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PARM-1 promotes cardiomyogenic differentiation through regulating the BMP/Smad signaling pathway

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ABSTRACT

PARM-1, prostatic androgen repressed message-1, is an endoplasmic reticulum (ER) molecule that is involved in ER stress-induced apoptosis in cardiomyocytes. In this study, we assessed whether PARM-1 plays a role in the differentiation of stem cells into cardiomyocytes. While PARM-1 was not expressed in undifferentiated P19CL6 embryonic carcinoma cells, PARM-1 expression was induced during cardiomyogenic differentiation. This expression followed expression of mesodermal markers, and preceded expression of cardiac transcription factors. PARM-1 overexpression did not alter the expression of undifferentiated markers and the proliferative property in undifferentiated P19CL6 cells. Expression of cardiac transcription factors during cardiomyogenesis was markedly enhanced by overexpression of PARM-1, while expression of mesodermal markers was not altered, suggesting that PARM-1 is involved in the differentiation from the mesodermal lineage to cardiomyocytes. Furthermore, overexpression of PARM-1 induced BMP2 mRNA expression in undifferentiated P19CL6 cells and enhanced both BMP2 and BMP4 mRNA expression in the early phase of cardiomyogenesis. PARM-1 overexpression also enhanced phosphorylation of Smads1/5/8. Thus, PARM-1 plays an important role in the cardiomyogenic differentiation of P19CL6 cells through regulating BMP/Smad signaling pathways, demonstrating a novel role of PARM-1 in the cardiomyogenic differentiation of stem cells.

1. Introduction

The heart is one of the first functional organs to develop during embryogenesis in vertebrates. Cardiomyogenic differentiation of stem cells is a multistep process in which undifferentiated cells commit to mesodermal cells and subsequently differentiate into cardiac mesoderm cells, which become terminally differentiated cardiomyocytes [1,2]. This process involves highly ordered signaling pathways and their intersection with transcriptional regulatory networks [2,3]. The directed differentiation of pluripotent stem cells into cardiomyocytes provides not only novel insights into the pathways that regulate lineage selection but also medical applications such as drug discovery and cell transplantation therapies for various heart diseases. The Wnt and BMP signaling pathways have been shown to be involved in developmental events such as stem cell maintenance, cell fate specifications, and organogenesis, through regulation of transcription via Smad and TCF/LEF transcription factors [4]. However, the molecular mechanisms governing differentiation of pluripotent stem cells into cardiomyocytes are not fully understood, and their comprehension could improve the efficiency of differentiation into cardiomyocytes.

PAPM-1 (prostatic androgen repressed message-1), which was originally identified as a gene overexpressed in the prostate of castrated rats, is a single-pass type 1 transmembrane molecule with a signal sequence that is highly expressed in cardiomyocytes [5–7]. In cardiomyocytes, PARM-1 is predominantly localized in endoplasmic reticulum (ER), and ER stress induces its expression, which protects against ER stress-induced apoptosis through regulating expression of PKR-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and C/EBP homologous protein transcription factor (CHOP) [7]. ER is a multifunctional intracellular organelle, where protein synthesis and folding of secreted and transmembrane proteins take place [8]. ER-resident proteins, such as ATF6 and GRP78, are expressed in embryonic hearts and implicated in embryonic development [9–11]. PARM-1 expression in the mouse heart is detectable at embryonic day 10.5, and it increases until neonatal stages and thereafter remains unchanged through adult stages [7]. However, whether PARM-1 is involved in cardiac

Abbreviations: PARM-1, prostatic androgen repressed message-1; BrdU, 5-bromodeoxyuridine; cTnI, cardiac troponin I; αMHC, α myosin heavy chain; BMP, bone morphogenetic protein; EGFP, enhanced green fluorescent protein.

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development remains unknown. In the present study, we sought to identify the role of PARM-1 in the differentiation of stem cells into cardiomyocytes.

