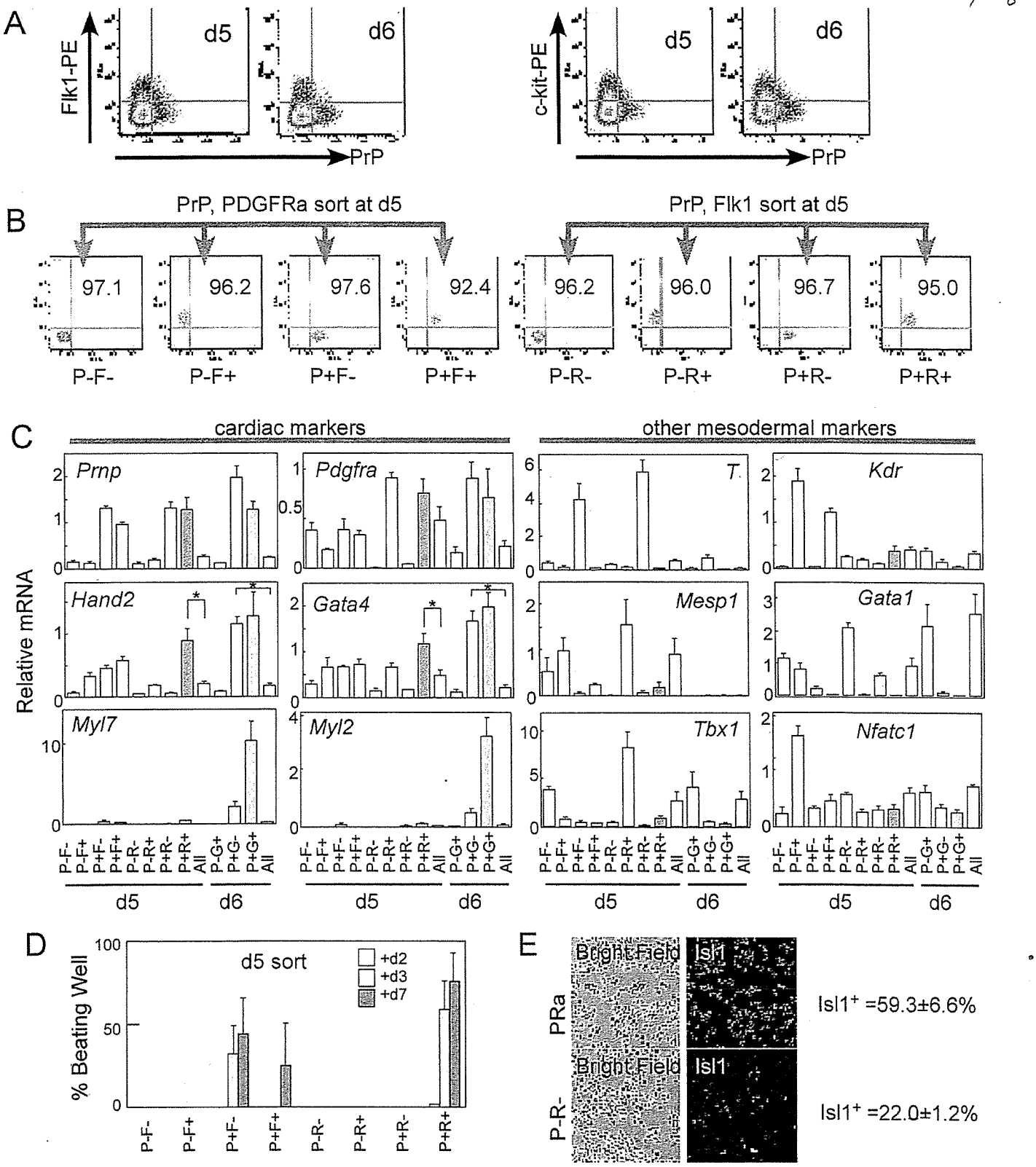
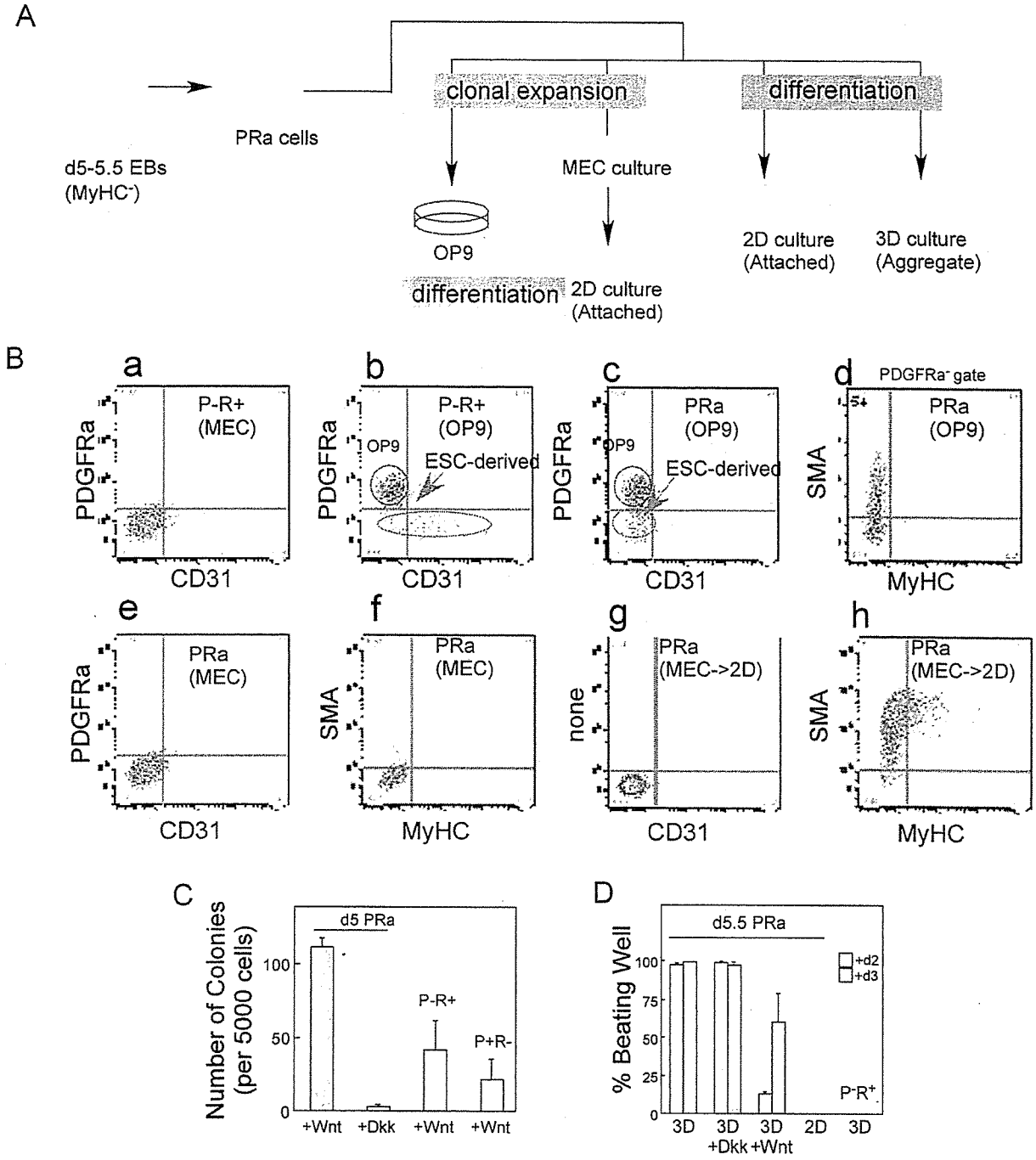
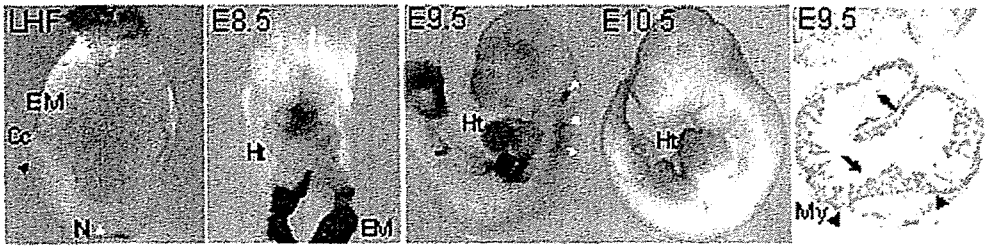


