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Protection of Cardiomyocytes from Ischemic/Hypoxic Cell Death via Drbp1 and pMe_2GlyDH in Cardio-specific *ARC* Transgenic Mice*

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The ischemic death of cardiomyocytes is associated in heart disease and heart failure. However, the molecular mechanism underlying ischemic cell death is not well defined. To examine the function of apoptosis repressor with a caspase recruitment domain (ARC) in the ischemic/hypoxic damage of cardiomyocytes, we generated cardio-specific *ARC* transgenic mice using a mouse α-myosin heavy chain promoter. Compared with the control, the hearts of *ARC* transgenic mice showed a 3-fold overexpression of *ARC*. Langendoff preparation showed that the hearts isolated from *ARC* transgenic mice exhibited improved recovery of contractile performance during reperfusion. The cardiomyocytes cultured from neonatal *ARC* transgenic mice were significantly resistant to hypoxic cell death. Furthermore, the ARC C-terminal calcium-binding domain was as potent to protect cardiomyocytes from hypoxic cell death as ARC. Genome-wide RNA expression profiling uncovered a list of genes whose expression was changed (>2-fold) in *ARC* transgenic mice. Among them, expressional regulation of developmentally regulated RNA-binding protein 1 (Drbp1) or the dimethylglycine dehydrogenase precursor (pMe_2GlyDH) affected hypoxic death of cardiomyocytes. These results suggest that ARC may protect cardiomyocytes from hypoxic cell death by regulating its downstream, Drbp1 and pMe_2GlyDH, shedding new insights into the protection of heart from hypoxic damages.

Programmed cell death, or apoptosis, is an evolutionarily conserved process that plays a critical role in embryonic development and adult tissue homeostasis. In humans and mice, dysregulated apoptosis has been implicated in the pathogenesis of cancer and in autoimmune, cardiovascular, and neurodegenerative diseases (1). Recently, apoptosis of cardiomyocytes has been recognized as a cellular mechanism of ischemic injury in the heart. Furthermore, a large body of research has focused on identifying the signaling molecules that might protect the myocardium from ischemic damage (2). For example, signaling molecules enhance apoptosis of cardiomyocytes, such as p38, c-Jun N-terminal kinase, tumor necrosis factor-α, p53, β-adrenergic receptors, and nitric oxide (2–4). In contrast, other signaling pathways have been demonstrated to protect the heart from apoptosis, such as cardiotrophin-1 through the gp130 receptor, p38β, insulin-like growth factor-1, Akt/protein kinase B, protein kinase C, and extracellular signal-regulated kinase 1/2 (2, 3, 5–9). Thus, an increased understanding of the signaling pathways that are regulated during ischemia/reperfusion is important for the development of effective therapies (10).

ARC[3] (apoptosis repressor with CARD) is a caspase recruitment domain (CARD) protein that is expressed almost exclusively in long-lived tissues, such as heart, skeletal muscles, and brain. ARC selectively interacts with the initiator caspases-2 and -8, and significantly attenuates death receptor-induced apoptosis dependent on the activation of these caspases (11). In the H9c2 cell line, ectopic expression of *ARC* suppressed apoptosis, the protection being mediated, in total or in part, through the blockade of hypoxia-induced cytochrome *c* released from the mitochondria (12). Therefore, specific interference with both receptor and mitochondria death pathways, as well as its high cardiac expression, makes ARC a unique and central cardiac death repressor. This supposition is supported by the observation that viral gene transfer or TAT-mediated transduction of ARC reduces infarct size after the ischemia/reperfusion injury of isolated rat hearts and blocks the develop-

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[3] The abbreviations used are: ARC, apoptosis repressor with a caspase recruitment domain; CARD, caspase recruitment domain; H & E, hematoxylin and eosin; LVDp, left ventricular development pressure; LVEDp, left ventricular end-diastolic pressure; MHC, myosin heavy chain; Tg, transgenic; shRNA, short hairpin RNA; WT, wild type; RT, reverse transcriptase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling.

ment of post-ischemic cardiomyopathy (13, 14). Recently, *ARC* knock-out mice also exhibited enhanced sensitivity to hypoxic injury in the heart (15). However, the detailed function of ARC as a suppressor of ischemic damage in the heart needs to be clarified. In this study, by generating cardio-specific *ARC* transgenic (Tg) mice and global gene expression analysis, we found that ARC protects cardiomyocytes from ischemic/hypoxic damages via its C-terminal Pro/Glu-rich region and expressional regulation of Drbp1 and pMe_2GlyDH.

MATERIALS AND METHODS

Plasmid Construction and shRNA Analysis—The mouse *ARC* cDNA was subcloned into an expression vector under the control of a cardio-specific mouse α-myosin heavy chain (α*MHC*) promoter (16). *ARC*, *NARC*-(1–98), and *CARC*-(99–208) were subcloned into *pcDNA3.1* (Invitrogen) and *pEGFP* (Clontech) (17). *Drbp1* cDNA was kindly provided by Dr. H. Endo (Jichi Medical School, Japan) and *Alas2* cDNA was subcloned into *pcDNA3.1*. For vector expressing shRNAs against *pMe_2GlyDH*, oligonucleotides (number 1 forward, 5′-GATCC CCGGG ATAAA CTTGA AGAAG ATTCA AGAGA TCTTC TTCAA GTTTA TCCCT TTTTG GAAA-3′ and reverse, 5′-AGCTT TTCCA AAAAG GGATA AACTT GAAGA AGATC TCTTG AATCT TCTTC AAGTT TATCC CGGG-3′; number 2 forward, 5′-GATCC CCCTG AAAGG GTGGA CGAAT TTTCA AGAGA AATTC GTCCA CCCTT TCAGT TTTTG GAAA-3′ and reverse, 5′-AGCTT TTCCA AAAAC TGAAA GGGTG GACGA ATTTC TCTTG AAAAT TCGTC CACCC TTTCA GGGG-3′) containing the target sequence (Dharmacon) were synthesized, annealed, and cloned into BglII and HindIII sites of pSuper (OligoEngine).