2. Materials and methods

2.1. Cell culture and induction of cardiomyocyte differentiation

P19CL6 cells were cultured as previously described [12–14]. In brief, P19CL6 cells were grown in a 100-mm culture dish with α-minimal essential medium (GIBCO) containing 10% fetal bovine serum (FBS, GIBCO), penicillin (100 U/ml), and streptomycin (100 μg/ml) (growth medium) and were maintained in a 5% CO_2 atmosphere at 37 °C. To induce cardiomyocyte differentiation, 1.85×10^5 cells were plated in a 6-well culture dish with the growth medium supplemented with 1% DMSO (differentiation medium). The differentiation medium was changed every 2 days. Days of differentiation were numbered consecutively after the first day of the DMSO treatment (day 0). P19E25 cells were established by transfecting the α myosin heavy chain (αMHC)-enhanced green fluorescent protein (EGFP) vector into P19CL6 cells as previously described [15,16].

2.2. Plasmids

The corresponding cDNA fragment for mouse PARM-1 was cloned by PCR with a mouse heart cDNA template. PCR was performed using the following primers: PARM-1, forward primer 5′-TGGTCAGACCCAGGAAACTC-3′ and reverse primer 5′-CAGAGAAGGGGAAGGGAAAA-3′. cDNA encoding mPARM-1 and LacZ were cloned into the pMSCVpuro Retroviral Vector (Clontech) to generate pMSCVpuro-PARM-1 and pMSCVpuro-LacZ, respectively.

2.3. Recombinant retroviruses and gene transfer

Recombinant retroviruses were generated as previously described [17]. GP2–293 cells (Clontech) were cotransfected with the helper vector pVSV-G, pMSCVpuro-LacZ, and pMSCVpuro-mouse PARM-1, using FuGENE6 (Roche). pMSCVpuro-LacZ was used as a control. The medium supernatant was collected and centrifuged to concentrate virus stocks according to the manufacturer's instructions. P19CL6E25 cells were infected with the retrovirus in the presence of 4 μg/ml polybrene for 24 h, and the medium was changed to fresh medium. Infected cells were selected with 2.5 μg/ml puromycin and analyzed.

2.4. RNA isolation and real-time PCR

Total RNA was isolated from cells using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Total RNA was exposed to RNase-free DNase I (QIAGEN) and reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems). Synthesized cDNA was analyzed by quantitative real-time PCR using the LightCycler (Roche Applied Science) with SYBR Premix Ex Taq (Takara). Mouse GAPDH was used for normalization. Primers used for the PCR were as follows (forward and reverse primers): PARM-1 (5′-TAGCCAGTGCCACT CACAAC-3′ and 5′-GATGTTGGCCACCGTAGAGT-3′); Oct3/4 (5′-AGGCAGGAGCACGAGTGGAAAGCA-3′ and 5′-GGAGGGCTTCGGG-CACTTCAGAAA-3′); Nanog (5′-AAGTACCTCAGCCTCCAGCA-3′ and 5′-GTGCTGAGCCCTTCTGAATC-3′); Eras (5′-CCCTCATCAGACTGC-TACTCCTGG-3′ and 5′-CACGCAGAGCCCGGTGAATATCCT-3′); Wnt3 (5′-GCGACTTCCTCAAGGACAAG-3′ and 5′-AAAGTTGGGGGAGTT CTCGT-3′); Wnt3a (5′-CCCTTTCCAGTCCTGGTGTA-3′ and 5′-CTTGA AGAAGGGGTGCAGAG-3′); Brachyury (5′-AAGGAACCACCGGTCAT C-3′ and 5′-GTGTGCGTCAGTGGTGTGTAATG-3′); Mesp1 (5′-CCTTC GGAGGGAGTAGATCC-3′ and 5′-AAAGCTTGTGCCTGCTTCAT-3′); GATA4 (5′-TCTCACTATGGGCACAGCAG-3′ and 5′-CGAGCAGGAA TTTGAAGAGG-3′); Nkx2.5 (5′-CAGTGGAGCTGGACAAAGCC-3′ and 5′-TAGCGACGGTTCTGGAACCA-3′); Tbx5 (5′-ATGGTCCGTAACTGG-CAAAG-3′ and 5′-TTTCGTCTGCTTTCACGATG-3′); cardiac troponin I (cTnI), (5′-TAAGATCTCCGCCTCCAGAA-3′ and 5′-CGGCATAAGT CCTGAAGCTC-3′); αMHC (5'-GAGGACCAGGCCAATGAGTA-3′ and 5′-GCTGGGTGTAGGAGAGCTTG-3′); bone morphogenetic protein 2 (BMP2) (5′-TGGAAGTGGCCCATTTAGAG-3′ and 5′-TGACGCTTTTC TCGTTTGTG-3′); BMP4 (5′-TGAGCCTTTCCAGCAAGTTT-3′ and 5′-CTTCCCGGTCTCAGGTATCA-3′); and GAPDH (5′-TTGTGATGGGT GTGAACCACGAGA-3′ and 5′-CATGAGCCCTTCCACAATGCCAAA-3′).

