Inhibition of Cardiomyocyte Differentiation by cADPR

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Background: The role and mechanism of cADPR, an endogenous Ca²⁺ mobilizing nucleotide, in cardiomyogenesis remain to be determined.

Results: We found that inhibition of the cADPR cascade facilitated cardiomyocyte differentiation of mouse ES cells.

Conclusion: The CD38-cADPR-Ca²⁺ signaling pathway antagonizes the cardiomyocyte differentiation of mouse ES cells.

Significance: Inhibition of cADPR signaling should provide a good approach to enrich functional cardiomyocytes from ES cells.

Cyclic adenosine diphosphoribose (cADPR) is an endogenous Ca²⁺ mobilizing messenger that is formed by ADP-ribosyl cyclases from nicotinamide adenine dinucleotide (NAD). The main ADP-ribosyl cyclase in mammals is CD38, a multi-functional enzyme and a type II membrane protein. Here we explored the role of CD38-cADPR-Ca²⁺ in the cardiomyogenesis of mouse embryonic stem (ES) cells. We found that the mouse ES cells are responsive to cADPR and possess the key components of the cADPR-signaling pathway. In vitro cardiomyocyte (CM) differentiation of mouse ES cells was initiated by embryoid body (EB) formation. Interestingly, beating cells appeared earlier and were more abundant in CD38 knockdown EBs than in control EBs. Real-time RT-PCR and Western blot analyses further showed that the expression of several cardiac markers, including GATA4, MEF2C, NKX2.5, and α-MLC, were increased markedly in CD38 knockdown EBs than those in control EBs. Similarly, FACS analysis showed that more cardiac Troponin T positive CMs existed in CD38 knockdown or 8-Br-cADPR, a cADPR antagonist, treated EBs compared to that in control EBs. On the other hand, overexpression of CD38 in mouse ES cells significantly inhibited CM differentiation. Moreover, CD38 knockdown ES cell derived CMs possess the functional properties characteristic of normal ES cell derived CMs. Last, we showed that the CD38-cADPR pathway negatively modulated the FGF4-Erks1/2 cascade during CM differentiation of ES cells, and transiently inhibition of Erk1/2 blocked the enhance effects of CD38 knockdown on the differentiation of CM from ES cells. Taken together, our data indicate that the CD38-cADPR-Ca²⁺ signaling pathway antagonizes the CM differentiation of mouse ES cells.

Embryonic stem (ES) cells hold great promise for tissue engineering (1,2). Their remarkable capacity to self-renew and their pluripotency properties make these cells ideal candidates for regenerative medicine. Studies have already demonstrated the potential of ES cell-derived cardiomyocytes (CMs) in recolonizing the scar of infarcted rat heart (3-5). Several methods have been developed to direct ES cells toward cardiac lineage. The most popular one is the use of embryoid bodies (EB), generated by aggregating ES cells in suspension, thereby initiating the differentiation of cells toward the three embryonic layers (6,7). Directed CM specification of ES can be promoted by biological molecules, such as BMP2, activin-A, and BMP4, or by co-culture with an endodermal...
cell line. Increased CM specification of ES cells can also be enhanced by small biological and non-biological molecules, including sulfonylhydrazone, an inducer of Nkx2.5, and BIO, a GSK-3 inhibitor (2,8-13). Yet, the efficiency for ES-directed cardiac lineage is still low.

Accumulated evidence has established Ca\(^{2+}\) as a major second messenger in directing the fate of stem cells and patterning of the heart in the embryo. Ca\(^{2+}\) exerts multiple functions in the processes of cardiac cell differentiation and in early heart development upon the amplitude, space, and time of Ca\(^{2+}\) signaling (2,8,9,14). Decoding the molecular mechanisms of the multifaceted roles of Ca\(^{2+}\) signaling will certainly help in achieving a high efficiency of directed CM differentiation of ES cells and make them suitable for cell replacement therapy.

Cyclic adenosine diphosphoribose (cADPR) is an endogenous second messenger for mobilizing Ca\(^{2+}\) release in a wide variety of cell types and species. cADPR is formed from nicotinamide adenine dinucleotide (NAD) by ADP-ribosyl cyclases. cADPR elicits Ca\(^{2+}\) release through ryanodine receptors (RyRs). The cADPR-mediated Ca\(^{2+}\) signaling has been indicated in a variety of cellular processes (15,16). Six homologues of ADP-ribosyl cyclase have been identified so far: Aplysia ADP-ribosyl cyclase, three sea urchin homologues (17), and two mammalian homologues, CD38 and CD157 (18). CD38 is a membrane-bound protein and the main mammalian ADP-ribosyl cyclase. As a novel multi-functional enzyme, CD38 catalyzes the synthesis and hydrolysis of both cADPR and NAADP, two structurally and functionally distinct Ca\(^{2+}\) messengers. Virtually all mammalian tissues ever examined have been shown to express CD38. CD38 knockout mice exhibited multiple physiological defects, ranging from impaired immune responses to metabolic disturbances (15,18).

Since its discovery, cADPR has been found to regulate cardiac functions. cADPR induces Ca\(^{2+}\) release in a variety of cardiac tissues and cells. cADPR was indicated as a regulator of excitation-contraction coupling in cardiac myocytes since cADPR enhances Ca\(^{2+}\) transients in cardiac myocytes whereas a cADPR antagonist reduces Ca\(^{2+}\) transients and contractions (19-23). Moreover, cardiac hypertrophy developed in CD38 knockout male mice. The RyR protein was increased only in female CD38 knockout mice compared with wildtype, suggesting that the CD38/cADPR signaling plays an important role in intracellular Ca\(^{2+}\) homeostasis in cardiac myocytes in vivo, although its deficiency was compensated differentially according to gender (24,25).

