Calreticulin reveals a critical Ca\(^{2+}\) checkpoint in cardiac myofibrillogenesis

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Introduction

Cardiogenesis is one of the earliest processes during vertebrate embryogenesis (Olson and Srivastava, 1996; Lin et al., 1997b; Black and Olson, 1998; Charron and Nemer, 1999; Harvey, 1999; Harvey and Rosenthal, 1999; Schwartz and Olson, 1999; Frey et al., 2000; Srivastava and Olson, 2000). Although intracellular Ca\(^{2+}\) is a critical second messenger in the heart, regulating diverse functions from gene expression to cell contraction and relaxation (Pozzan et al., 1994; Clapham, 1995), the potential contribution of Ca\(^{2+}\) signaling during early stages of cardiac development remains largely unexplored.

Within the ER, the Ca\(^{2+}\) reservoir of nonmuscle cells, Ca\(^{2+}\) is mainly bound to calreticulin (Michalak et al., 1999). Calreticulin is a highly conserved, 46-kD protein that modulates many cellular functions, including Ca\(^{2+}\) homeostasis (Liu et al., 1994; Bastianutto et al., 1995; Camacho and Lechleiter, 1995; Mery et al., 1996; Coppolino et al., 1997; Fasolato et al., 1998; John et al., 1998), protein folding (Helenius et al., 1997; Krause and Michalak, 1997; Ellgaard and Helenius, 2001), integrin-dependent cell adherence (Coppolino et al., 1995; Opas et al., 1996; Coppolino et al., 1997), and steroid-dependent gene expression (Burns et al., 1994; Dedhar et al., 1994).

Calreticulin is essential for proper cardiac development because knockout of the calreticulin gene is embryonically lethal due to impaired cardiac development (Mesaeli et al.,...
Calreticulin-null mouse embryos show a marked decrease in ventricular wall thickness and deep intertrabecular recesses in the ventricular walls (Mesaeli et al., 1999). Importantly, at early stages of cardiac development, calreticulin is highly expressed in differentiating cardiomyocytes (Mesaeli et al., 1999). It is subsequently downregulated in the mature heart (Mesaeli et al., 1999) because elevated expression of calreticulin in newborn hearts of transgenic mice leads to complete heart block and sudden death (Nakamura et al., 2001a).

Mechanisms responsible for impaired cardiac development in calreticulin-deficient (crt<sup>−/−</sup>) mice are not well understood. Here we used calreticulin-deficient crt<sup>−/−</sup> embryonic stem (ES)* cells to investigate the role of the protein in early events of cardiac differentiation.

We show that calreticulin-deficient cardiomyocytes derived from ES cells had an impaired myofibrillogenesis due to decreased ventricular myosin light chain 2 (MLC2v) expression and phosphorylation. Nuclear translocation of myocyte enhancer factor C2 (MEF2C), a transcription factor responsible for activation of several cardiac specific embryonic genes, including the MLC2v, was inhibited in crt<sup>−/−</sup> cardiomyocytes. Furthermore, calreticulin-deficient myocytes featured disorganized myofilaments and inhibited contractile activity. Most importantly, this phenotype was reversed by transient elevation of cystolic-free calcium ([Ca<sup>2+</sup>]<sub>c</sub>) with a Ca<sup>2+</sup> ionophore. This reveals a Ca<sup>2+</sup>-dependent checkpoint in early events associated with cardiac myofibrillogenesis.

### Results

**Phenotype of calreticulin-deficient embryoid bodies**

Fig. 1 shows that crt<sup>−/−</sup> embryoid bodies (EBs) cultured in suspension between day 2 (D2) and D6 had a significantly larger size than wild-type (wt) EBs (669 ± 60 μm, vs. 359 ± 26 μm, respectively), as measured at D6 (Fig. 1 A, left). Despite the size difference, both cell types had similar protein content as measured for 20 pooled EBs (4.66 ± 0.55 and 4.82 ± 1.06 μg/μl in wt and crt<sup>−/−</sup> EBs, respectively; n = 3; Fig. 1 A, right). In addition, no difference in the growing capacity (mitotic rate) of cultured wt versus crt<sup>−/−</sup> undifferentiated ES cells was observed (unpublished data).