2.5. Western blotting analysis

Cells of each differentiation day were lysed with RIPA buffer (20 mM HEPES pH 7.7, 1% TritonX-100, 20 mM β-glycerophosphate, 75 mM NaCl, 2.5 mM $MgCl_2$, 1 mM NaF, 0.5 mM DTT, 100 μg/ml PMSF, 1× protease inhibitor cocktail (Pierce), 0.1 mM EDTA pH8.0, 0.2 mM Na_3VO_4). Lysates normalized by protein concentration were subjected to SDS–PAGE, and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were immunoblotted with the primary antibody against pSmad1/5/8, Smad1, pSmad2, Smad2/3 (Cell Signaling), and GAPDH (Millipore), followed by horseradish peroxidase-labeled rabbit or mouse secondary antibody (GE Healthcare Life Sciences). Chemiluminescence was detected using ChemiDoc™ XRS+ System (BIO-RAD).

2.6. Flow cytometry

P19E25 cells of each differentiation day were detached from the culture dish using 0.8 mg/ml collagenase type II (Worthington). 1.0×10^6 cells were resuspended in Dulbecco's Phosphate-Buffered Saline containing 3% FBS. The percentages of EGFP-positive cells were analyzed using FACSCalibur Flow Cytometer (Becton Dickinson).

2.7. Proliferation assay

Cell proliferation was assessed using Cell Proliferation ELISA, 5-bromodeoxyuridine (BrdU) (colorimetric) (Roche Applied Science) according to the manufacturer's instructions as described previously [14,18]. In brief, 10,000 cells were seeded in 96-well plates and incubated in growth medium at 37 °C for 24 h. BrdU reagent was added and the cells were incubated at 37 °C for 4 h. The absorbance of the samples was measured using a microplate reader at 450 nm.

2.8. Statistical analysis

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

3. Results

3.1. PARM-1 expression is induced during cardiomyogenesis

We assessed the expression of PARM-1 during cardiogenic differentiation in P19CL6 cells. As shown in Fig. 1, PARM-1 expression was undetectable in undifferentiated P19CL6 cells, while upon induction of cardiomyogenesis, upregulation of PARM-1 expression

began at day 4 and its expression reached a peak at day 8, which followed expression of Brachyury, a panmesodermal marker gene, and Mesp1, a cardiogenic mesodermal marker gene, and preceded expression of cardiac transcription factors, such as Nkx2.5 and Tbx5, and cardiac sarcomeric proteins, such as αMHC and cTnI.

3.2. PARM-1 promotes cardiomyogenic differentiation

To examine the role of PARM-1 in cardiomyogenesis, we established P19CL6E25 cells stably expressing PARM-1, P19CL6-PARM-1, using recombinant retrovirus expressing PARM-1. P19CL6E25 cells are P19CL6 cells harboring a cardiac αMHC promoter-driven EGFP gene, whose expression is correlated with αMHC expression during cardiomyogenesis as previously reported [15,16]. Fluorescent microscopy revealed that the number of EGFP-positive cells among P19CL6-PARM-1 cells after day 10 was greater than that in P19CL6E25 cells stably expressing LacZ, P19CL6-LacZ cells, which were used as a control (Fig. 2A). Flow cytometry confirmed that the rate of EGFP-positivity was significantly increased in P19CL6-PARM-1 cells compared with that in P19CL6-LacZ cells (Fig. 2B). Consistent with these observations, αMHC and cTnI mRNA expression in P19CL6-PARM-1 cells was also increased compared with that in P19CL6-LacZ cells (Fig. 2C). These results indicated that PARM-1 promotes cardiomyogenic differentiation.