Along this line, the involvement of cADPR and the cyclase in the alteration of Ca\(^{2+}\) homeostasis during the ischemia/reperfusion injury of the heart have been reported (22,23). Yet, the role and mechanism of CD38/cADPR signaling in cardiomyogenesis remain to be determined.

Here we examined the role of CD38 in the CM differentiation of mouse ES cells and found that the CD38-cADPR-Ca\(^{2+}\) cascade antagonizes the cardiogenesis of mouse ES cells.

**EXPERIMENTAL PROCEDURES**

**Cell culture-** ES cells, D3 from Prof. Tsang SY at Chinese University of Hong Kong, and R1 from ATCC, were normally maintained with feeders (mouse embryonic fibroblasts) in Dulbecco’s Modified Eagle Medium plus15% FBS (ES qualified, Invitrogen), 1% nonessential amino acids, 1% penicillinstreptomycin, 0.2% 2-mercaptoethanol, 1000 U/ml leukemia inhibitory factor (LIF). Cells are passaged every two days. Prior to any experimental procedures, ES cells are cultured in feeder-free ES medium containing LIF on gelatin-coated plates for two passages. The pluripotency of ES cells are periodically assessed by alkaline phosphatase assay and Oct4 immunostaining.

**Cardiomyocyte differentiation of mouse ES cells-** CM differentiation of mouse ES cells was performed as described previously (6,7). To start differentiation, mouse ES cells were cultured in the differentiation medium (standard medium without LIF) in hanging drops to form embryoid bodies (EBs) (800 cells/drop) for 2 days, and then kept in suspension for 2 days. The EBs were finally plated into gelatin-coated cell culture dishes (day 0) to allow the cells to go through the entire differentiation process. Cardiomyocytes (CMS) present in EBs started to rhythmically contract around 6-8 days after differentiation.

**Isolation of ES-derived cardiomyocyte-** CMs derived from ES cells were isolated as described previously (26). Briefly, beating areas in EBs were microscopically dissected from EBs and then incubated in 1 mg/ml collagenase II (Life technologies) plus 1 mg/ml pancreatin (Sigma) in enzyme buffer (120 mM NaCl, 5.4 mM KCl, 5 mM MgSO\(_4\), 5 mM Na pyruvate, 20 mM
glucose, 20 mM taurine, 0.03 mM CaCl₂, 10 mM HEPES, pH 6.9) at 37°C for 30 minutes. After digestion, the isolated cells were incubated in KB solution (85 mM KCl, 30 mM K₂HPO₄, 5 mM MgSO₄, 1 mM EGTA, 2 mM Na₂ATP, 5 mM Na pyruvate, 5 mM creatine, 20 mM taurine, 20 mM glucose, pH 7.2) with gentle shaking at room temperature for 30 minutes. Finally, the isolated cells were plated onto gelatin coated cover glasses to grow overnight for immunohistochemistry or Ca²⁺ measurement.

CD38 shRNA and CD38-GFP lentivirus production and infection- Five optimal 21-mers were selected in the mouse CD38 genes (Table S1). One 21-mer was selected in the GFP gene as a control. These sequences were then cloned into pLKO.1 vector for expressing shRNA. Likewise, a human CD38 cDNA was cloned into pCHMWS-GFP, a lentiviral vector with GFP at its C-terminal. The shRNA or CD38GFP lentivirus production was performed in 293T cells. For infection, ES cells were plated at a density of 3×10⁵ cells/well in 6-well plates. On the next day, 100 µL pools of shRNAs lentivirus were added to the cells in fresh medium containing 8 µg/mL polybrene. Two days later, cells infected with shRNA lentiviruses were selected in fresh medium containing puromycin (3 µg/mL) for 3–5 days. The puromycin-resistant cells were pooled and the knockdown efficiency was verified by both quantitative real-time RT-PCR and/or Western blot analyses. For ES cells infected with CD38-GFP lentiviruses on day five after infection, cells were trypsinized with 0.05% trypsin-EDTA, washed with PBS and resuspended in DMEM, and filtered through 70 µm cell strainer (BD Bioscience) to discard any cell aggregates. Sorting selection of CD38-GFP expressed cells was then performed using BD FACSARia I Cell Sorter (BD biosciences).

Western blot analysis- Western blot analysis was performed as described previously (27,28). In brief, cells were lysed in an ice-cold EBC lysis buffer (50 mM HEPES at pH 7.5, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, 150 µM PMSF, 10 mM NaF, 10 ng/ml leupeptin, 1 mM DTT, and 1 mM sodium vanadate) and passed through a 21-gauge needle several times to disperse any large aggregates. Protein concentrations of the cell lysates were determined by Bradford protein assay. 30 µg of protein per lane was diluted in the standard SDS-sample buffer and subjected to electrophoresis on 8 or 10% SDS polyacrylamide gels. Proteins were then transferred to an Immobilon PVDF membrane (Millipore, Billerica, MA), blocked with 5% milk in TBST (20 mM Tris, 150 mM NaCl, pH 7.6), and incubated with the primary antibody (CD38, SC-7049, Santa Cruz Biotechnology, 1:500 dilution; phosphor-specific Erk1/2, #9106, Cell Signaling Technology, 1:1000 dilution; NKX2.5, A7732, Santa Cruz, 1:1000 dilution; Cardiac Troponin I, ab19615, Abcam, 1:1000 dilution; myosin light chain 3, ab680, Abcam, 1:500 dilution; active β-catenin, #05-665, 1:1000 dilution, Millipore; Connexin 43, #3512, cell signaling Technology, 1:1000 dilution; Raf-B, SC-9002, Santa Cruz Biotechnology, 1:1000 dilution; GAPDH, #G8795, Sigma, 1:2000 dilution) overnight. After washing with TBST, the blots were probed with a secondary antibody (1:3000 dilution) for detection by chemiluminescence.