Removal of leukemia inhibitory factor (LIF) from the culture medium induces ES cell differentiation (Maltsev et al.,...
Calreticulin and Ca\(^{2+}\) in cardiogenesis

Li et al. 105

1994) into EBs containing clusters of contracting cardiomyocytes after 7 d of culture (Meyer et al., 2000). Therefore, we analyzed for the presence of the contractile activity and the beating frequency of cardiomyocytes derived from wt and \(crt^{-/-}\) ES cells at D8 of culture. Approximately 70% of the adherent wt EBs were strongly contracting and contained more than two beating areas, each beating with a frequency of 72 \(\pm\) 8 contractions/min. In contrast, 90% of \(crt^{-/-}\) EBs did not feature any contracting foci (Fig. 1 B). Wild-type EBs loaded with the Ca\(^{2+}\)-indicator dye fluo3 exhibited normal \([Ca^{2+}]_{c}\) oscillations (Fig. 1 C, left) (Bony et al., 2001), whereas \(crt^{-/-}\) EBs did not show any measurable \([Ca^{2+}]_{c}\) spikes (Fig. 1 C, right).

The myofibrillogenesis was evaluated in 8-d-old EBs by in situ immunostaining of sarcomeric proteins \(\alpha\)-actinin and MLC2v. As expected (Meyer et al., 2000), in wt ES-derived cardiomyocytes, \(\alpha\)-actinin and MLC2v appeared organized as an interconnected network with the myofibrils stretching throughout the cytoplasm even by running from one cell into another (Fig. 2 A, a and b). These newly formed sarcomeres featured a length of 2 \(\mu\)m, similar to that of adult cardiac sarcomeres, indicating that the contractile apparatus was fully organized at this stage of EBs development. In contrast, \(crt^{-/-}\) cardiomyocytes showed an anomalous distribution of both \(\alpha\)-actinin and MLC2v (Fig. 2 A, c and d). \(\alpha\)-actinin was not incorporated into the Z-disks but remained localized in cytosolic spots. MLC2v also failed to form and localize to the A bands. It was distributed within the cytosol as short disorganized filaments. In addition, a severe reduction in MLC2v mRNA and protein was also observed as revealed by reverse transcription–PCR (unpublished data) and Western blot analysis (Fig. 2 B), respectively. This indicated that calreticulin deficiency interferes with myofibril formation.

**Effect of extracellular Ca\(^{2+}\) chelation, intracellular \([Ca^{2+}]_{c}\) buffering, and inhibition of calmodulin-dependent protein kinases on ES cell differentiation**

Calreticulin-deficient mouse embryonic fibroblasts (MEFs) have impaired Ca\(^{2+}\) release from ER (Nakamura et al., 2001b). To test whether an altered Ca\(^{2+}\) homeostasis in cal-
The activation of Ca\(^{2+}\)/calmodulin-dependent protein kinases (CaMks) is essential for myofibrillogenesis (Zhang et al., 2002) and sarcomere organization (Aoki et al., 2000). To test the involvement of CaMks in EGTA or BAPTA-dependent inhibition of myofibrillogenesis we used KN93, an inhibitor of CaMkII (Sumi et al., 1991). wt EBs treated with KN93 from D4 to D8 had impaired distribution of both \(\alpha\)-actinin and MLC2v, similar to the EGTA- or BAPTA-treated cells. Most importantly, they exhibited a phenotype similar to \(crt^{-/-}\) cardiomyocytes. MLC2v was spread within the cytosol as short disorganized filaments (Fig. 3 C, d, f, and h vs. b for control), whereas \(\alpha\)-actinin showed a spotted distribution (Fig. 3 C, c, e, and g). We concluded that elevation of \([Ca^{2+}]_{c}\) and activation of CaMK are essential for early stages of myofibrillogenesis, and that these processes are impaired in the absence of calreticulin.

### Ca\(^{2+}\)-dependent rescue of the myofibrillogenesis in calreticulin-deficient cardiomyocytes

The phenotype of wt cells differentiated in the absence of extracellular Ca\(^{2+}\) or under Ca\(^{2+}\) buffering conditions was reminiscent of the phenotype of \(crt^{-/-}\) cells. Therefore, we tested whether a transient elevation of \([Ca^{2+}]_{c}\) prior to myofibrillogenesis (i.e., appearance of recognizable cardiac cells) would initiate myofibril formation and restore the beating activity in the calreticulin-deficient EBs.