3.3. PARM-1 enhances expression of cardiac transcription factors

We then investigated whether PARM-1 affects the undifferentiated state in P19CL6 cells. The proliferative capacity assessed by the BrdU assay did not differ between P19CL6-PARM-1 and P19CL6-LacZ cells (Fig. 3A). Furthermore, mRNA expression of Oct3/4 and Nanog, undifferentiated markers, at day 0 was not different from that in P19CL6-LacZ cells (Fig. 3B). Eras, which is expressed in embryonic stem cells [19], was expressed in both undifferentiated P19CL6-LacZ and P19CL6-PARM-1 cells, and expression of Eras in P19CL6-PARM-1 cells was not different from that in P19CL6-LacZ cells. These observations indicate that P19CL6-PARM-1 cells maintain the undifferentiated state.

Expression levels of Oct3/4, Nanog, and Eras mRNA rapidly declined after induction of cardiomyogenesis in both P19CL6-LacZ and P19CL6-PARM-1 cells, and there was no difference in mRNA expression of Oct3/4, Nanog, and Eras during cardiomyogenesis between P19CL6-LacZ and P19CL6-PARM-1 cells. The expression of cardiac transcription factors, such as GATA4, Nkx2.5, and Tbx5, at day 8 was augmented in P19CL6-PARM-1 cells compared with that in P19CL6-LacZ cells (Fig. 3C), while the expression of mesodermal markers, Brachyury and Mesp1, was not altered between P19CL6-LacZ and P19CL6-PARM-1 cells (Fig. 3D). These findings suggest the involvement of PARM-1 in enhanced expression of cardiac transcription factors.

3.4. PARM-1 enhances BMP/Smad signaling during cardiomyogenesis

Since the Wnt and BMP pathways are involved in cardiac differentiation [1], we examined whether the expression of signaling mediators involved in the Wnt and BMP pathways is altered in P19CL6-LacZ and P19CL6-PARM-1 cells. Wnt3 expression was not altered by PARM-1 overexpression, while Wnt3a expression in P19CL6-PARM-1 cells peaked at day 2 and its expression at day 4 was lower than that in P19CL6-LacZ cells (Fig. 4A). PARM-1 overexpression increased BMP2 expression at day 0 in P19CL6-PARM-1 cells and augmented BMP2 and -4 expression at days 2–6 (Fig. 4B). To assess the functional significance of increased expression of BMP2 and -4, we examined the activation of Smads. Phosphorylation of Smad1/5/8, downstream of BMP2 and -4 signals, was enhanced at day 0 and 2 by PARM-1 overexpression, while phosphorylation of Smad2, a downstream mediator of TGF-β signaling, was unchanged (Fig. 4C).

4. Discussion

Terminal differentiation of cardiomyocytes is a highly ordered process involving specification, differentiation, and specialization [2]. We showed in the present study that PARM-1 expression during cardiomyogenesis followed Brachyury and Mesp1 expression and preceded Nkx2.5 and Tbx5 expression. Brachyury is a direct transcriptional target for the canonical Wnt pathway and is required for mesodermal formation [20–23]. Mesp1, whose expression is positively regulated by the Wnt pathway, is the earliest marker of cardiovascular progenitors and promotes cardiovascular differentiation during embryonic development and pluripotent stem cell differentiation [24]. Overexpression of PARM-1 in