RNA isolation, RT-PCR, and quantitative real-time RT-PCR- Total RNA of ES cells and EBs at specified differentiated days was extracted using RNA extraction kit (Life Technologies). RT-PCR of ryr and CD38 using SuperScript® One-Step RT-PCR kit (Life Technologies) was performed with Takara PCR Thermal Cycler Dice (Takara). The quantitative real-time RT-PCR using the SuperScript® III Platinum® One-Step Q-RT PCR Kit (Invitrogen Life Technologies) was performed in MiniOpticon™ Real-time PCR Detection System (Bio-RAD) according to the manufacturer’s instructions. The primers for detecting CD38, ryr1, ryr2, ryr3, mef2c, GATA4, α-MHC, and Gapdh mRNAs are listed in Table S1. Relative gene expression was normalized to Gapdh expression.

Immunohistochemistry- Cells on cover glasses were fixed for 15 minute with 4% paraformaldehyde at room temperature, washed twice with PBS, and permeabilized with PBS containing 0.1% Triton X-100 for 30 minutes. Thereafter, the cover glasses were blocked with 1% normal donkey serum, 1% BSA, 0.1% Triton X-100 in PBS for 1 hour, and incubated with primary antibodies (cardiac Troponin T, MS-295-P, thermo, 1:500 dilution; α-actinin, sigma, 1:500 dilution) for 2 hours, followed by secondary antibody (Alexa Fluor® 488 Goat Anti-Mouse IgG, A11008, Life Technologies, 1:500 dilution) incubation for 1 hour. DAPI was used to stain the nuclei. Alexa Fluor® 568
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Phalloidin (A12380, Life technologies) staining was performed according to the manufacture’s recommendation. Cells were imaged using an inverted Olympus IX81 fluorescence microscope with a CellR image system. Flow cytometry analysis- EBs were digested into single cells by incubating with 1 mg/ml collagenase II (Invitrogen) plus 1 mg/ml pancreatin (Sigma) at 37°C for 30 minutes. The isolated single cells were then immunostained with cardiac Troponin T as described above and filtered through 40 µm cell strainer (BD Bioscience) to discard cell aggregates. Cardiac Troponin T positive cells were analyzed by the BD FACS Canto II analytic flow cytometer. Cytosolic Ca²⁺ in ES cells were measured using an Olympus epifluorescence microscope as described previously (29,30). Briefly, ES cells were cultured in 24-well plates at the density of 7x10⁶ cells /well in regular medium overnight and were labeled with 4 µM Fura-2 AM (Invitrogen) in HBSS at room temperature for 30 min. The cells were then washed with HBSS three times and incubated at room temperature for another 10 min. Cells were put on the stage of an Olympus inverted epifluorescence microscope and visualized using a 20x objective. Fluorescence images were obtained by alternate excitation at 340 nm and 380 nm with emission set at 510 nm. Images were collected by a CCD camera every 3 or 6 seconds and analyzed by a Cell R imaging software.

Cytosolic Ca²⁺ transients in isolated CMs were measured using a confocal imaging system (Olympus Fluoview System version 4.2 FV300 TIEMPO) connected with an inverted Olympus microscope (IX71). Briefly, cells were loaded with 2µM Fluo-4 AM (Life technologies) in Hanks’ balanced salt solution (HBSS) (Life technologies) for 30 min at room temperature. The cells were then washed once with HBSS and incubated for another 10 min before measurement. Line scan of Ca²⁺ transients within single CMs were recorded in line vs. time mode (XT) using the uplanAPO 20X objective at 378 frames per second. Spontaneously Ca²⁺ oscillation was recorded by area vs. time mode (XYT) as a line plot with the excitation at 488 nm. Images were collected by a CCD camera every 2 s and analyzed by a Cell R imaging system. For Ca²⁺ mobilization in single cell, a 60X oil immersion objective was used.

Electrophysiology- Action potentials in ES-derived cardiomyocytes were recorded with the conventional electrophysiological procedure as described previously (31). Briefly, single spontaneously contracting EB outgrowths from both control and CD38 knock down cells were microdissected and plated onto glass cover slips and maintained in differentiation medium for 2 to 3 days before electrophysiological recording. Cell clusters were superfused (2 mL/min, 37 °C) with Tyrode’s solution (140 mM NaCl, 5.0 mM KCl, 1.8 mM CaCl₂, 1mM MgCl₂, 10 mM Hepes, 10 mM glucose, pH 7.3 adjusted with NaOH). Electrical signals were recorded using an EPC-10 amplifier and Pulse software (Heka Elektronik, Lambrecht, Germany). Borosilicate glass electrodes were pulled with a Brown/Flaming puller (model P-97, Sutter Instrument, Nato, CA) and had resistances of 30-50 MΩ when filled with 3-M KCl. The tip potential was zeroed before the patch pipette contacted the cell. Cardiomyocyte activity was recorded in a current clamp mode with the sharp electrode using an EPC-10 amplifier and Pulse software (Heka Elektronik, Lambrecht, Germany). Endogenous cADPR measurement- EBs were extracted with 300 µL of cold 0.6 M perchloric acid, followed by neutralization with a solution (3:1) of chloroform and tri-n-octylamine by vortexing for 1 min. The neutral extracts were then supplemented with 20 mM sodium phosphate, pH 8, and incubated with an enzyme mixture (0.015 unit/ml NADase, 2 mM MgCl₂) for 18 h at 37 °C, followed by ultrafiltration with 96-well Immobilon-P plates to remove the NADase. The concentration of cADPR was finally measured by an enzymatic cycling assay as described previously (32).