Online Fig. IV. A, Flow cytometry analysis of cell fractions sorted using PrP (P), PDGFRα (R), and Flk1 (F). B, RT-PCR analysis of cell fractions. Relative mRNA levels are shown (Day-4 EB = 1.0 for T and Mesp1; Day-8 EB = 1.0 for the rest of the genes). Expression levels of *Nkx2.5*, *Tbx5*, *Isl1* (see Fig 3), *Hand2*, and *Gata4* were enriched in the PrP$^+$PDGFRα$^+$ (PRa) fraction (pink bars) on Day 5, whereas cardiac contractile protein markers were not expressed. *Kdr* (also known as *Flk1*), T (primitive streak marker), *Mesp1* (mesoderm marker), *Gata1* (hematopoietic cell marker), and *Nfatc1* (endocardial cell marker) were not expressed in the PRa cell fraction. The P-R+ fraction may contain progenitors that are earlier in the developmental process than PRa cells, because *Mesp1* expression was detected in this fraction. Error bars represent the standard errors of the mean (n = 3). C, The percentage of beating reaggregates of sorted cells. Cells were sorted on Day 5 and plated in a 96-well ultra-low binding plate. Wells containing beating aggregates were counted. The PRa cell fraction frequently gave rise to beating aggregates. Error bars represent the standard errors of the mean (n = 5).