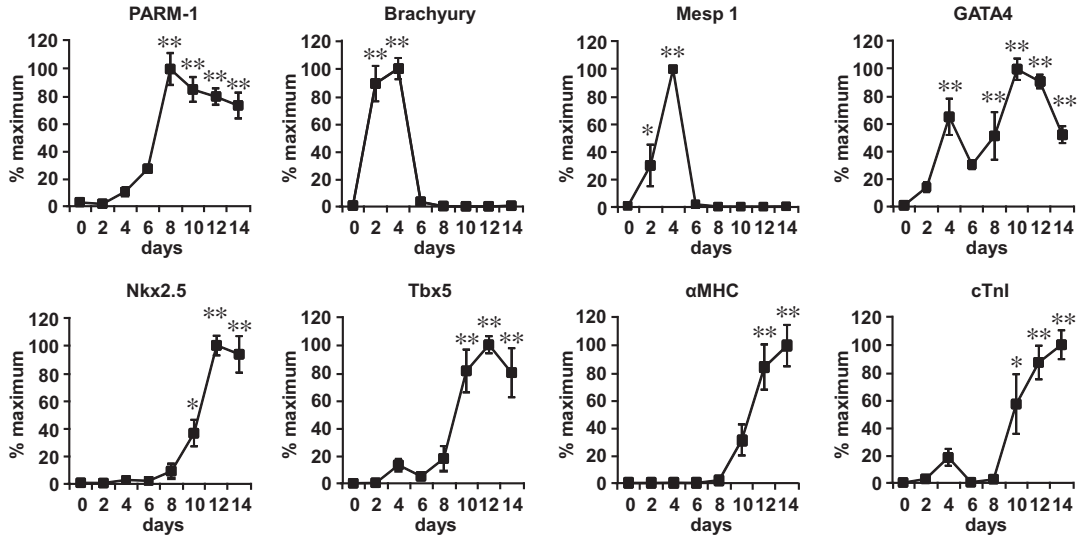

Fig. 1. PARM-1 expression during cardiomyogenesis. Cardiomyogenesis was induced by 1% DMSO in P19CL6 cells. Expression of PARM-1, Brachyury, Mesp1, GATA4, Nkx2.5, αMHC, and cTnI mRNA was analyzed by quantitative real-time PCR with total RNA isolated from P19CL6 cells on the indicated days. GAPDH was used as a control for assessing RNA loading. $^{*}P < 0.05$ compared with day 0, and $^{**}P < 0.01$ compared with day 0.