RESULTS

Characterization of the CD38/cADPR/Ca²⁺ signaling in the mouse ES cells. To assess a potential contribution of the cADPR signaling to ES cell fate decisions, we surveyed the expression of CD38 and ryanodine receptors (RyRs) in several types of ES cells by RT-PCR. As shown in Figure 1A, CD38, and ryanodine receptors (RyRs) were expressed in several other mouse ES cells (Figures S1A, S1B, and S1C). Moreover, a cell permeant cADPR agonist, cIDPRE (33), at a concentration of 500 µM
induced Ca\textsuperscript{2+} release in intact D3 ES cells, which was significantly inhibited by pretreatment of the cells with a cADPR-antagonist, 8-Br-cADPR (200 μM), or a RyR antagonist, ryanodine (20 μM) (Figure 1B). These data indicate that the mouse ES cells are responsive to cADPR and possess the key components of the cADPR-signaling pathway.

To further establish the role of endogenous cADPR in extracellular stimuli-induced Ca\textsuperscript{2+} release in mouse ES cells, we screened a series of cytokines, growth factors, and neurotransmitters for their ability to induce Ca\textsuperscript{2+} release in the cells and the involvement of cADPR in the process. Indeed, we found that cADPR is required for Ca\textsuperscript{2+} spikes induced by a few of these stimuli in ES cells (data not shown). For example, bradykinin-induced Ca\textsuperscript{2+} changes were inhibited significantly by 8-Br-cADPR, high concentrations of nicotinamide, which is commonly used to inhibit the ADP-ribosyl cyclase activity thereby preventing cADPR production, or ryanodine, a RyR antagonist (Figure 1C). These data indicate that cADPR is responsible for mediating the bradykinin-induced Ca\textsuperscript{2+} increase in mouse ES cells. That cADPR is the messenger for the bradykinin-induced Ca\textsuperscript{2+} release has also been reported in several other cell types (34). Taken all the data together, it is clear that the cADPR signaling is functioning endogenously in mouse ES cells.

**CD38 knockdown in mouse ES cells.** Next, we examined the expression pattern of CD38 during the process of in vitro differentiation of ES cells initiated by EB formation. Increasing amounts of CD38 were expressed (Figures 2A and S1B) by the cells during the process, concomitant with the increase in the number of beating EBs (Figure 3A). However, CD38 expression in beating CMs was slightly decreased compared to that in undifferentiated ES cells (Figure S1D). These data indicated that the CD38/cADPR signaling might play a role in the CM differentiation of ES cells.

Thus, we knocked-down the expression of CD38 in mouse ES cells by infecting the cells with the lentiviruses carrying expression cassettes that encode short hairpin RNAs (shRNA) to generate gene-specific siRNAs against the mouse CD38. We have designed 5 distinct shRNA constructs against the mouse CD38 gene. As shown in Figure 2B, CD38 proteins were efficiently knocked-down by different CD38 shRNA constructs. Accordingly, endogenous cADPR levels in CD38 knockdown ES cells were markedly decreased (Figure S1E); whereas NADP levels remained unchanged (Figure S1F), and NAADP, another potential CD38 product, was not even detected in ES cells. Compared with the control scramble-shRNA infected ES cells, CD38 knock-down cells proliferated normally (Figure 2D) and exhibited no differences in the expression of Nanog, SSEA-1, and Oct3/4, the key transcription factors for the pluripotency of ES cells (Figure 2E), and in the activity of alkaline phosphatase compared to control (Figure S2), indicating that the CD38 knockdown ES cells are undifferentiated when maintained in regular ES medium.

**CD38-GFP expressed D3 ES cells.** Likewise, a stable CD38-GFP expressed ES cell line was established by infecting cells with lentiviruses carrying expression cassettes that encode human CD38 gene. As shown in Figure 2C, CD38-GFP indeed was expressed as a membrane protein. CD38-GFP overexpressing cells also exhibited no growth defects (Figure 2D), or differences in the expression of Nanog, SSEA-1, and Oct3/4 (Figure 2E), and in the activity of alkaline phosphatase (Figure S2), indicating that the CD38 overexpression also has no effects on the pluripotent properties of ES cells when maintained in regular ES medium.