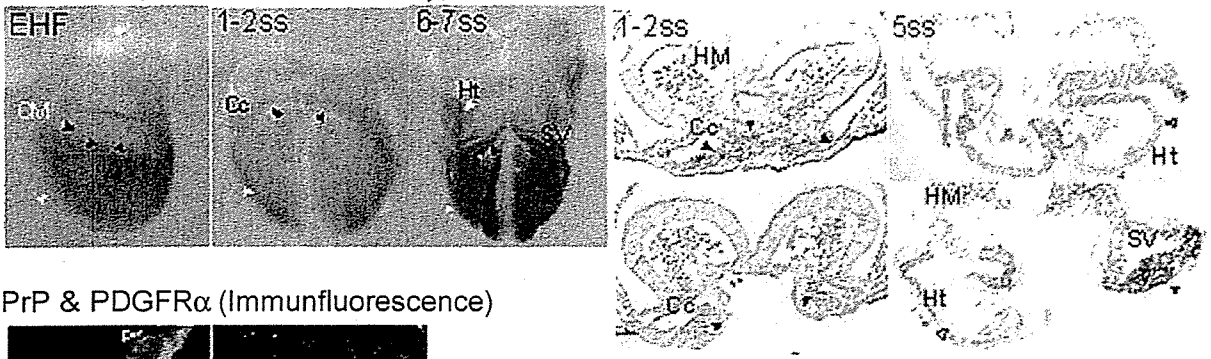


Online Fig. V. A, Schematic diagram of the culture methods for PRa cells. Sorted PRa cells were plated on OP9 stromal cells or in methylcellulose (MEC) at a clonal density to obtain colonies derived from single cells. Colonies grown in MEC were picked on Day 6 and plated on gelatin-coated plates. To differentiate cells directly, cells were cultured on gelatin-coated plates (2D cultures) or after reaggregation in ultra-low binding multiwell plates (3D cultures). B, Analysis of the ability of the OP9 or MEC culture system to support the undifferentiated state. PRa or P-R+ cell fractions were sorted and cultured on OP9 cells (b, c, d) or in MEC (a, e, f, g, h) for 8-9 days. After cell dissociation, cell surface and intracellular markers were analyzed using flow cytometry. We found that OP9 cells expressed PDGFRα (data not shown) and could be distinguished from ES cell-derived cells (pink open circles in b and c; the PDGFRα+ fraction was gated out in d). Whereas OP9 cells induced the differentiation of both endothelial ($CD31^+$, b) and smooth muscle cells (SMA^+, d), MEC did not (a, c, e), suggesting that MEC supported the undifferentiated state. When MEC colonies were cultured on gelatin-coated plates for 3 days (MEC → 2D), they started to differentiate into smooth muscle cells and cardiomyocytes (h). In contrast to P-R+ cells, the PRa cell fraction did not give rise to endothelial cells (d, g), suggesting that the PRa cell fraction did not contain multipotent cardiovascular progenitors. C, Colony formation from single PRa cells in MEC cultures depended on Wnt signaling. The colonies derived from Day-5.5 PRa cells were counted. D, Differentiation of Day-5.5 PRa cells after 2D or 3D cultures. Bars represent standard errors of the mean from three independent experiments.

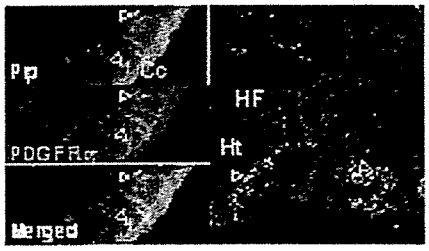
A PrP (Immunohistochemistry)



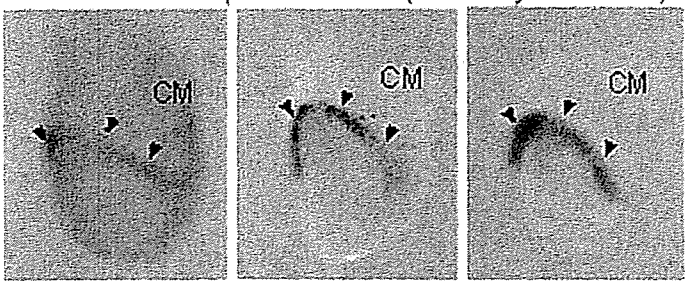
B PDGFRα (Immunohistochemistry)