Generation of ARC Tg Mice—The mouse *ARC* expression construct was injected into embryos and positive F_0 mice were identified by PCR analysis using a synthetic oligonucleotide (forward, 5′-CCACA TTCTT CAGGA TTCTC-3′) corresponding to the *MHC* promoter and a oligonucleotide (reverse, 5′-CTTCT GGCGT CCAGT GG-3′) of *ARC* cDNA (Macrogen Inc., Korea). Three independent founder lines were identified and mated to FVB wild type (WT) mice to generate a pure FVB genetic background WT for the F_2 generation, and then F_3 Tg offspring were then backcrossed to Balb/c 3T3 mice under a 12:12 h light:dark cycle with access to food and water *ad libitum*. The animal protocols were approved by the Gwangju Institute of Science and Technology and Seoul National University Standing Committees on Animals.

Genomic DNA PCR and Reverse Transcription-PCR (RT-PCR)—Genomic DNA PCR from the tails of Tg mice and RT-PCR from the whole tissues of Tg mice were carried out as described previously (18, 19). Gene-specific oligonucleotides were synthesized: *Alas2* forward, 5′-GTATT GGACG CTGCC CCATC C-3′ and reverse, 5′-CTTCA GGGTC TCCTC TATGG C-3′; *Drbp1* forward, 5′-GTGAT CAGCA AGCAC ACATC C-3′ and reverse 5′-CGCAC GTAGC CCAAG CCTTT A-3′; *pMe_2GlyDH* forward, 5′-GACAG AGCAG AGACT GTGAT-3′ and reverse, 5′-CATCT CCTGG GTTAT ACAGT C-3′; *ARC* forward, 5′-ATGGG CAACG TGCAG GAG-3′ and reverse, 5′-CTTCT GGCGT CCAGT GG-3′; and β-*actin* forward, 5′-GAGCT GCCTG ACGGC CAGG-3′ and reverse, 5′-CATCT GCTGG AAGGT GGAC-3′; atrial natriuretic factor forward, 5′-CCATA TTGGA GCAAA TCCTG-3′ and reverse, 5′-CGGCA TCTTC TCCTC CAGG-3′; β-*MHC* forward, 5′-TGCAA AGGCT CCAGG TCTGA GGGC-3′ and reverse, 5′-GCCAA CACCA ACCTG TCCAA GTTC-3′; *MMP2* forward, 5′-GATAC CCTCA AGAAG ATGCA GAAGT-3′ and reverse, 5′-ATCTT GGCTT CCGCA TGGT-3′ (20).

Western Blot Analysis—Samples were homogenized in lysis buffer (120 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM Na_3VO_3, 10 mM $Na_4P_2O_7$, 10 mM sodium fluoride, 1% Triton, 10% glycerol, and 50 mM Tris-HCl, pH 8.0) and the amounts of protein were determined using the method of Bradford (Bio-Rad). Total 30–50 μg of cell extracts in a buffer (60 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, and 0.5% β-mercaptoethanol) were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes using the Semi-Dry Transfer system (Bio-Rad). The membranes were blocked with TBST buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 0.2% Tween 20) containing 3% bovine serum albumin. Proteins were then visualized using Enhanced Chemiluminescence. Anti-ARC antibody was purified by ARC affinity chromatography. Mouse anti-FADD and anti-poly(ADP-ribose)polymerase antibody were purchased from Pharmingen.

Histological Analysis and Immunohistochemistry—After perfusion, the hearts were fixed with 4% formaldehyde, embedded, and thin-sectioned, followed by deparaffinization and rehydration. The paraffin sections were blocked in 5% rabbit serum and then incubated overnight at 4 °C with anti-ARC antibody. For the preparation of frozen sections, hearts were isolated after perfusion and immediately frozen in liquid nitrogen-cooled OCT embedding medium (Tissue-Tek). The frozen sections were cut to a 5-μm thickness and mounted on silane-coated slides.

Langendoff Preparation of Isolated Mouse Hearts—The mice were anesthetized with pentobarbital (50 mg/kg, intraperitoneal injection) and their hearts were excised and perfused with oxygenated buffer (21, 22). The hearts were retrogradely perfused on a Langendoff apparatus with Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, 1 mM $CaCl_2$, and 10 mM HEPES) with a gas mixture of 95% O_2 and 5% CO_2 at 37 °C. Through a left atrial incision, a latex balloon connected to a pressure transducer was inserted into the left ventricular (LV) cavity for the measurement of the LV isovolumic pressure. The LVEDp was identified as the lowest value from the LV pressure curve. The LVDp and heart rate were continuously monitored using a polygraph and a computer analysis system (PolyView, GRASS Co.).

Induction of Myocardial Infarction—After 1 h of ischemia and 24 h of reperfusion, the left anterior descending coronary artery was reoccluded, and 1 ml of 1.0% Evans blue was injected into the apex of each heart to stain nonischemic tissue. The hearts were then excised, washed with phosphate-buffered saline, embedded in agarose, and cut into five transverse slices for 15 min of incubation at room temperature with 1.5% 2,3,5-triphenyltetrazolium chloride to measure viable myocardium

(red staining). Slices were photographed (each side) under a microscope and the left ventricular area, the area at risk, and the infarct area were determined by digital planimetry (14).

TUNEL Assay—Accumulated internucleosomal DNA fragments (apoptosis) were detected using an *in situ* apoptosis detection kit (Roche Applied Science). Percentages of positively stained cells were determined by counting the numbers of labeled cells and total cells in cross-sections (15).

Isolation of Neonatal Cardiomyocytes and DNA Transfection—*ARC* homozygous Tg mice for this study were produced by mating heterozygous *ARC* Tg mice in the Balb/c 3T3 background to heterozygous *ARC* Tg mice and used for primary culture. A procedure for culturing ventricular cardiomyocytes from neonatal mice was modified (23). For transfection, cardiomyocytes isolated from neonatal *ARC* Tg mice were allowed to stabilize for 3 days and then transfected with plasmids using PolyFectamine reagent according to the manufacturer's instruction (Qiagen).