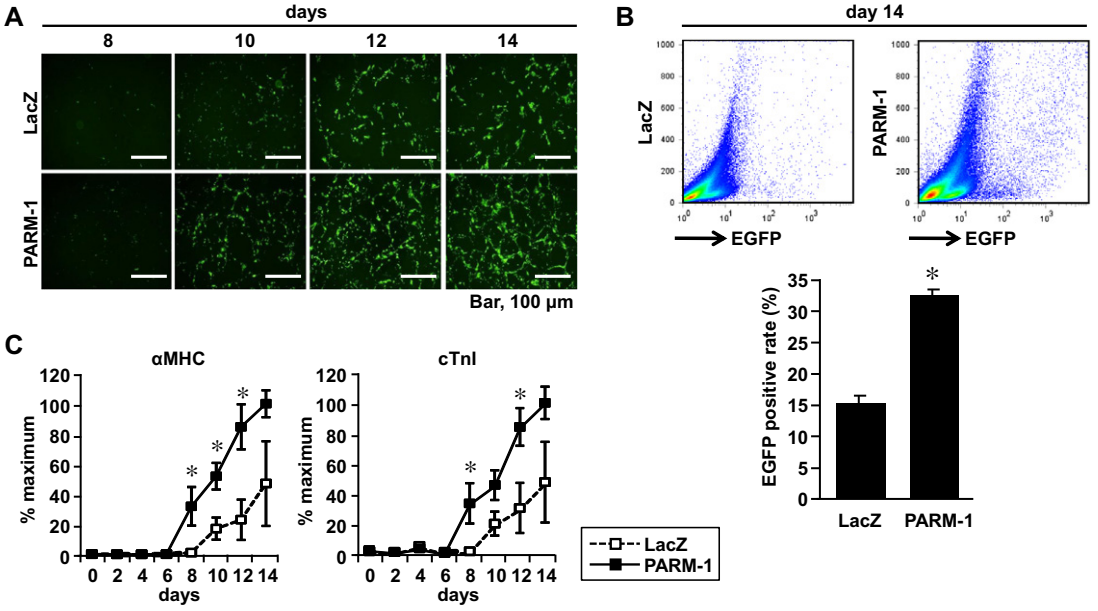

Fig. 2. Enhanced cardiomyogenesis by PARM-1 overexpression. (A) Fluorescence microscopic images of P19CL6-LacZ and P19CL6-PARM-1 cells during cardiomyogenesis. (B) Flow cytometric analysis for EGFP-expressing cells among P19CL6-LacZ and P19CL6-PARM-1 cells at day 14. *$P < 0.05$ compared with P19CL6-LacZ cells. (C) Expression of αMHC and cTnI mRNA was analyzed by quantitative real-time PCR with total RNA isolated from P19CL6-LacZ and -PARM-1 cells on the indicated days. GAPDH was used as a control for assessing RNA loading. *$P < 0.05$ compared with P19CL6-LacZ cells at the corresponding days. LacZ, P19CL6-LacZ cells. PARM-1, P19CL6-PARM-1 cells.

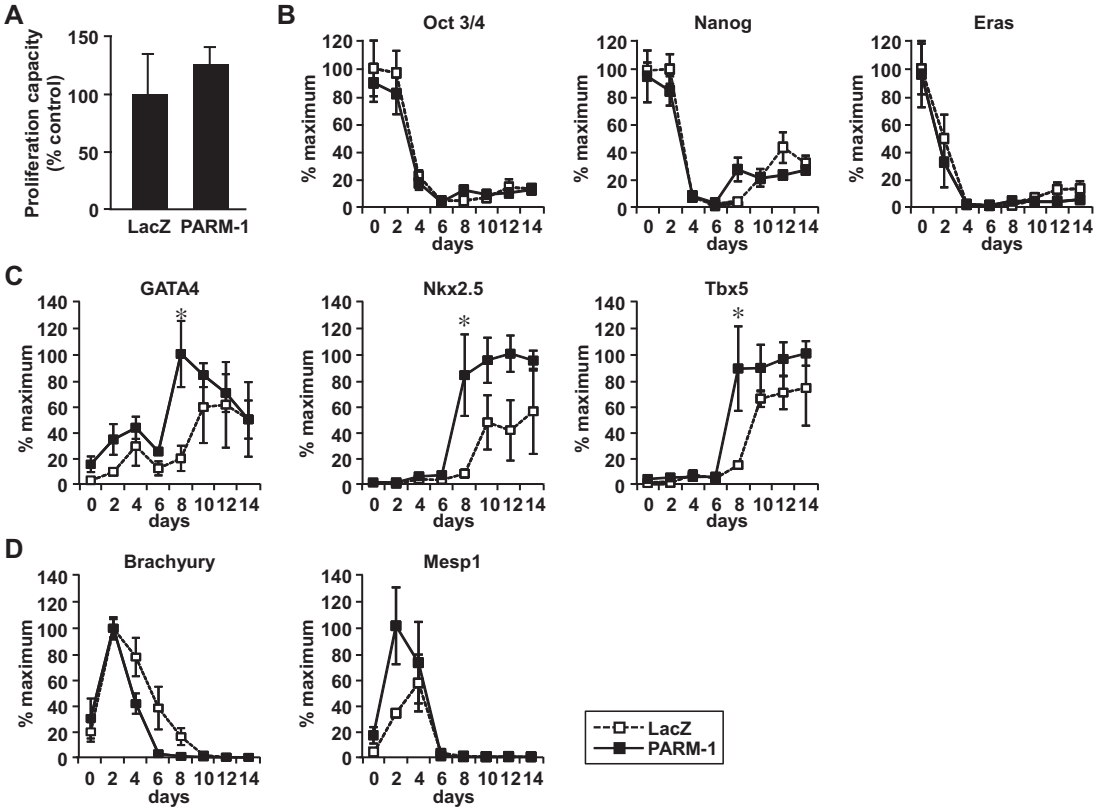

Fig. 3. Upregulated expression of cardiac transcription factors by PARM-1 overexpression. (A) BrdU incorporation was assessed in undifferentiated P19CL6-LacZ and -PARM-1 cells. (B) Oct3/4, Nanog, and Eras mRNA expression was analyzed by quantitative real-time PCR with total RNA isolated from P19CL6-LacZ and -PARM-1 cells on the indicated days. (C) Expression of GATA4, Nkx2.5, and Tbx5 mRNA was analyzed by quantitative real-time PCR with total RNA isolated from P19CL6-LacZ and -PARM-1 cells on the indicated days. *$P < 0.05$ compared with P19CL6-LacZ cells at the corresponding days. (D) Expression of Brachyury and Mesp1 mRNA was analyzed by quantitative real-time PCR with total RNA isolated from P19CL6-LacZ and -PARM-1 cells on the indicated days. LacZ, P19CL6-LacZ cells. PARM-1, P19CL6-PARM-1 cells.