**Requirement of the CD38/cADPR signaling for cardiomyocyte differentiation of mouse ES cells.** The control scramble-shRNA infected, CD38-knockdown, or CD38-GFP overexpressing cells were then plated to form EB to start differentiation. Surprisingly, CD38-knockdown EBs showed a much higher number of beating EBs and larger beating areas than control EBs, whereas few beating EBs appeared in CD38-GFP overexpressing cells (Figure 3A and data not shown). Immunofluorescence staining of the beating EBs also revealed the expression of cardiac specific Troponin T was much higher in the CD38-knockdown, or CD38-GFP overexpressing cells, respectively, compared to those in control cells. In addition, Western blot analyses showed that the expression of other cardiac protein markers, including NKX2.5 (Figure 3F),...
Inhibition of cardiomyocyte differentiation by cADPR Troponin I (Figure 3G), α-MLC (Figure 3H), and Connexin (Figure S3A) were markedly increased or decreased in CD38 knockdown or CD38-GFP overexpressing EBs, respectively, than those in control EBs. Flow cytometry analyses further showed that more cardiac Troponin T positive CMs existed in CD38 knockdown EBs compared to those in control EBs (Figures 4A and 4B). These results indicate that CD38 knockdown accelerates and enhances cardiomyocyte differentiation of mouse ES cells.

CD38 is a type II membrane protein and can also act as a signaling receptor unrelated to its enzymatic activity. Ligation by agonistic antibodies against CD38 can trigger a wide range of responses in various types of blood cells (18). Therefore, we examined whether the effects of CD38 on the differentiation of mouse ES is related to its cyclase activity. We first applied a potent CD38 inhibitor, CZ27 (35), to ES cells to inhibit CD38 cyclase activity. Indeed, CZ27 significantly inhibited the endogenous cADPR levels in ES cells (Figure 5A). The differentiation of CMs was then initiated in ES cells treated with or without CZ27. We found that CZ27 markedly increased the expression of several cardiac genes, including α-MLC (Figure 5B), α-MHC (Figure 5C), MEF2C (Figure 5D), and GATA4 (Figure 5E). Alternatively, the differentiation of CMs was initiated in ES cells treated with or without 8-Br-cADPR, a cADPR antagonist. More cardiac Troponin T positive cardiomyocytes were found in 8-Br-cADPR treated EBs compared to those in control EBs (Figures 5F and S3B). Taken together, our data indicated that the CD38/cADPR signaling cascade inhibits CM differentiation of mouse ES cells.

Functional characterization of CD38 knockdown and CD38-GFP overexpressing cardiomyocytes. We next tried to isolate CMs derived from wildtype, CD38 knockdown, and CD38-GFP overexpressing EBs. The beating CMs were purified from wildtype and CD38 knockdown EBs, but only few unbeating CMs were isolated from CD38-GFP expressing EBs (data not shown). Thus, the Ca\(^{2+}\) handling was only assessed in rhythmically beating wildtype or CD38-knockdown CMs. Both groups of CMs exhibited spontaneous Ca\(^{2+}\) oscillations assessed by confocal microscopy (Figure 6A). Quantifications of the Ca\(^{2+}\) oscillations showed no significant differences in either amplitudes or frequencies between these two groups (Figures 6B and 6C). In addition, immunofluorescence staining of α-actin and Troponin T showed that CD38-knockdown or CD38-overexpressing CMs had intact contractile apparatuses, exhibiting the classic striated appearance as the controls (Figures 6D and S4). Furthermore, action potentials (APs) of wildtype or CD38-knockdown CMs were measured, and all 3 major CM subtypes, namely atrial-like, pacemaker-like, and ventricular-like cells, were detected in similar percentage in both groups (Figures 7A and 7B). Both groups of CMs also responded similarly to drug applications, i.e. a reduced activity to nifedipine, a L-type Ca\(^{2+}\) channel antagonist, and an increased activity to the sympathetic neurotransmitter norepinephrine (Figure 7C). Collectively, these data indicated that the CMs derived from CD38 knockdown ES cells possess the functional properties characteristic of CMs derived from wildtype ES cells.

The CD38-cADPR pathway inhibits FGF4/MAPK during cardiomyocyte differentiation of mouse D3 ES cells. We finally analyzed the signaling pathways which CD38/cADPR might regulate to inhibit the CM differentiation of ES cells. Several growth factors or cytokines, including fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), wingless-related MMTV integration sites (Wnts), and Notch, have been shown to regulate early cardiogenesis (36). Therefore, western blot analyses on these known cardiac differentiation modulators were performed in wildtype and CD38 knockdown CMs. We found CD38 knockdown had little effect on Wnts, Notch, and BMPs signaling during the CM differentiation of ES cells (data not shown). Interestingly, from day 5 to day 10 after CM differentiation, Erk1/2 activation was markedly increased in CD38 knockdown EBs as compared with control EBs, yet Erk1/2 activation was decreased in CD38 knockdown EBs by day 15 after differentiation (Figure 8A). We then assessed whether FGFs are regulated by CD38/cADPR to modulate Erk activation during CM differentiation of mouse ES cells. Targeted enhancement with FGF4 promoted cardiogenic output from ES cells (37-39). Thus, real-time RT-PCR analyses of FGF4 were performed in wildtype and CD38 knockdown CMs. Consistently, the expression of FGF4 correlated with the activation of Erk1/2 in CD38 knockdown EBs, in that FGF4 mRNA in CD38
knockdown EBs was increased significantly from day 5 to day 10, but went down sharply by day 15 after differentiation, as compared to control EBs (Figure 8B). Thus, CD38 knockdown EBs were treated with U0126 (Figure S5), a MEK1 inhibitor, from day 5 to day 10 during CM differentiation. As shown in Figure 8C, treatment of CD38 knockdown EBs with U0126 markedly reversed the enhancement effects of CD38 knockdown on CM differentiation of ES cells. Taken together, these data indicated that the cADPR/CD38/Ca²⁺ pathway negatively modulates FGF4-Erk1/2 during CM differentiation of ES cells.