In Vitro Hypoxia of Cultured Cardiomyocytes and Cell Death Assays—Cardiomyocytes were cultured in fetal bovine serum/minimal essential medium. Hypoxia was simulated by incubating cells in a hypoxic buffer (125 mM NaCl, 8 mM KCl, 1.2 mM KH_2PO_4, 1.25 mM $MgSO_4$, 1.2 mM $CaCl_2$, 6.25 mM $NaHCO_3$, 20 mM 2-deoxyglucose, 5 mM sodium lactate, and 20 mM HEPES, pH 6.6) and by placing the cells under hypoxic pouches (GasPak^{TM} EZ, BD Biosciences) at 37 °C (10). Cell death assays were performed using the Live/Dead cell viability kit (Molecular Probes).

Microarray Using GenePix 4000B Gene Expression Profiling—Total RNA samples were prepared from the hearts of 3- and 30-week-old mice by using an RNeasy Mini Kit (Qiagen). RNA samples (30 μg) were labeled with cyanine (Cy3) or cyanine (Cy5)-conjugated dCTP (Amersham) by a reverse transcription reaction using SuperScript II (Invitrogen). The labeled cDNA mixture was resuspended and mixed in 10 μl of hybridization solution (GenoCheck, Korea) and placed on an OpArray mouse genome 35K (OPMMV4, Operon Biotechnologies, GmbH). The hybridized slides were washed in a buffer (2× SSC, 0.1% SDS for 2 min, 1× SSC for 3 min, and then 0.2× SSC for 2 min) at room temperature.

Microarray Data Analysis—Microarray data analysis was carried out as described previously (24).

Statistical Analyses—All statistical analyses were performed using a two-tailed Student's *t* test or one-way analysis of variance followed by SigmaStat software.

RESULTS

Generation of Tg Mice Overexpressing ARC in the Heart—To examine the molecular function of *ARC* in the hypoxic injury of heart, we generated cardio-specific Tg mice overexpressing mouse *ARC* using the promoter of mouse *MHC* gene (Fig. 1). *ARC* Tg mice were born normal and grew to adulthood without any abnormalities in their health and appearance. Compared with WT, an analysis of tissue extracts using Western blotting (Fig. 1*A*) and polymerase chain reaction (Fig. 1*B*) demonstrated an increased level of ARC protein and RNA with about 3-fold in the whole heart tissues of *ARC* Tg mice, but not in the kidney as well as in liver and brain.[4] Similar expression levels of ARC were observed in the other two lines of *ARC* Tg mice we generated.[4]

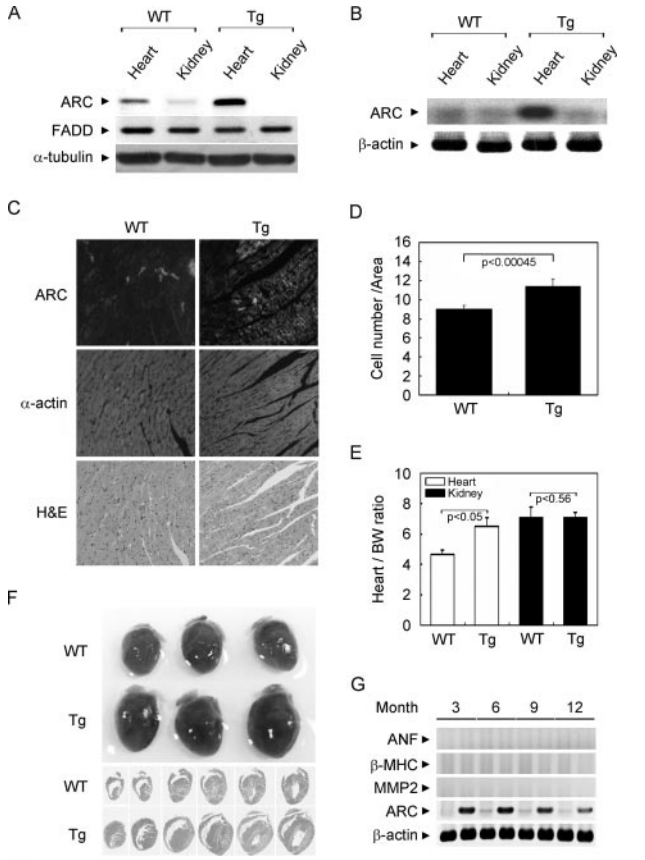

FIGURE 1. **Generation of cardio-specific *ARC* Tg mice.** *A*, Western blot analysis of *ARC* in the heart of ARC Tg mice. Tissue extracts (30 μg) were prepared from the heart and kidney isolated from 8–12-week-old *ARC* Tg and age-matched WT mice, and proved with the indicated antibodies after separation on SDS-polyacrylamide gel. FADD and α-tubulin were used as internal controls. *B*, RT-PCR analysis. Total RNA was extracted from the hearts and *ARC* was amplified by RT-PCR using mouse *ARC*-specific oligonucleotides. *C*, immunohistochemistry of ARC. Heart frozen sections were prepared and left ventricle (*LV*) sections of the hearts were probed with anti-ARC or anti-α-actin antibodies. Cardiac histological sections proved with anti-ARC antibodies were stained with hematoxylin and eosin (H & E). *D*, determination of cell numbers in the hearts. Cells were counted from each of 10 *ARC*-positive areas in paraffin sections of *ARC* Tg mice or the same areas of WT mice. *Bars* indicate mean ± S.E. *E*, heart/body and kidney/body weight ratios of WT (n = 10) and age-matched *ARC* Tg mice (n = 9). *Bars* depict mean ± S.E. *F*, gross morphology of the hearts in the 8–12-week-old WT and *ARC* Tg mice (*upper panel*). Hematoxylin and eosin (H & E) staining of serial longitudinal sections (*lower panel*). *G*, expression analysis of hypertrophy-associated genes in *ARC* Tg mice. Total RNAs were obtained from the hearts of *ARC* Tg mice at the indicated months and examined for the expression of atrial natriuretic factor (*ANF*), *β-MHC*, and *MMP-2* by RT-PCR analysis.