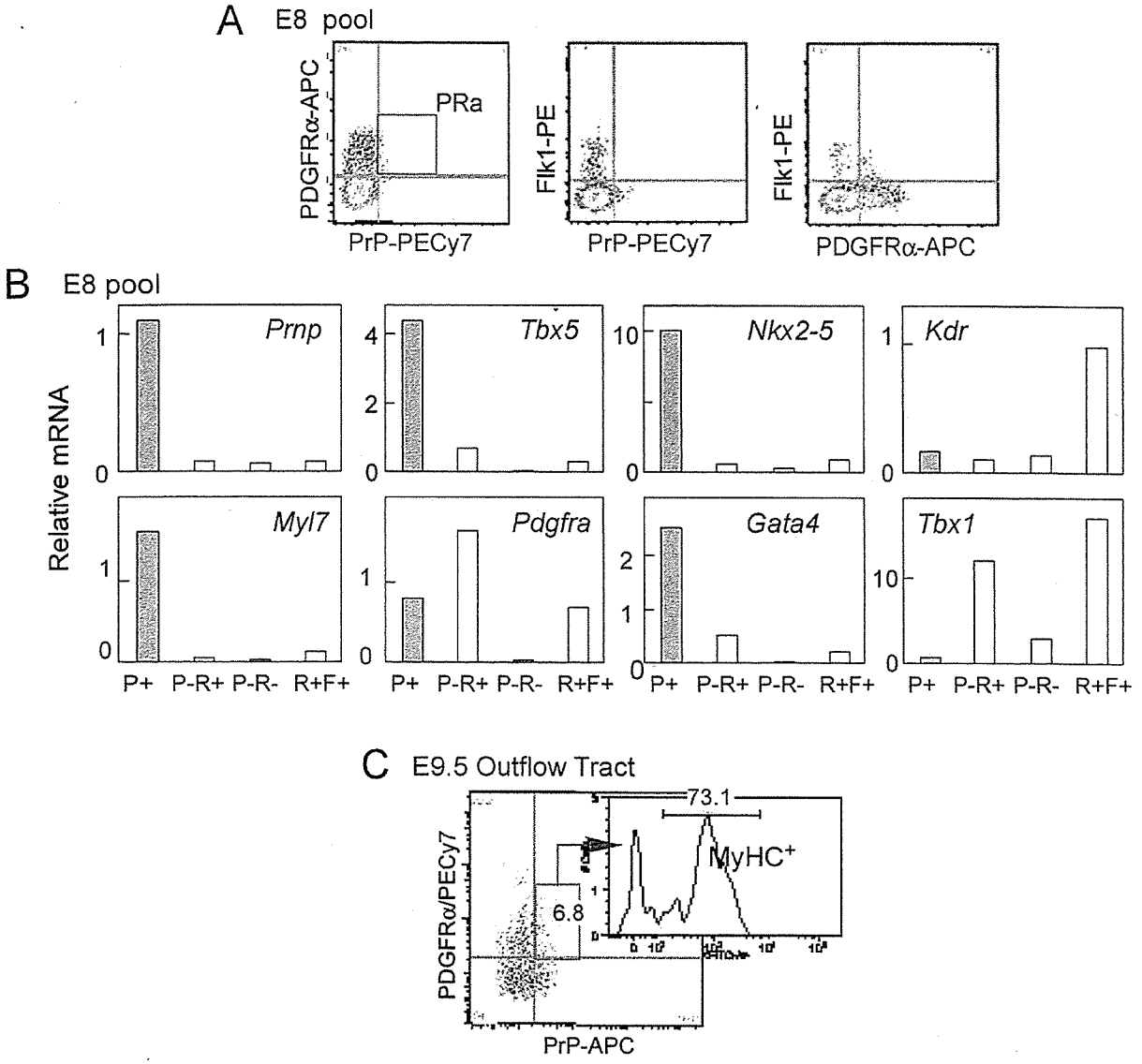
C PrP & PDGFRα (Immunfluorescence)



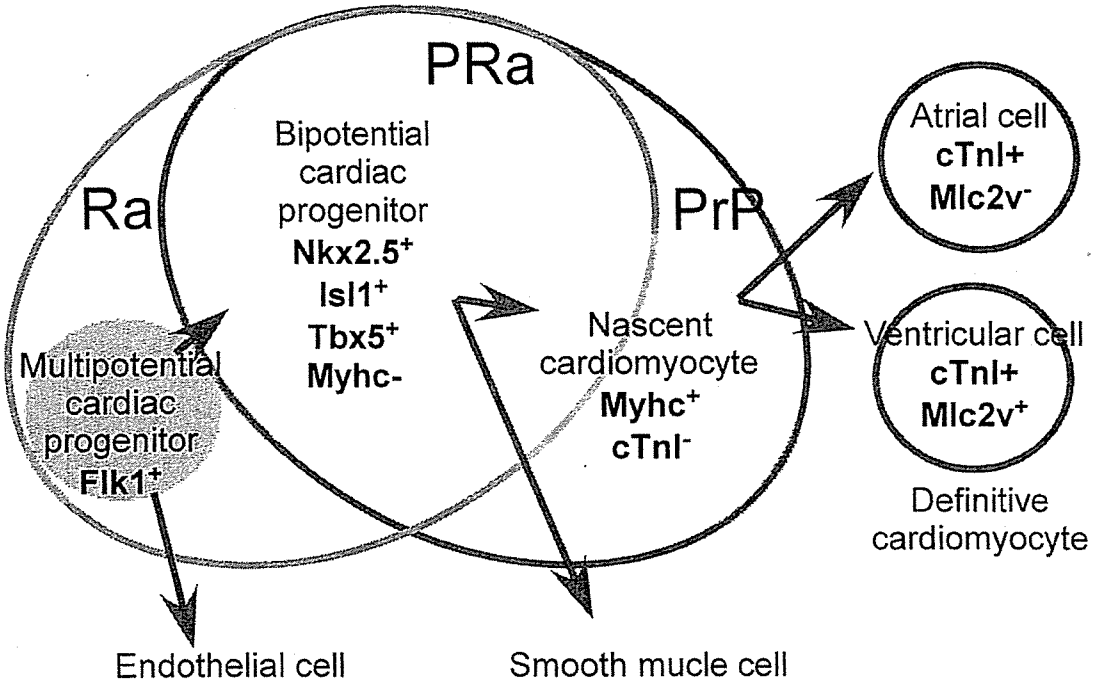
D Cardiac Transcription Factors (in situ hybridization)