To elicit a transient increase in \([Ca^{2+}]_{c}\), we incubated \(crt^{-/-}\) EBs at D4 with the Ca\(^{2+}\) ionophore ionomycin (10–1,000 nM) for 2 h, as indicated in Fig. 4 A. Only a 2-h treatment (followed by a washout) was used to exclude any potential toxic effect of ionomycin. Western blot analysis revealed no
significant change in the level of GRP94, a heat-shock ER chaperone of the HSP90 family normally upregulated after prolonged exposure to increased intracellular Ca²⁺ (Mitani et al., 1996) (unpublished data). This confirmed that this exposure of crt⁻/⁻ cells to Ca²⁺ ionophore did not induce notable cell stress. Furthermore, we did not detect any cell death after ionomycin treatment, as determined by cell viability using trypan blue exclusion (3.8 ± 0.2% positive cells in control EBs vs. 3.4 ± 0.3% in ionomycin-treated EBs) and by extracellular release of cytosolic lactate dehydrogenase (1.5 ± 0.1% from control EBs vs. 2.0 ± 0.2% from ionomycin-treated EBs; n = 3).

Fig. 4 B shows that addition of 100 nM ionomycin to wt and crt⁻/⁻ EBs induced a transient elevation in [Ca²⁺], in both types of EBs. After washout, [Ca²⁺] returned to basal level within 5 h.

Crt⁻/⁻ ES-derived EBs were incubated with 10–100 nM ionomycin, at D4 (Fig. 4, C and D) or D7 (Fig. 4 D), followed by washout and analysis of their beating activity 4 d after addition of the ionophore. Fig. 4 C shows that crt⁻/⁻ EBs treated with ionomycin had fully restored beating activity as rapidly as 4 d after ionomycin treatment. Partial recovery of EBs (20% beating EBs) was already observed the first day after incubation with ionomycin (unpublished data).

Next, we tested MLC2v expression in crt⁻/⁻ cardiomyocytes within ionomycin-treated EBs. There was a correct insertion of both α-actinin and MLC2v into sarcomeric structures (Fig. 5 A), indicating that myofibrillogenesis was restored in crt⁻/⁻ cells as a result of ionomycin treatment. Importantly, crt⁻/⁻ EBs treated with ionomycin expressed high levels of MLC2v protein as observed for wt (Fig. 5 B).

Phosphorylation of MLC2v by the Ca²⁺/calmodulin-dependent myosin light chain kinase (MLCK) is a prerequisite for its insertion into the A-band of the sarcomere (Sanbe et al., 1999). The phosphorylated and unphosphorylated forms of MLC2v can be readily identified by two-dimensional gel electrophoresis and followed by Western blot
analysis with specific antibodies. As expected, wt EBs contained both phosphorylated and unphosphorylated forms of MLC2v. In contrast, in \textit{crt}^{-/-} cardiomyocytes, there was no detectable phosphorylated MLC2v (Fig. 5 C, middle). Most importantly, treatment of \textit{crt}^{-/-} EBs with ionomycin resulted in appearance of phosphorylated MLC2v (Fig. 5 C, bottom). This indicated that \textit{crt}^{-/-} cardiomyocytes feature impaired Ca^{2+}-dependent phosphorylation of MLC2v.

Interestingly, the content of atrial MLC2 (MLC2a) was not significantly affected by treating wt or \textit{crt}^{-/-} EBs with ionomycin (Fig. 5 D), and it was correctly inserted into sarcomeres in \textit{crt}^{-/-} cardiomyocytes (Fig. 5 E). We concluded that \textit{crt}^{-/-} EBs are deficient in a specific Ca^{2+} signal (“checkpoint”) required for elevated expression of MLC2v (but not MLC2a), its phosphorylation, and assembly into functional sarcomeres.

**Nuclear translocation of MEF2C is impaired in calreticulin-deficient cardiomyocytes**

To investigate mechanisms responsible for downregulation of MLC2v in the absence of calreticulin, we measured expression and nuclear targeting of MEF2C, GATA4, and Nkx2.5, a group of cardiac transcription factors known to regulate the MLC2v gene expression. MEF2C, GATA4, and Nkx2.5 are detected as early as D4 to D5 of EB culture (Meyer et al., 2000). Real-time quantitative PCR analysis revealed no differences in the expression of all three transcription factors at D8 in wt and \textit{crt}^{-/-} EBs (Fig. 6 A). Ionomycin treatment had no significant effect on expression of MEF2C, GATA4, and Nkx2.5 in wt cells (Fig. 6 A); however, Western blot analysis revealed an increase in MEF2C levels in ionomycin-treated \textit{crt}^{-/-} cells (Fig. 6 B). In contrast, GATA4 and NKx2.5 mRNA were decreased to 40 and 50%, respectively, in ionomycin-treated \textit{crt}^{-/-} EBs (Fig. 6 A). The increased level of MEF2C in ionomycin-treated cells was observed at the protein level in both wt and \textit{crt}^{-/-} EBs, whereas we did not detect any significant difference of MEF2C content in wt versus \textit{crt}^{-/-} under control conditions (Fig. 6 B).