Immunostaining analysis also revealed the increased expression of *ARC* in the left ventricle of the heart of ARC Tg mice (Fig. 1*C*). In *ARC* Tg mice, strong immunoreactivity against ARC was restricted to ~40% of individual cardiomyocytes, which is consistent with the previous report demonstrating the expression pattern of heme oxygenase-1 under the control of MHC promoter (21). Although histological examination with

[4] J.-O. Pyo, J. Nah, H.-J. Kim, J.-W. Chang, Y.-W. Song, D.-K. Yang, D.-G. Jo, H.-R. Kim, H.-J. Chae, S.-W. Chae, S.-Y. Hwang, S.-J. Kim, H.-J. Kim, C. Cho, C.-G. Oh, W. J. Park, and Y.-K. Jung, unpublished observations.

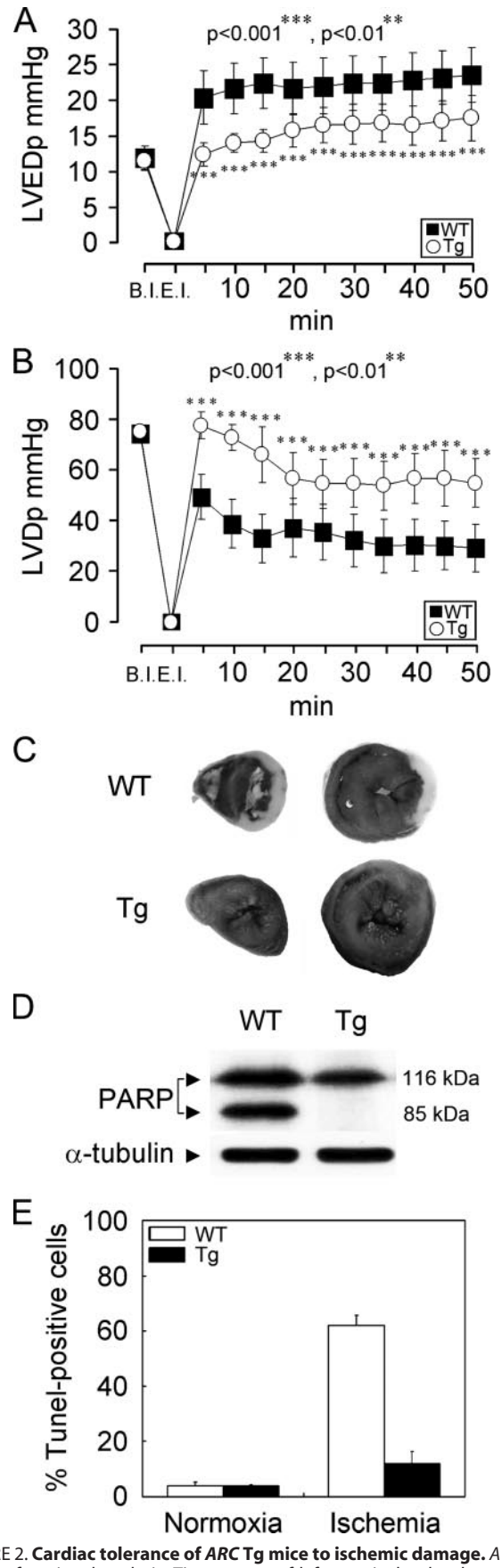

FIGURE 2. **Cardiac tolerance of *ARC* Tg mice to ischemic damage.** *A* and *B*, cardiac functional analysis. Time course of left ventricular-developed pressure (*LVDp*) (*A*) and left ventricular end diastolic pressure (*LVEDp*) (*B*). Hearts were isolated from 8–12-week-old WT (*filled square*) and *ARC* Tg (*open circle*) mice. After stabilization for 20 min, the hearts were subjected to global ischemia for 30 min and then reperfused for the indicated times. Pressure gradients are representative for $n = 7$ per group. *B.I.*, before ischemia; *E.I.*, end ischemia. *p* values denote statistic significance with Student's *t* test. ***, $p < 0.0001$; **, $p < 0.01$. *C–E*, ischemic damage analysis. Representative infarct-injured hearts of WT and *ARC* Tg mice. After 1 h of ischemia followed by 24 h of reperfusion, the hearts were isolated and stained with 2,3,5-triphenyltetrazolium chloride (*C*). *D*, Western blot analysis of poly(ADP-ribose)polymerase (*PARP*) cleavage. The lysates were prepared from infarct-injured hearts as described in *C*. Frozen sections of the injured heats were stained with TUNEL assay and TUNEL-positive cells were quantitated. *Bars* depict mean ± S.E. (*E*).