P19CL6 cells did not affect Brachyury and Mesp1 expression, but augmented GATA4, Nkx2.5, and Tbx5 expression during cardiomyogenesis, resulting in enhanced cardiomyogenesis. These results suggest that PARM-1 promotes cardiomyogenesis through

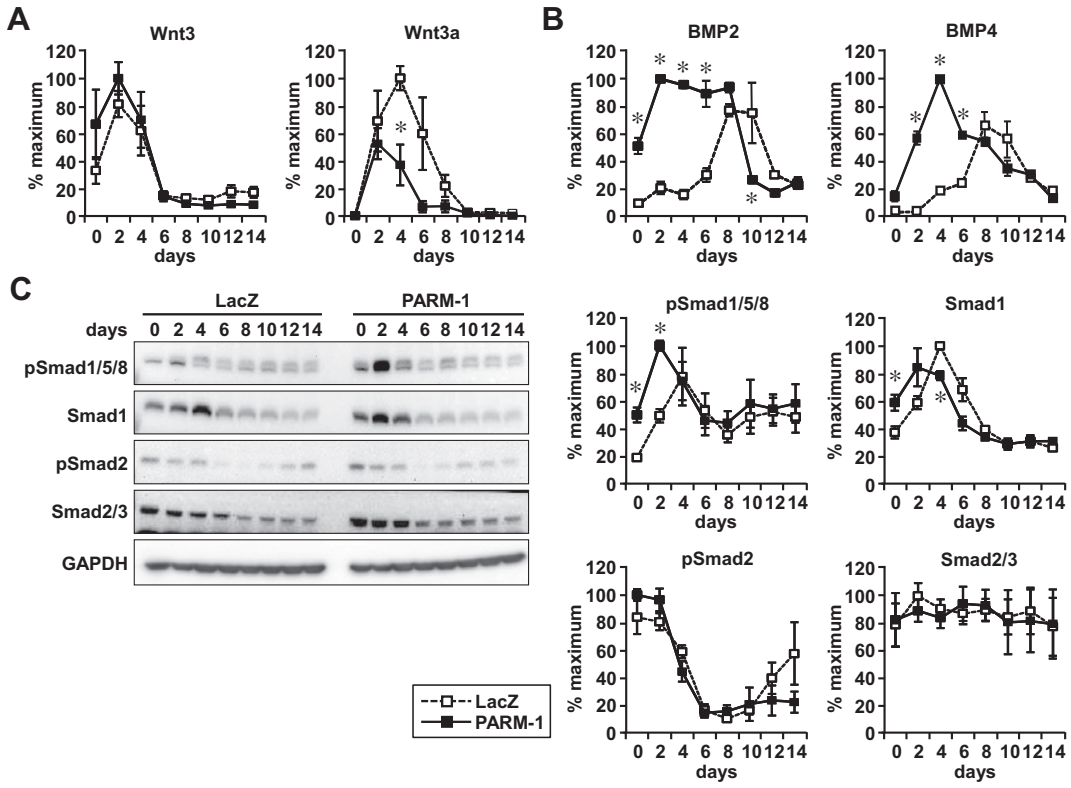

Fig. 4. Enhanced activation of BMP/Smad signaling by PARM-1 overexpression. (A) Wnt3 and Wnt3a mRNA expression was analyzed by quantitative real-time PCR with total RNA isolated from P19CL6-LacZ and -PARM-1 cells on the indicated days. (B) BMP2 and BMP4 mRNA expression was analyzed by quantitative real-time PCR with total RNA isolated from P19CL6-LacZ and -PARM-1 cells on the indicated days. (C) Expression of pSmad1/5/8, Smad1, pSmad2, and Smad2/3 was analyzed by Western blots with cell lysates prepared from P19CL6-LacZ and -PARM-1 cells on the indicated days. GAPDH was used as a loading control. $^{*}P < 0.05$ compared with P19CL6-LacZ cells at the corresponding days. LacZ, P19CL6-LacZ cells. PARM-1, P19CL6-PARM-1 cells.

enhancement of cardiac specification but not mesodermal induction.