Next, we investigated the localization of MEF2C, which regulates the activity of MLC2v promoter (Zou and Chien, 1995). Fig. 7 illustrates the intranuclear distribution of MEF2C within EBs. Distribution of nuclei within EBs was identified by Hoechst staining (Fig. 7, inset). In wt EBs, nuclei were strongly stained by the anti-MEF2C antibody, as visualized by the intranuclear three-dimensional distribution of MEF2C using the shadow projection mode (Fig. 7 A, a), as

![Figure 5. MLC2v and myofibrillogenesis in ionophore-treated EBs.](image-url)

(A) Reestablishment of the myofibrillogenesis in ionomycin-treated EBs. α-actinin (left) and MLC2v distribution (right), visualized as shadow projection after three-dimensional reconstruction of z-series images. Bars, 10 μm. (B) Control and ionomycin-induced increase of MLC2v expression during EB differentiation, as analyzed by Western blot with the anti-MLC2v antibodies. (C) Two-dimensional gel electrophoresis of wt and \textit{crt}^{-/-} detergent-insoluble contractile proteins. The phosphorylated (MLC2v-P) and unphosphorylated forms of MLC2v were identified by Western blot analysis using the anti-MLC2v antiserum. (D) Effect of ionomycin treatment on MLC2a expression in wt and \textit{crt}^{-/-} EBs, as assessed by Western blot analysis with the anti-MLC2a antibodies. (E) Immunolocalization of MLC2a in \textit{crt}^{-/-} EBs, visualized as shadow projection after three-dimensional reconstruction of z-series images. Bar, 10 μm.
well as by the summed fluorescence across the nuclei (Fig. 7 A, a’). In contrast, MEF2C failed to accumulate within the nuclei of crt/−/− EBs and remained perinuclear (Fig. 7 A, b–b’). Importantly, the ionomycin-induced [Ca^{2+}]_{i} elevation resulted in the restoration of MEF2C translocation into nuclei of crt/−/− EBs (Fig. 7 A, d–d’), as well as in an increased accumulation of MEF2C into nuclei of wt cells (Fig. 7 A, c–c’). The impaired nuclear transport was specific to MEF2C because the intracellular distribution of another cardiac transcription factor, GATA4, was not affected. Fig. 7 C shows that GATA4 was localized to the nuclei in both wt and crt/−/− cells, indicating that the nuclear import of this transcription factor was not affected by the absence of calreticulin. This indicates that Ca^{2+}-dependent processes are involved in the specific translocation of MEF2C to the nucleus of wt and crt/−/− cells.

Next, we tested for a role of CaMKs in nuclear translocation of MEF2C. wt EBs were treated with KN93 from D4 to D8 to inhibit CaMK. In KN93-treated EBs, MEF2C was not targeted into the nucleus, resulting in the absence of contractile foci in these 8-d-old EBs (unpublished data; Fig. 3 A). KN92 was used as a negative control, and as expected, it did not affect MEF2C nuclear targeting or EB contractility.

Discussion

The generation of mice deficient in calreticulin has uncovered a critical role of the ER Ca^{2+}-binding protein (Mesaeli et al., 1999). Calreticulin-deficient mice display defects in heart development and function due to reduced thickness of the ventricular wall and impaired trabeculation (Mesaeli et al., 1999). Here we used calreticulin-deficient ES cells to investigate the molecular mechanisms responsible for the heart failure of crt/−/− mice. We demonstrated that during the differentiation process, crt/−/− ES-derived cardiomyocytes feature a severe disruption of myofibrillogenesis due to insufficient expression and Ca^{2+}-dependent phosphorylation of MLC2v. This is likely due to insufficient availability and mobilization of Ca^{2+} from ER in crt/−/− cells as manifested by inhibited CaMK-dependent nuclear translocation of MEF2C. The importance of Ca^{2+} in these processes is documented by rescue of crt/−/− phenotype with Ca^{2+} ionophore. We show that specific Ca^{2+}-dependent checkpoint is required for myofibrillogenesis. This signal is not generated in the absence of calreticulin.