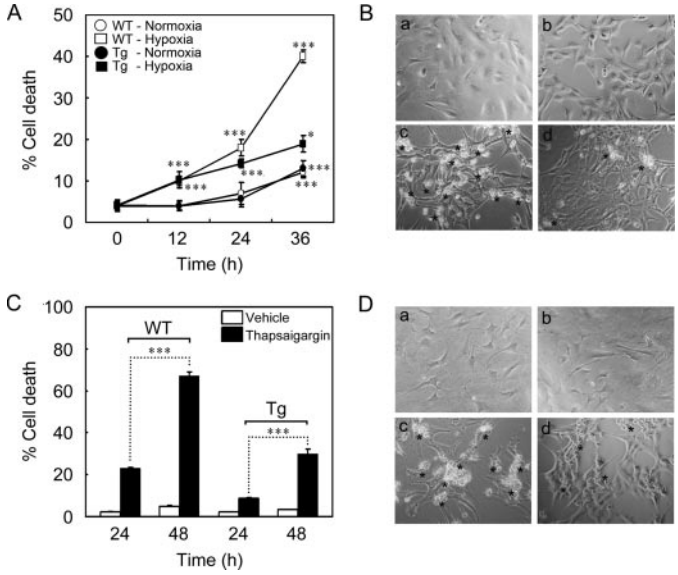

FIGURE 3. **Increased resistance of the primary cardiomyocytes cultured from *ARC* Tg mice to hypoxic damages.** *A* and *B*, hypoxic cell death of primary cardiomyocytes. Cardiomyocytes were prepared from neonatal WT and *ARC* homozygous Tg mice, cultured for 3 days *in vitro*, and then left untreated or exposed to the hypoxic condition for the indicated times. *Asterisks* indicate dying cells under hypoxia at 36 h (*B*). Cell death assays were performed using Live/Dead viability kit as described under "Materials and Methods." *C* and *D*, tolerance of ARC cardiomyocytes to thapsigargin-induced cell death. Cardiomyocytes cultured from neonatal WT and *ARC* Tg mice were exposed to thapsigargin (3 μM) for the indicated times and death rates were determined (*C*) as described in *A*. *Asterisks* indicate dying cells at 48 h (*D*). *Bars* represent mean ± S.E. of three independent experiments. *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$ *versus* control.

hematoxylin and eosin (H & E) staining revealed no severe cardiac morphological defects, cardiac remodeling was evident in the hearts of *ARC* Tg mice with the relative increase in cell numbers (Fig. 1*D*). Comparison of heart sizes revealed that under resting conditions, the hearts of *ARC* Tg mice were apparently larger than those of age-matched WT mice (Fig. 1*F*). There was a 20% difference in the ratios of heart/body weights between 8-week-old ARC Tg and WT mice, whereas no detectible difference was observed with respect to kidney weight (Fig. 1*E*). In addition, examination of the expression of hypertrophic markers, such as atrial natriuretic factor and *β-MHC*, and *MMP2*, the sign of fibrosis, with RT-PCR analysis revealed no obvious increase in the hearts of *ARC* Tg mice (Fig. 1*G*). These findings suggest that heart enlargement observed in *ARC* Tg mice may result from the principal cause of the increased cell numbers.

Hemodynamic Function of the Isolated Hearts of ARC Tg Mice—Under normal living conditions, *ARC* Tg mice displayed no apparent failure of the heart after a 12-month follow-up. To

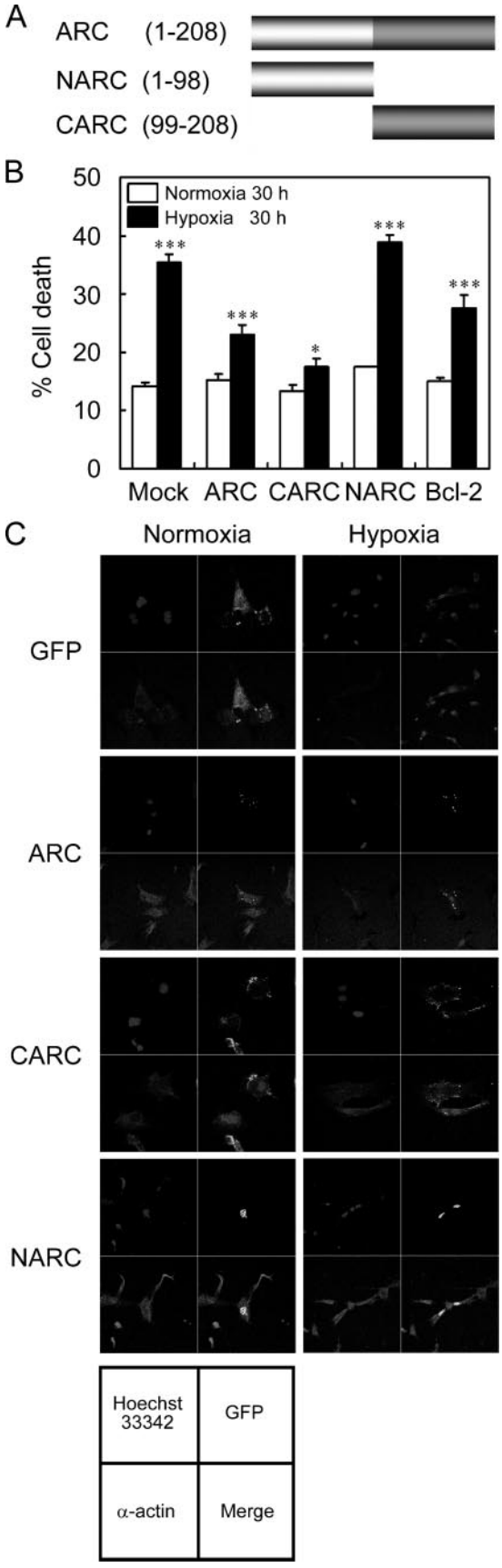

FIGURE 4. **Domain mapping of ARC regulating hypoxia-induced death of cardiomyocytes.** *A*, schematic representation of *ARC* and its deletion mutants (*NARC* and *CARC*). *CARD* and Pro/Glu-rich domain of *ARC* are illustrated and the *numbers* indicate amino acid residues. *B*, suppression of hypoxia-induced death of cardiomyocytes by ARC Pro/Glu-rich domain. Cardiomyocytes were cultured for 3 days *in vitro* and then transfected with GFP (Mock) and GFP-fused *ARC*, *CARC*, *NARC*, or *Bcl-2* for 24 h. Cells were then exposed to the hypoxic condition for 30 h and examined for cell viability based on the morphology of *GFP*-positive cells under fluorescence microscope. *Bars* represent mean ± S.E. of three independent experiments (*B*). *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$ *versus* control. *C*, expression pattern of GFP-fused *ARC* and its deletion mutants. Cardiomyocytes were transfected with either *GFP* or *GFP*-fused *ARC*, *CARC*, and *NARC* for 24 h and then left untreated or exposed to the hypoxic condition for 30 h. Cells were immunostained with anti-α-actin antibodies, a positive control of cardiac myocytes, and Hoechst 33342 (nuclei). The samples were examined under a confocal laser-scanning microscope. Green fluorescent protein, *green*; α-actin; *red*, nuclei, *blue*.

investigate the cardiac function of ARC, the hemodynamic parameters were recorded for 50 min of reperfusion after global ischemia. The baseline heart rates were similar between two groups, WT and *ARC* Tg mice.[4] Compared with WT mice, however, the left ventricular development pressure (LVDp) and the left ventricular end-diastolic pressure (LVEDp) were significantly improved in the hearts of *ARC* Tg mice (Fig. 2, *A* and *B*). These findings indicate that ARC functions to suppress ischemia/reperfusion-induced deterioration of the Langendoff preparation exhibiting cardiac dysfunction and overt heart failure.