Canonical Wnt signaling is required for mesodermal induction, while its downregulation is subsequently required for specification of precardiac mesoderm [25]. In the canonical Wnt pathway, Wnt3 and Wnt3a play critical roles in mesodermal development [26–29], and BMPs are responsible for inducing cardiac differentiation through induction of the myocardial gene program, such as Nkx2.5, GATA4, and ventricular MHC expression, after the canonical Wnt pathway is downregulated [30,31]. Wnt3 expression in P19CL6-PARM-1 cells did not differ from that in P19CL6-LacZ cells, while the peak of Wnt3a expression in differentiating P19CL6-PARM-1 cells was earlier than that of P19CL6-LacZ cells, suggesting that downregulation of the canonical Wnt pathways after mesodermal induction may be accelerated in P19CL6-PARM-1 cells. Overexpression of PARM-1 induced BMP2 expression and pSmad1/5/8 phosphorylation in undifferentiated P19CL6 cells, and enhanced BMP2 and -4 expression and pSmad1/5/8 phosphorylation during the early phase of cardiomyogenesis, which preceded augmentation of GATA4, Nkx2.5, and Tbx5 expression. These data indicate that the promotion of cardiac specification in P19CL6-PARM-1 cells is likely attributable to BMP/Smad signaling enhanced by PARM-1. Accelerated downregulation of canonical Wnt signaling after mesodermal induction may also contribute to this process in P19CL6-PARM-1 cells. Our results also suggest that the expression of endogenous PARM-1 during cardiomyogenesis partly regulates cardiac specification through the activation of BMP/Smad signaling.

We previously demonstrated that PARM-1 is localized to ER in cardiomyocytes [7]. Perturbation of ER functions results in ER stress via the activation of a complex of cytoplasmic and nuclear signaling pathways termed the unfolded protein response (UPR) [32]. The initial response of the UPR reestablishes ER homeostasis and enhances survival, but prolonged UPR leads to apoptosis. In the heart, ER stress response has been demonstrated to be activated in several pathological models including myocardial infarction, ischemia/reperfusion, and pressure overload-induced hypertrophy [32]. During development, inhibition of ER stress by tauroursodeoxycholic acid has been shown to improve the rate of two-cell embryo development to blastocysts [33]. Deficiency of X-box binding protein (XBP)-1, which regulates a subset of ER resident chaperone genes in the UPR, causes embryonic lethality [34]. ATF6α- or ATF6β-deficient mice are viable, but double-knockout mice are embryonic lethal [11]. Thus, these reports suggest the involvements of ER stress and UPR signaling molecules in developmental processes. PARM-1 regulated PERK, ATF6, and CHOP expression in cardiomyocytes [7], suggesting the possibility that PARM-1-induced cardiomyogenesis is also mediated by the UPR signaling molecules. Furthermore, considering the protective role of PARM-1 against ER stress-induced apoptosis [7], the promotion of cardiomyogenesis in P19CL6-PARM-1 cells may reflect PARM-1-mediated increases in both cardiac specification in differentiating cells and cell viability in differentiated cells. BMP2 has been shown to activate UPR signaling molecules, including ATF6, PERK, and CHOP, during osteoblast differentiation [35,36]. BMP2 also regulates the expression of ATF6 and the spliced form of XBP1 in chondrogenesis [37]. Although it remains unknown how PARM-1 regulates the expression of BMP2 and -4, PARM-1 may also regulate PERK, ATF6, and CHOP expression through BMP2 expression. Further studies are needed to determine its molecular mechanisms.

In the present study, we investigated cardiomyogenesis using P19CL6 cells and revealed the novel role of PARM-1 in cardiac

differentiation of stem cells. PARM-1 regulates cardiomyogenesis through the activation of BMP/Smad signaling. These findings provide a novel insight into cardiac specification, which might contribute to improvement of the efficiency of differentiation into specific cell types.

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