Early cardiogenesis is regulated by three families of transcription factors (i.e., Nkx2.5, MEF2, GATA) (Srivastava and Olson, 2000). Among these factors, MEF2C is an essential regulator of ventricular development (Black and Olson, 1998). The targeted disruption of the MEF2C gene in the mouse results in embryonic lethality due to cardiac developmental arrest and severe downregulation of a number of cardiac markers (Durocher et al., 1996; Lin et al., 1997a; Charon and Nemer, 1999) including MLC2v (Liu et al., 2001). MEF2C is a major cardiac transcription factor regulating the activity of MLC2v promoter (Chen et al., 1998; Nguyen-Tran et al., 1999). Therefore, it is not surprising that impaired Ca^{2+}-dependent nuclear translocation of MEF2C in crt/−/− cells results in low expression of MLC2v, and consequently impaired myofibrillogenesis.

MEF2 is at the crossroad of multiple [Ca^{2+}] signals, which affect transcriptional processes (Olson and Srivastava, 1996). Here we show that MEF2C function depends on the presence of a calreticulin-dependent signal. MEF2 integrates Ca^{2+}-dependent signals involving calcineurin, CaMKI, CaMKIV, MMK6/p38 MAPK, and extracellular signal-regulated kinase 5 (Yang et al., 1998; Blaese et al., 2000; Friday et al., 2000; Han and Molkentin, 2000; Passier et al., 2000). Crt/−/− ES-derived cardiomyocytes have normal expression of MEF2C, GATA4, or Nkx2.5, but have an impaired nuclear translocation of MEF2C. MEF2C remains in the cytosol and in perinuclear area in crt/−/− ES-derived cardiomyocytes, whereas it is inside the nucleus in wt cells. This is specific for MEF2C because nuclear localization of GATA4 is not affected in crt/−/− cardiomyocytes. Indeed, GATA4 was correctly targeted to the nucleus of these cells. Therefore, our data do not favor the hypothesis that the absence of calreticulin, by affecting the Ca^{2+} filling state of the stores and the nuclear cisternae, could indiscriminately impair the nuclear import of transcription factors through a conformational closure of nuclear pores after an upward shift of the transporter (Perez-Terzic et al., 1996). How nuclear import of macromolecules including MEF2C is impaired in crt/−/− cardiomyocytes is currently under investigation.

As expected, MLC2a expression is significantly less affected in crt/−/− cardiomyocytes, as it is likely activated by GATA4 and
Figure 7.  
Study of the intracellular localization of MEF2C in clustered cardiomyocytes within EBs.  

(A) Three-dimensional representation of MEF2C immunolocalization in cardiomyocytes within EBs at D8 of differentiation.  
(a to d) Shadow projections.  

(a to d) Profiles on the x–y dimension of MEF2C intranuclear localization visualized as the summed fluorescence distribution across the nuclei (fluorescence of middle plane crossing the nuclei in the z-axis).  
(a and a’ and c and c’) wt EBs ± ionomycin respectively.  
(b and b’ and d and d’) crt−/− EBs ± ionomycin.  
(a and b) wt and crt−/− cells EB, respectively, in control conditions (addition of DMSO).  
(c and d) wt and crt−/− cells treated with ionomycin, respectively.  
(Inset) Hoechst staining of nuclei within an EBs.  

(B) Profile on the x–y dimension of GATA4 intranuclear localization visualized as the summed fluorescence distribution across the nuclei (fluorescence of middle plane crossing the nuclei in the z-axis).  
Bars, 10 μm (100 nuclei analyzed in four different experiments).
Nkx2.5 factors (Gruber et al., 1998; Franco et al., 1999; Nguyen-Tran et al., 1999; Doevendans et al., 2000). The presence of correctly inserted ML2v is likely to account for some contractility observed in 5–10% of 

\[ \text{Ca}^{2+} \]

EBs. This is in line with calreticulin-knockout gene studies which showed that calreticulin deficiency primarily affects ventricular development and has no significant effect on atrial cells (Mesaél et al., 1999).