We then assessed the myocardial infarct after occluding left anterior descending coronary artery followed by reperfusion for 24 h. Total left ventricular area, the area at risk, and the infarct area were measured. Large infarcts were observed in the hearts of WT mice (Fig. 2*C*). Infarct size (infarct/risk area) was significantly reduced in Tg mice compared with WT mice (about 40%).[4] This was accompanied by a significant decrease in apoptosis as measured with proteolytic cleavage of poly-(ADP-ribose)polymerase, a hallmark of apoptosis (Fig. 2*D*). Similarly, an evaluation of death rates using the TUNEL assay revealed reduced cell death (5.2-fold decrease) in the hearts of *ARC* Tg mice compared with WT mice during ischemic damages (Fig. 2*E*).

Suppression of Hypoxia-induced Cell Death by ARC in Cardiomyocytes—We then addressed sensitivity of the primary cardiomyocytes isolated from *ARC* Tg mice to hypoxia-induced cell death. Although the incubation of control cardiomyocytes in the hypoxic conditions induced 42% of cell death at 36 h, the hypoxic death rate in the same condition decreased to 20% in the cardiomyocytes prepared from *ARC* Tg mice (Fig. 3, *A* and *B*). These results show that the primary cardiomyocytes expressing ARC derived from *ARC* Tg mice are resistant to hypoxic cell death *in vitro*.

Previously, we found that ARC may be a calcium-binding protein (25). Thus, we addressed whether ARC affects calcium overload-induced cell death of the primary cardiomyocytes. Incubation with thapsigargin, an inhibitor of Ca^{2+}-ATPase in the endoplasmic reticulum, induced 67% of cell death in WT cardiomyocytes but 30% in the cardiomyocytes cultured from the neonatal *ARC* Tg mice, indicating that ARC suppresses calcium overload-induced death of cardiomyocytes (Fig. 3, *C* and *D*). We then analyzed the domain(s) of ARC responsible for the anti-hypoxic cell death activity using its deletion mutants (Fig. 4). The primary cardiomyocytes transiently transfected with

TABLE 1

Relative expression ratios of the selected groups of genes assessed by RNA microarray analysis

Cardiac RNA was collected from two wild type and two *ARC* Tg mice (Tg 1, 3-week-old; Tg 2, 30-week-old) and subjected to expression profiling using the GenePix 4000B array. –Fold change filters include the requirement that the genes be present in at least 200% of the controls for up-regulated genes and in less than 50% of the controls for down-regulated genes. Absolute normalized expression data are shown for each sample in the right-hand columns along with the GenBank accession number.

	Relative gene expression (ratio)		
	Tg 1	Tg 2	GenBank™
Enzyme activity			
N-Acylsphingosine amidohydrolase (acid ceramidase)-like (Asahl)	0.212107421	0.178740707	NM_025972
Carbonic anhydrase 3	2.805291579	2.709155955	NM_007606
Methionine aminopeptidase-like 1	2.21796869	1.83970689	NM_025633
Dimethylglycine dehydrogenase precursor (pMe_2GlyDH)	0.048853625	0.052353731	NM_028772
Adipsin	2.954059736	1.977203728	NM_013459
Aminolevulinic acid synthase 2, erythroid (Alas2)	2.160885548	2.036045855	NM_009653
6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (Pfkfb3), transcript variant 1	0.412229347	0.450426963	NM_133232
Unknown			
Mus musculus RIKEN cDNA 4930550L24 gene	0.252136945	0.466819994	XM_621012
Bromodomain and WD repeat domain containing 3	0.0485131	0.056798788	XM_356350
Bardet-Biedl syndrome 4 homolog (human)	0.331905826	0.359647179	NM_175325
Protein binding			
Neurofibromatosis 2	2.433380622	1.551598644	NM_010898
PREDICTED: DMRT-like family B with proline-rich C-terminal	0.365815777	0.433494475	XM_205469
WW domain binding protein 1	0.302490345	0.373305013	NM_016757
Fras1-related extracellular matrix 2	2.064505125	1.519267093	NM_172862
Protocadherin β4 (Pcdhb4)	2.004928795	1.566438906	NM_053129
Vitamin D receptor interacting protein (Vdrip)	0.230963855	0.145157177	NM_026119
FYVE, RhoGEF, and PH domain containing 1	0.180371462	0.239611358	NM_008001
Ligand of numb-protein X 1 (Lnx1)	0.389637699	0.367762517	NM_010727
Synuclein, α (Snca)	2.01965798	1.90034372	NM_009221
Signal transducer			
Period homolog 3 (*Drosophila*) (Per3)	2.586591092	1.821002595	NM_011067
Regulator of G protein signaling 7 (Rgs7)	2.1900124	1.738080612	NM_011880
Other			
RIKEN cDNA 1600012F09 gene	2.617133021	2.601587207	NM_025904
Ribosomal protein S6 kinase polypeptide 3	2.388833873	1.727797544	NM_148945
UDP-GlcNAc:βGal β-1,3-*N*-acetylglucosaminyltransferase 1	2.197476071	1.886600148	NM_175383
Signal recognition particle 54	2.070249964	1.75692098	NM_028527
PREDICTED: *M. musculus* similar to surface sperm protein P26h	0.414372494	0.475688281	CK137444
RIKEN cDNA 1810008A14 gene	0.412743742	0.499033244	NM_025457
Sad1 and UNC84 domain containing 1	0.395520299	0.300412406	NM_177576
Hypothetical protein 4931417A20	0.356462299	0.458295539	NM_145380
Par-3 (partitioning defective 3) homolog (*Caenorhabditis elegans*)	0.327191914	0.354896792	NM_031235
Similar to transmembrane protein induced by tumor necrosis factor α	0.237574687	0.283793964	AK_132283
RIKEN cDNA 2310056P07 gene	0.234790079	0.175357973	NM_027342
RIKEN cDNA 4930451I11 gene	0.233646847	0.2583342	NM_183131
RIKEN cDNA 4930563C04 gene, mRNA	0.106973251	0.160551176	NM_029231
Transcription activity			
Hairy and enhancer of split 7 (*Drosophila*)	0.331072736	0.428150795	NM_033041
Ankyrin repeat and SOCS box-containing protein 12	0.327740074	0.367856146	NM_080858
Nucleic acid binding			
Developmentally regulated RNA binding protein 1 (Drbp1)	4.843207045	4.596722765	NM_153405
G patch domain containing 3, mRNA	0.230790527	0.329046637	NM_172876
Receptor activity			
Leucine-rich repeat-containing G protein-coupled receptor 7 (Lgr7)	2.405636205	2.106995516	NM_212452