**DISCUSSION**

Here we studied the role of the CD38/cADPR/Ca²⁺ cascade in CM differentiation of mouse ES cells. We found that inhibition of the cADPR cascade by either CD38 knockdown, or treatment with 8-Br-cADPR, a cADPR antagonist, or CZ27, a CD38 inhibitor, facilitated the cardiogenesis, whereas overexpression of CD38 inhibited it. We further showed that CD38/cADPR negatively modulated the FGF4-ERK1/2 cascade to inhibit cardiomyocyte differentiation of ES cells. Interestingly, the CMs derived from CD38 knockdown ES cells were functionally similar to CMs derived from wildtype ES cells. Taken together, our data indicated that the CD38/cADPR signaling antagonizes the cardiogenesis of mouse ES cells. Therefore, applying cADPR antagonists might be a good approach to enrich the generation of CMs from ES cells.

Initiated by EB formation, CD38 expression was markedly increased in total EB compared to that in ES cells (Figures 2A and S1B). Yet, in purified cardiomyocytes, CD38 expression was similar, or even decreased, compared to that in undifferentiated ES cells (Figure S1D). Interestingly, during the neural differentiation of mouse ES cells initiated by monolayer cell culture (40), the expression of CD38 was decreased initially, and there was a mobility shift of CD38 during the late neuronal differentiation of ES cells (unpublished data). These data indicate that CD38 expression was dynamically regulated during ES cell differentiation and might differentially regulate the fate of ES cells during differentiation. Along this line, we found that the CD38/cADPR pathway is required for neural differentiation of ES cells, in that, perturbation of the CD38/cADPR pathway markedly inhibited neural entry of mouse ES cells (unpublished data). The roles of the CD38/cADPR pathway in ES cells differentiation into other cell lineages remain to be determined.

The essential role of Ca²⁺ in cardiac contraction and relaxation has been well established (14). Several Ca²⁺ handling genes knockout mice exhibited the defects in heart development (41-43). Similar results have also been observed in Drosophila (44), Xenopus (45), and Zebrafish (46), showing the evolutionary conserved role of Ca²⁺ signaling in heart development. Recent burgeoning research of ES cells further documented the multifaceted roles of Ca²⁺ signaling and the underlying mechanisms during different stages of cardiogenesis. It is well accepted that the Ca²⁺ signaling temporally and spatially regulates cardiogenesis in a dose dependently manner by fine tuning both cardiac gene expression and myofibrillogenesis (47-49). Here we added another role to this versatile ion: the CD38/cADPR mediated Ca²⁺ mobilization antagonizes cardiogenesis by inhibiting the FGF4/MAPK cascade. Although a majority of Ca²⁺ signaling genes promote CM differentiation from ES cells (50,51), similar results have been observed in calreticulin-null ES cells, in which cardiomyogenesis was accelerated yet dysregulated (26).

We also found that the CD38/cADPR cascade inhibited the FGF4-Erk1/2 pathway during the CM differentiation of ES cells, yet the underlying molecular details remain unclear. It has been shown that inhibition of the CD38/cADPR/Ca²⁺ signaling abolished local reactive oxygen species (ROS) production from sarcoplasmic reticulum (SR) via NAD(P)H oxidase in coronary arterial myocytes (52). Interestingly, oxidizing agents inhibited the ability of Oct-1, Oct-3, Sp1, and several Sp1-related nuclear proteins to bind important cis-regulatory elements located in the FGF-4 gene (53). Thus, it is possible that the CD38/cADPR/Ca²⁺ signaling regulate the redox state of the cell to inhibit gene transcription of FGF4, thereby inhibiting Erk1/2 activation during CM differentiation of mouse ES cells. It is also noteworthy that the expression of FGF4 and the activation of Erk1/2 were upregulated during early CM differentiation (from day 5 to day 10) but downregulated at late differentiation (after day 15) in CD38 knockdown EBs as compared to the control (Figures 8A and 8B).
The significance of the temporally differential regulation of the FGF4-Erk1/2 by the CD38/cADPR remains unknown. Yet, the effects of several known signaling events on CM differentiation of ES cells are temporally dependent. For example, transient inhibition of BMP signaling by Noggin induces CM differentiation of mouse ES cells (12).

Although cardiac differentiation of ES cells is regulated by the interplay among VEGF, BMPs, FGFs, and Wnts (14,54), other unknown signaling events might also participate in the cardiomyogenesis in the embryo. Along this line, it has been shown recently that granulocyte colony-stimulating factor receptor signaling promotes cardiac differentiation of ES cells (55). Therefore, it is possible that the cADPR signaling may also temporally relate to other signaling events besides FGF4 to regulate the fate of ES cells.