Although inhibited nuclear translocation of MEF2C in 

\[ \text{crt}^{-/-} \]

cardiomyocytes explains the reduced expression of ML2v, it cannot account for the lack of insertion of the existing light chains into functional sarcomeres. We show that the latter defect is likely attributed to low active 

\[ \text{Ca}^{2+} \]

and synaptic transmission, more sustained 

\[ \text{Ca}^{2+} \]

events such as muscle contraction, secretion, adhesion, and oscillations from the ER and it does not require Ca 2

\[ \text{crt} \]

triggered by inositol 1,4,5-trisphosphate–dependent [Ca2+]c signals induce a defect in osinol 1,4,5-trisphosphate–dependent [Ca2+]c spikes, suggesting a defect in osinol 1,4,5-trisphosphate signaling in calreticulin-deficient cardiomyocytes. Although rapid [Ca2+]c signals induce events such as muscle contraction, secretion, adhesion, and synaptic transmission, more sustained [Ca2+]c signals are known to be involved in cell proliferation and differentiation. We rescued the expression, the phosphorylation, and the assembly of ML2v into sarcomeric units by triggering a transient but long-lasting [Ca2+]c increase in 

\[ \text{crt}^{-/-} \]

EBs before the appearance of contractions (i.e., D4 or D7) using the Ca2+ ionophore, ionomycin. This treatment led to a recovery of contractility in 

\[ \text{crt}^{-/-} \]

ES-derived cardiomyocytes. Although applied for a brief period (2 h), ionomycin induced a transient but long-lasting Ca2+ influx into the embryoid body as previously shown in other cell types (Timmerman et al., 1996; Durham and Russo, 2000). This ionophore-dependent Ca2+ increase mimics receptor-medi
dated Ca2+ elevation into the cytosol, an event missing in 

\[ \text{crt}^{-/-} \]

EBs. It further resets the cardiac differentiation program, paused after an early stage of cardiac commitment.

In summary, although 

\[ \text{crt}^{-/-} \]

cells seem to undergo a normal cardiac commitment up to D4 (time of maximal expres-
RNA extraction and reverse transcription reaction
Total cellular RNA was isolated from EBs using the RNA Extraction Kit (Promega). RNA was reverse-transcribed using the Superscript II kit (GIBCO BRL). The cDNA was diluted 10-fold prior to PCR amplification.

Real-time quantitative PCR by SYBR green detection
The nucleotide sequences of the PCR primers used were: MEF2C forward 5′-AGATTCCACACACACACCGGCC and reverse 5′-ATCTCCGAGGACGTCCAGCCGT; GATA4 forward 5′-CGAGTGGAGGCGGACT and reverse 5′-CTCACCCTCGGCCATTACGA; and Nkx2.5 forward 5′-TGGAGAAGGCGCTGATCAGAACC and reverse 5′-TTGACTCTGAGCCCAGGCTGACAAG; β-tubulin forward 5′-CCGGAGACCTGCGGCAACGCGG; and reverse 5′-TGGCCCAAAGACCTGGACGAAGCC. Real-time quantitative PCR was performed using a LightCycler rapid thermal cycler (Roche). Amplification was carried out as recommended by the manufacturer. 12 μl reaction mixture contained 1 μl of LightCycler-DNA Master SYBR Green 1 mix (FAST Start Kit, containing Taq DNA polymerase, reaction buffer, deoxynucleoside trisphosphate mix, and SYBR Green I mix (FAST Start Kit, containing Taq DNA polymerase, reaction buffer, deoxynucleoside trisphosphate mix, and SYBR Green I mix (FD Biotech, Inc.) and 200 nM concentration of appropriate primer and 0.5 μM of cDNA. The relative cDNA concentrations were established by a standard curve using serial dilutions of corresponding PCR fragments. The data were normalized by PCR analysis of the β-tubulin. The amplification program included the initial denaturation step at 95°C for 8 min, and 40 cycles of denaturation at 95°C for 3–5 s, annealing at 60–65°C for 10–5 s, and extension at 72°C for 5–10 s. The temperature transition rate was 20°C/s. Fluorescence was measured at the end of each extension step. After amplification, a melting curve was acquired by heating the product at 20°C/s to 95°C, cooling it at 20°C/s to 70°C, keeping it at 70°C for 20 s, and then slowly heating it at 0.1°C/s to 95°C. Fluorescence was measured through the slow heating phase. Melting curves were used to determine the specificity of PCR products, which were further confirmed using conventional gel electrophoresis.

The Student’s t test was used to analyze statistical significance. All p values corresponded to two-tailed tests, and a P < 0.05 was considered statistically significant.

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Calcineurin and Ca2

in cardiologyogenesis | Li et al. 113