ARC or ARC C terminus rich in proline and glutamate (Pro/Glu-rich domain) (CARC, amino acid residues, 99–208) were resistant to hypoxic cell death to a degree comparable with that of Bcl-2, an anti-apoptotic protein, whereas the ARC N terminus (CARD domain) (NARC, amino acid residues, 1–98) failed to show such inhibitory effects on hypoxic cell death (Fig. 4, *B* and *C*). These results indicate that ARC exhibits anti-hypoxic cell death activity in cardiomyocytes through its C-terminal Pro/Glu-rich region, a calcium-binding region.

Altered Profiles of Gene Expression in the Heart of ARC Tg Mice—Given the vast number of effectors that might potentially influence the survival *versus* the apoptotic decision of myocardial cells after ischemia-reperfusion injury, we performed a large-scale genomic screening for altered gene expression. Specifically, hearts harvested from 3- and 30-week-old control and *ARC* Tg mice were analyzed for gene expression profiling using the GenePix 4000B array. This array contains all of the genes in the murine Unigene data base that have been functionally characterized (~40,000). Heart samples were cross-compared between WT and *ARC* Tg mice, resulting in 170 genes being significantly detected in one or more groups after internal normalization. Of these genes, 39 genes exhibited significantly altered expression between the hearts of *ARC* Tg and WT mice (Table 1).

The raw data shown in Table 1 represent the 2n relationship of expression between 3- and 30-week-old *ARC* Tg mice hearts compared with those of WT mice. The expressions of 23 genes, including the dimethylglycine dehydrogenase precursor (*pMe_2GlyDH*), *bromodomain*, *WD repeat domain containing 3*, *RIKEN cDNA 4930563C04*, and *FYVE RhoGEF* were significantly down-regulated in the hearts of *ARC* Tg mice. On the contrary, 16 genes, including the developmen-

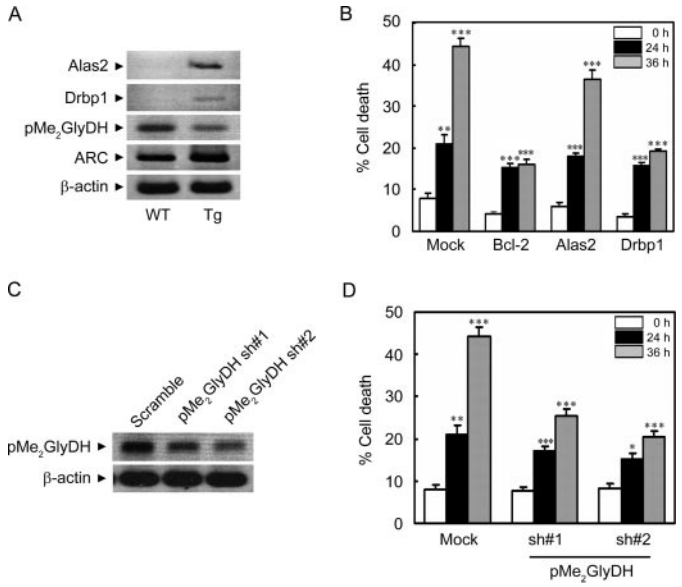

FIGURE 5. **Regulation of ischemic/hypoxic cell death by Drbp1 and pMe_2GlyDH.** *A*, RT-PCR analysis showing expressional regulation of *Alas2*, *Drbp1*, and *pMe_2GlyDH* in the hearts of *ARC* Tg mice. Total RNA was isolated from the hearts of 6-week-old WT and *ARC* Tg mice. RT-PCR analysis was performed using gene-specific synthetic oligonucleotides. β-Actin was used as an internal control. *B*, suppression of hypoxic death of the primary cardiomyocytes by the ectopic expression of Drbp1. Cardiomyocytes were cultivated from neonatal mice, cotransfected with *pGFP*, and *pcDNA* (Mock), *pBcl-2*, *pAlas2*, or *pDrbp1* for 48 h, and then left untreated or exposed to the hypoxic condition for the indicated times. Determination of cell death was assessed based on the morphology of green fluorescent protein-positive cells under a fluorescence microscope. Bcl-2 was employed as a positive control. *Bars* represent mean ± S.E. of three independent experiments. *C* and *D*, reduced expression of pMe_2GlyDH protects cardiomyocytes from hypoxic cell death. Neonatal cardiomyocytes were cultured from WT mice, cultivated for 3 days *in vitro*, and transfected with *pMe_2GlyDH*-shRNA (sh) numbers 1 or 2 for 48 h. Total RNA was isolated and subjected to RT-PCR analysis to examine the expression of *pMe_2GlyDH* (*C*). After transfection with *pMe_2GlyDH*-shRNA, the cells were left untreated or exposed to the hypoxic condition for 24 or 36 h (*D*). Cell death were examined as described in *B*. *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$ *versus* control.

tally regulated RNA-binding protein 1 (*Drbp1*), *Alas2*, and *carbonic anhydrase 3*, exhibited increased expression more than 2-fold in the hearts of *ARC* Tg mice. The data of the microarray analysis were confirmed by RT-PCR analysis (Fig. 5*A*). Compared with control, the level of *Alas2* and *Drbp1* mRNAs was increased in the hearts of *ARC* Tg mice, whereas the precursor form of *pMe_2GlyDH*, the substrate for mitochondria-associated flavinylation reaction, was decreased.