Online Fig. VI. A, Immunohistochemical analysis of PrP protein. PrP was detected in cardiac crescent (Cc, black arrowhead) and node (N, yellow arrowhead) at the late headfold stage (LHF). Expression of PrP persisted in heart tube (Ht) from E8.5 to E10.5. PrP expression was detected in ganglia at E9.5 (yellow arrowheads). Background labeling was observed in extraembryonic mesoderm (EM). B, Immunohistochemical analysis of PDGFRα. PDGFRα expression was observed in mesodermal tissues, including cardiac mesoderm (CM) at the early headfold stage (EHF) and in cardiac crescent (Cc) at E8.0. At E8.25, PDGFRα was expressed in the sinus venosus (SV, black arrowheads), whereas expression levels were downregulated in the heart tube (white arrowhead). C, Immunofluorescence analysis of PrP and PDGFRα. Overlapping expression was observed in cardiac crescent (Cc) at the late headfold stage (LHF) and in heart tube (Ht) at the 6-7 somite stage (6-7ss). D, *In situ* hybridization analysis of cardiac transcription factor mRNA at the early headfold stage. *Nkx2.5*, *Tbx5*, and *Isl1* mRNA was detected cardiac mesoderm (CM).



Online Fig. VII. A, Flow cytometry analysis of surface proteins on mouse embryo-derived cells. Mouse embryos were dissected and the extraembryonic region was removed. E8 pools (containing embryos from LHF to 7 somite stage) were labeled with antibodies specific for PrP, PDGFRα, and Flk1. B, RT-PCR analysis of sorted cells from E8 pool embryos. Note that the PrP^+ fraction was specifically enriched for cardiac markers. Representative results from three independent experiments are shown. C, Flow cytometry analysis of PrP, PDGFRα, and Myhc in the outflow tract (OFT) segment derived from E9.5 embryos. The OFT was dissected, dissociated, and analyzed using flow cytometry. Overlapping expression of PrP and PDGFRα was observed. Intracellular staining revealed that PRa cells included $Myhc^+$ cells.



Online Fig. VIII. Potential and committed cardiac progenitor cells based on ES cell studies. The earliest cardiomyogenic population was the PRa cells. PRa cells include $Myhc^+$ nascent cardiomyocytes as well as $Myhc^-$ bipotential progenitors that can differentiate into cardiac or smooth muscle cells. PrP can also be used to isolate nascent cardiomyocytes that differentiate into atrial or ventricular cardiomyocytes.

ORIGINAL ARTICLE

Genetic Link Between Obesity and *MMP14*-Dependent Adipogenic Collagen Turnover

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OBJECTIVE—In white adipose tissue, adipocytes and adipocyte precursor cells are enmeshed in a dense network of type I collagen fibrils. The fate of this pericellular collagenous web in diet-induced obesity, however, is unknown. This study seeks to identify the genetic underpinnings of proteolytic collagen turnover and their association with obesity progression in mice and humans.