Another unexpected result of the current study is that CMs derived from CD38 knockdown ES cells were functionally comparable to normal ES cells derived CMs, considering that cADPR is tightly linked to contractility of adult CMs (19-23,56). In adult CMs, the major source of Ca\textsuperscript{2+} for excitation-contraction coupling is RyR-mediated Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) from SR initiated by Ca\textsuperscript{2+} entry via L-type Ca\textsuperscript{2+} channels (14). In contrast, the SR Ca\textsuperscript{2+} stores in embryonic CMs are present but immature, and RyR-mediated Ca\textsuperscript{2+} releases only plays a minor role in contractility of ES cells-derived CMs (47,57-59). It was suggested that the spontaneous Ca\textsuperscript{2+} currents in embryonic CMs depend on IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release to promote depolarization of the plasma membrane, thereby triggering voltage-dependent Ca\textsuperscript{2+} influx (57). We reasoned that the minor role of RyRs in the Ca\textsuperscript{2+} handling in embryonic CMs might explain why CMs derived from CD38 knockdown ES cells are functional similar to wildtype CMs. However, given that a matured and functional SR is essential for heart development, defects in the CD38/cADPR cascade could eventually harm the maturation of normal heart. Therefore, it is essential to add back cADPR agonists or remove cADPR antagonists after generation of CMs from ES cells by inhibiting cADPR signaling in order to obtain matured CMs. In addition, as mentioned previously that cardiac hypertrophy developed in CD38 knockout male mice (25), it is of interest to assess whether cADPR agonists could rescue the defected heart development in CD38 null male mice.

REFERENCES

FOOTNOTES
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The abbreviations used are: cADPR, cyclic adenosine diphosphate ribose; CM, cardiomyocyte; ES, embryonic stem cell; RyRs, ryanodine receptors; sarcoplasmic reticulum (SR); cIDPRE, N^1-[(5''-O-Phosphorylethoxy)methyl]-5'-O-phosphorylinosine 5',5''-cyclicpyrophosphate.

FIGURE LEGENDS
Figure 1. The cADPR signaling in mouse D3 ES cells. (A) Expressions of CD38, ryr1, ryr2, and ryr3 in D3 ES cells were determined by RT-PCR. (B) Ca^{2+} increase in ES cells induced by a cell-permeant cADPR agonist, cIDPRE (500 μM), in the presence or absence of 8-Br-cADPR (200 μM) or ryanodine (50 μM). (C) Pretreatment of Fura-2 loaded D3 ES cells with either 8-Br-cADPR (200 μM), nicotinamide (20 mM), or ryanodine (50 μM) inhibited the bradykinin (10 μM)-induced Ca^{2+} increase. The graph represents data from three independent experiments expressed as means ± S.E., n=20 to 30.
Inhibition of cardiomyocyte differentiation by cADPR

Figure 2. The effects of CD38 knockdown and overexpression on mouse D3 ES cells. (A) CD38 expression during the differentiation of D3 ES cells initiated by EB formation was determined by anti-CD38 immunoblot analysis. (B) CD38 knockdown by five distinctive shRNAs in D3 ES cells was verified by anti-CD38 immunoblot analysis. (C) The DIC and fluorescence images of D3 ES cells that stably express CD38-GFP. (D) Growth curves of wildtype, CD38 knockdown, and CD38-GFP expressing D3 ES cells were determined by the WST assay. (E) The expression of Oct3/4, Nanog, and SSECA in wildtype, CD38 knockdown, and CD38-GFP expressing D3 ES cells. Scale bars, 10 μm.

Figure 3. The antagonistic effects of CD38 on cardiomyocyte differentiation of mouse ES cells. The control scramble-shRNA infected, CD38-knockdown, or CD38-GFP overexpressing cells were plated to form EB to start CM differentiation as described in “EXPERIMENTAL PROCEDURES”. (A) Ratio of beating EB derived from wildtype, CD38 knockdown, and CD38-GFP overexpressing D3 ES cells for the indicated times. The results plotted represent the means ± S.D. for three independent experiments. (B) Immunofluorescent staining of Troponin T in EBs (day 10) derived from wildtype and CD38 knockdown D3 ES cells, scale bars, 10 μm. Representative of three independent experiments. (C) to (E) Quantitative RT-PCR analyses of α-MHC (C), Mef2C (D), and GATA 4 (E) in EBs derived from control, CD38 knockdown, and CD38-GFP expressing D3 ES cells for the indicated times. The results plotted represent the means ± S.D. for three independent experiments. The * symbols indicate the results of t Test analysis, p < 0.05. (F) to (I) Western blot analyses of Nkx2.5 (F), Troponin I (G), and α-MLC (H) in EBs derived from control, CD38 knockdown, and CD38-GFP expressing D3 ES cells for the indicated times. Representative of three independent experiments.

Figure 4. Promotion of the cardiomyocyte differentiation of mouse ES cells by CD38 knockdown. Flow cytometry analyses of Troponin T immunostaining of day 9, 13, and 20 EBs derived from wildtype and CD38 knockdown D3 (A) and R1 (B) ES cells. Data in (A) and (B) were representative of three and two independent experiments, respectively. The * symbols indicate the results of t Test analysis, p < 0.05.

Figure 5. Enhancement of the cardiomyocyte differentiation of mouse ES cells by CZ27 and 8-Br-cADPR treatment. (A) CZ27 (100 μM) significantly inhibited endogenous cADPR concentration in D3 cells as determined by a cycling assay. (B) Western blot analysis of α-MLC in EBs derived from control and CZ27 (100 μM) treated D3 ES cells. (C) to (E) Quantitative RT-PCR analyses of α-MHC (C), Mef2c (D), and GATA 4 (E) in EBs derived from control and CZ27 (100 μM) treated D3 ES cells. The results plotted represent the means ± S.D. for three independent experiments. (F) Flow cytometry analyses of Troponin T immunostaining of day 9 and 13 EBs derived from wildtype and 8-Br-cADPR (100 μM) treated D3 ES cells. Data in (F) were representative of three independent experiments. The * symbols indicate the results of t Test analysis, p < 0.05.