We then examined the contribution of the expressional regulation of *Alas2*, *Drbp1*, and *pMe_2GlyDH* to hypoxic cell death. Interestingly, ectopic expression of *Drbp1*, but not *Alas2*, protected the primary cardiomyocytes from hypoxic cell death as much as Bcl-2 especially at the later time point (36 h) (Fig. 5*B*). On the contrary, the reduction of pMe_2GlyDH expression using shRNAs (numbers 1 and 2) effectively suppressed hypoxic death of cardiomyocytes, reducing death rates from 45 to 21% at 36 h (Fig. 5, *C* and *D*). These results suggest that the cardioprotective effects of ARC in the *ARC* Tg mice may be contributed by the expressional regulation of its downstream mediators, Drbp1 and pMe_2GlyDH.

DISCUSSION

Much progress has been made over the last decade in understanding the genetic and biochemical functions of ARC *in vitro* and *in vivo* (11, 13, 14, 15, 25). In the present study, we demonstrated that ARC, especially the C terminus calcium-binding domain, and its downstream mediators play an important role in heart by protecting cardiomyocytes from ischemia/hypoxia-induced cell death. Major findings from our analysis using *ARC* Tg mice and cultured cardiomyocytes support this conclusion. First, the hearts from the *ARC* Tg mice exhibited improved recovery of contractile performance during reperfusion after ischemia. Second, consistent with the reduced ischemic area and cell death in the hearts of *ARC* Tg mice, cultured cardiomyocytes from neonatal *ARC* Tg were more resistant to hypoxic injury compared with control. Third, ectopic expression of the C-terminal Pro/Glu-rich region of ARC protected the cardiomyocytes from hypoxic cell death. Fourth, the expressional regulation of Drbp1 and pMe_2GlyDH in the heart of *ARC* Tg mice may be associated with the anti-ischemic/hypoxic cell death activity of ARC.

Ischemic/hypoxic cell death in neuronal cells is associated with an intracellular burst of calcium overload. However, a role of calcium in ischemic death of cardiomyocytes is not well established yet. Recently, we proposed that ARC binds to calcium *in vitro* and that it can lower the intracellular calcium transient and calcium accumulation triggered by treatments with ATP and thapsigargin, respectively (25). In particular, the C-terminal Pro/Glu-rich region of ARC binds to calcium, whereas the N-terminal CARD is believed to be involved in protein-protein interaction of the proteins containing CARD. Our observations that the C-terminal Pro/Glu-rich region of ARC suppressed cardiac cell death triggered by hypoxia suggest that the calcium binding ability of ARC may be responsible for its anti-ischemic/hypoxic activity in the cardiomyocytes. Similarly, the C terminus of ARC also exhibited neuroprotective activity against hypoxic injury (25). Also, our proposal may explain that Nop30, an alternative splicing variant of ARC containing the common N-terminal CARD but lacking the divergent C terminus, failed to protect cardiomyocytes from ischemic/hypoxic cell death.[4]

In an effort to find the downstream mediator(s) of ARC in hearts, we also found that ARC induced the alteration in gene expression profiles that may be associated with the increased resistance of *ARC* Tg cardiomyocytes to ischemic/hypoxic injury. Among them, *Drbp1* was increased 4–5-fold in the heart of *ARC* Tg mice and was very potent to suppress ischemic/hypoxic death of the primary cardiomyocytes. Drbp1 is relatively abundant in heart and known to bind tightly to RNA stability elements (AU-rich elements) in the 3′-untranslated region of various mRNAs through four RRM-type RNA-binding domains (26). Therefore, this protein may be induced to stabilize some fragile mRNAs that are required for cell survival of the cardiomyocytes in *ARC* Tg mice during hypoxic damage. In addition, the increased expression of Alas2 in the heart of *ARC* Tg mice reflects its HIF1α-independent increase in the brain during hypoxia (27). Although Alas2 regulates mRNAs encoding the p45 subunit of NF-E2,

β-globin, and enzymes in the heme biosynthetic pathway that shows resistance to cerebral and myocardial ischemia of Tg mice of neuroglobin (28, 29), it may not be relevant to support the tolerance of the cardiomyocytes of *ARC* Tg mice to ischemic/hypoxic cell death.

On the contrary, down-regulation of pMe_2GlyDH was evident in the heart of *ARC* Tg mice and *pMe_2GlyDH*-shRNA protected the primary cardiomyocytes from hypoxic damage. Interestingly, of patients with cardiomyopathy and myocarditis, 36 and 25%, respectively, were anti-flavoenzyme-positive by Western blot and enzyme-linked immunosorbent assay, but only 13% of patients with other heart diseases and none of healthy controls were positive (30). Thus, as a substrate for mitochondria-associated flavinylation reaction, pMe_2GlyDH deserves more characterization as a possible risk factor of heart diseases. Furthermore, together with detailed characterization, such global regulation of gene expression in the heart will be valuable to understand the signaling pathway of cardiac protection against ischemic injury. In the cases of ARC, the cardioprotective activity of ARC against ischemic/hypoxic cell death may then converge into blocking the release of cytochrome *c* from mitochondria (15).

Taken together, our results suggest that the cardiac expression of *ARC* suppresses ischemic/hypoxic injury of cardiomyocytes through its C-terminal calcium-binding domain and expressional regulation of Drbp1 and pMe_2GlyDH, adding valuable molecules to our understanding about the ischemic/hypoxic damages of cardiomyocytes.

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