RESEARCH DESIGN AND METHODS—The hydrolysis and degradation of type I collagen at early stages of high-fat diet feeding was assessed in wild-type or *MMP14 (MT1-MMP)*-haploinsufficient mice using immunofluorescent staining and scanning electron microscopy. The impact of *MMP14*-dependent collagenolysis on adipose tissue function was interrogated by transcriptome profiling with cDNA microarrays. Genetic associations between *MMP14* gene common variants and obesity or diabetes traits were examined in a Japanese cohort (n = 3,653).

RESULTS—In adult mice, type I collagen fibers were cleaved rapidly in situ during a high-fat diet challenge. By contrast, in *MMP14* haploinsufficient mice, animals placed on a high-fat diet were unable to remodel fat pad collagen architecture and display blunted weight gain. Moreover, transcriptional programs linking type I collagen turnover with adipogenesis or lipogenesis were disrupted by the associated decrease in collagen turnover. Consistent with a key role played by *MMP14* in regulating high-fat diet–induced metabolic programs, human *MMP14* gene polymorphisms located in proximity to the enzyme's catalytic domain were closely associated with human obesity and diabetes traits.

CONCLUSIONS—Together, these findings demonstrate that the *MMP14* gene, encoding the dominant pericellular collagenase operative in vivo, directs obesogenic collagen turnover and is linked to human obesity traits. ***Diabetes* 59:2484–2494, 2010**

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Obesity is closely linked to the development of diabetes and negatively affects life expectancy (1–3). At early stages of obesity, gene programs are activated within fat depots that support tissue expansion, which arises as a combined result of the hypertrophic growth of adipocytes and the recruitment of adipocyte precursors to the adipogenic process (4,5). Within adipose tissues, both adipocytes and adipocyte precursors are enveloped by a dense network of type I collagen (6,7). This collagenous web is presumed to provide adipose tissues with structural integrity and elasticity necessary to maintain optimal form and function (8). Matrix metalloproteinase (MMP)14 (MT1-MMP) is a pericellular type I collagenase that is critical for the postnatal development of the mesenchyme, including adipose tissues (7). Whether the developmental role played by *MMP14* during adipogenesis is relevant to the pathogenesis of adult obesity remains unclear. The premature morbidity and mortality observed in *MMP14* knockout mice (7,9) preclude attempts to assess the impact of nutritional interventions on adult obesity in these animals. Moreover, it has not been determined whether the diet-induced expansion of fat mass is functionally linked to the remodeling of the type I collagen network.

Here, we demonstrate that the type I collagen architecture of adipose tissues undergoes rapid degradative remodeling in response to a high-fat diet challenge in vivo via a proteolytic process mediated by MMP14 (10). A reduction in *MMP14* dosage not only prevents the diet-induced cleavage of the type I collagen network in vivo but also disrupts the regulation of the transcriptional programs linked to adipogenesis and lipogenesis. Consistent with insights obtained from analyses of genetically modified mice, human *MMP14* single nucleotide polymorphisms (SNPs) are identified that positively associate with human obesity and diabetes traits. Taken together, these data support a new model wherein *MMP14* not only participates in obesity pathogenesis but also serves as a potential genetic modifier of obesity and diabetes predisposition in humans.

RESEARCH DESIGN AND METHODS

Mice, diets, and metabolic phenotyping. *MMP14*$^{+/-}$ mice were kindly provided by Dr. Motoharu Seiki (University of Tokyo) and maintained in C57BL/6 background (11). *MMP14* haploinsufficient (*MMP14*$^{+/-}$) and wild-type (*MMP14*$^{+/+}$) male mice, aged 12 weeks, were used for all experiments. Each mouse was housed individually and placed on a high-fat (45% fat) or control (10% fat) diet (Research Diets) for the indicated periods of time. In selected studies, mice were individually housed in Oxymax/CLAMS metabolic chambers (Columbus Instrument) for metabolic studies. After 2 days' acclimation, Vo_2, food consumption, and x-y ambulation were measured consecutively for 4 days.