Figure 6. The Ca²⁺ handling and morphology of cardiomyocyte derived from wildtype, CD38 knockdown, and CD38 GFP expressing D3 ES cells. (A) The spontaneous oscillations of intracellular Ca²⁺ in CMs derived from wildtype and CD38 knockdown ES cells as assessed by line scan presenting a distance-time plot of Ca²⁺. (B) and (C) The amplitudes (B) and frequencies (C) of (A). (D) Immunofluorescence staining of α-actin and Troponin T in CMs derived from wildtype, CD38 knockdown and CD38 overexpressing ES cells.

Figure 7. Functional characterization of cardiomyocytes derived from wildtype and CD38 knockdown ES cells. Spontaneously beating CMs were isolated from wildtype and CD38 knockdown EBs (day 10) as described in “EXPERIMENTAL PROCEDURES”. (A) Representative AP recordings from spontaneously beating CMs derived from wildtype and CD38 knockdown ES cells. Based on AP shape and duration, the CMs were grouped into three different cardiac subtypes: atrial-like, pacemaker-like, and ventricular-like cells. (B) The distribution of cardiac subtypes in wildtype and CD38 knockdown ES cells derived CMs, n=46 and 41 CMs, respectively. (C) Both the wildtype and CD38 knockdown ES derived CMs showed a reduced activity to nifedipine, a L-type Ca²⁺
channel antagonist, and an increased activity to norepinephrine (NE), a sympathetic neurotransmitter treatment. Nifedipine or NE was perfused into the system once the AP recordings of ES-CMs were stable.

Figure 8. CD38/cADPR inhibits FGF2/MAPK during cardiomyocyte differentiation of mouse D3 ES cells. (A) Activation of the Erks1/2 during CM differentiation of wildtype and CD38 knockdown ES cells as determined by phospho-MAPK immunoblot analysis. (B) Quantitative RT-PCR analysis of FGF4 in control and CD38 knockdown ES cells derived EBs. The results plotted represent the means ± S.D. for three independent experiments. (C) Flow cytometry analyses of Troponin T immunostaining of day 9 and day 13 EBs derived from wildtype and CD38 knockdown D3 cells treated with or without MEK1 inhibitor, U0126 (20 μM). Data in (C) were representative of three independent experiments. The * symbols indicate the results of t Test analysis, p < 0.05.
Inhibition of cardiomyocyte differentiation by cADPR

Figure 1

A

Ryr1  Ryr2  Ryr3  CD38
300 bp- 200 bp- 100 bp-

B

B

C

pretreated with Ryanodine
pretreated with 8-Br-cADPR
pretreated with nicotinamide

BK addition
Figure 2

A

Days after differentiation

B

C

CD38-GFP

DIC

E

control

CD38-shRNA

CD38-GFP

Oct3/4

Nanog

SSEA-1
Inhibition of cardiomyocyte differentiation by cADPR

Figure 4

A  D3 cells

<table>
<thead>
<tr>
<th>Day</th>
<th>TnT+ (%)</th>
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<tbody>
<tr>
<td>9</td>
<td>0.7%</td>
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<tr>
<td>13</td>
<td>0.9%</td>
</tr>
<tr>
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negative control  Scramble-shRNA  CD38-shRNA

B  R1 cells

<table>
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<tr>
<th>Day</th>
<th>TnT+ (%)</th>
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<tbody>
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<tr>
<td>13</td>
<td>0.7%</td>
</tr>
<tr>
<td>24</td>
<td>0.3%</td>
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</tbody>
</table>

negative control  Scramble-shRNA  CD38-shRNA

TnT+ Cells (%)
Inhibition of cardiomyocyte differentiation by cADPR

Figure 5

A

B

days after differentiation
0 5 10 15
control

C

D

E

Ratio (α-MHC/GAPDH)

Ratio (Mef2C/GAPDH)

Ratio (GATA4/GAPDH)

F

Day 9

Day 13

negative control

DMSO treated D3

8-Br-cADPR treated

D3 cells

TnT+ 1.1%

TnT+ 2.1%

TnT+ 24.5%

TnT+ 0.9%

TnT+ 7.2%

TnT+ 33.6%

TnT positive CMs (%)

DMSO 8-Br-cADPR
Inhibition of cardiomyocyte differentiation by cADPR

Figure 6

A

Scramble

CD38 shRNA

B

C

D

Control

CD38-shRNA

CD38-GFP

α-Actin

Troponin T
Inhibition of cardiomyocyte differentiation by cADPR

Figure 7

A

Nodal-like

20 mV

0 -

Atrial-like

20 mV

0 -

Ventricular-like

25 mV

0 -

1s

100 ms

B

wildtype

CD38-shRNA

Embryonic atrial-like

Embryonic ventricular-like

Embryonic nodal-like

63%

21.8%

15.2%

61%

14.6%

24.4%

C

wildtype

CD38-shRNA

Control

Nifedipine 1 μM

Control

Nifedipine 1 μM

Control

NE 1 μM

Control

NE 1 μM

0 -

10 mV

200 ms

10 mV

100 ms

10 mV

200 ms

10 mV

200 ms
Inhibition of cardiomyocyte differentiation by cADPR

Figure 8

A

B

C

Ratio (FGF4/GAPDH)

Day 5

Day 10

Day 15

Day 9

Day 13

TnT+ Cells (%)

TnT+

TnT+

TnT+

TnT+

negative control

Scramble-shRNA

CD38-shRNA

CD38-shRNA + U0126

0.3%

1.91%

8.9%

3.4%

0.9%

4.3%

20.7%

5.1%

Day 9

Day 13

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