Sarcoplasmic reticulum calcium defect in Ras-induced hypertrophic cardiomyopathy heart


The nature of the secondary signaling pathway in the disease process is not clear. However, a quantitative correlation between the level of Ras gene expression and the severity of the hypertrophic phenotype in patients with HCM has been reported (20). Ras expression was also induced in the pressure-overloaded rat heart (22) and was revealed as part of the α1-adrenergic signaling pathway during hypertrophy in neonatal cardiac myocytes (24, 30).

The cellular mechanism involved in Ras-mediated contractile defects has been the subject of a number of studies. Using neonatal myocytes in culture coupled with the DNA transfection method, Chen et al. (6) have shown that activation of Ras results in reduced L-type calcium channel currents and sarco(plasmic reticulum (SR) calcium uptake associated with sarcomere disorganization in neonatal cardiomyocytes. In the present study, we investigated the in vivo effects of Ras activation on cellular calcium handling and sarcomere organization in adult ventricular myocytes using a newly established transgenic mouse model with targeted expression of the H-Ras-v12 mutant. The transgenic hearts expressing activated Ras developed significant hypertrophy and postnatal lethal heart failure. In adult ventricular myocytes isolated from the transgenic hearts, the calcium transient was significantly depressed but membrane L-type calcium current was unchanged compared with control littermates. The expressions of sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase (SERCA)2a and phospholamban (PLB) were significantly reduced at mRNA levels. The amount of SERCA2a protein was also modestly reduced. However, the expression of PLB protein and gross sarcomere organization remained unchanged in the hypertrophic hearts, whereas Ser$^{16}$ phosphorylation of PLB was dramatically inhibited in the Ras transgenic hearts compared with controls. Hypophosphorylation of PLB was also associated with a significant induction of protein phosphatase 1 expression. Therefore, our results from this in vivo model system suggest that Ras-induced contractile defects do not involve decreased L-type calcium channel activities or disruption of sarcomere structure. Rather, suppressed SR calcium uptake due to reduced SERCA2a expression and hypophosphorylation of PLB due to changes in protein phosphatase expression may play important roles in the diastolic dysfunction of Ras-mediated hypertrophic cardiomyopathy.

diastolic dysfunction; calcium regulation; protein phosphorylation; phosphatase

CARDIAC HYPERTROPHY is a common clinical manifest in heart diseases (7, 8) and is thought to be part of the compensatory process in response to cardiac injury and loss of contractility (35). The signaling mechanism underlying the development of cardiac hypertrophy and the transition to heart failure remains poorly understood (7, 26, 33). In humans, one distinct form of hypertrophy is familial hypertrophic cardiomyopathy (HCM), which is caused by mutations in a number of sarcomeric components that often lead to asymmetric ventricular hypertrophy, myocyte disarray, and arrhythmic sudden death (4). The nature of the secondary signaling pathway in the disease process is not clear. However, a quantitative correlation between the level of Ras gene expression and the severity of the hypertrophic phenotype in patients with HCM has been reported (20). Ras expression was also induced in the pressure-overloaded rat heart (22) and was revealed as part of the α1-adrenergic signaling pathway during hypertrophy in neonatal cardiac myocytes (24, 30).

The cellular mechanism involved in Ras-mediated contractile defects has been the subject of a number of studies. Using neonatal myocytes in culture coupled with the DNA transfection method, Chen et al. (6) have shown that activation of Ras in myocytes leads to suppressed sarcoplasmic reticulum (SR) calcium load and release. In a more recent study (14), Ho et al. also showed that activation of Ras results in reduced L-type calcium channel currents ($I_{Ca,L}$) and sarcomere disorganization. These studies implicated a Ras-Raf-MEK-ERK signaling cascade in both hypertrophy and calcium handling defects in cardiac myocytes, and the cellular mechanism of the contractile defect involves calcium entry and SR calcium uptake, which has causal effects on sarcomere organization. Although the proposed signaling scheme is plausible, supporting evidence from adult myocytes in vivo has not yet been established. In the present study, we developed a new transgenic model of targeted expression of the activated mutant H-Ras-v12 in ventricular myocytes to investigate the in vivo effects of Ras
activation on calcium handling, sarcomere organization, and expression of calcium-regulating proteins in adult myocytes. Results from whole heart studies demonstrated that Ras activation in vivo led to hypertrophic remodeling with severely impaired ventricular relaxation. Cellular physiology revealed preserved sarcomere organization and unchanged membrane calcium current but suppressed intracellular calcium transient in isolated ventricular myocytes, which was associated with a modest reduction in sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA)\(_{2a}\) protein expression but a remarkable decrease in Ser\(^{16}\) phosphorylation of phospholamban (PLB) associated with a significant induction of protein phosphatase expression. These results provide clear in vivo evidence to implicate specific defects in SR calcium handling in the development of diastolic dysfunction of the Ras-mediated hypertrophic cardiomyopathy heart.

**MATERIALS AND METHODS**

Transgenic construct and generation of transgenic animals. The experimental protocols performed on the animals were approved by the Institutional Animal Care and Use Committee of the University of Maryland and University of California-San Diego (UCSD). The green fluorescent protein (GFP) coding sequence and polyA fragment were cloned from pEGFP-N1 (Invitrogen) by Age I and Afl II digestion and inserted between the two loxP sequences at the BamHI site of the pUC1015loxlox vector (a kind gift from Dr. Jamy Marth, UCSD) to generate ploxGFP. The XbaI/SmaI fragment containing the loxP sequences and the GFP-polyA sequences was then inserted at the HindIII site of the pMHC vector behind the 5.5-kb mouse \(\beta\)-myosin heavy chain (MHC) promoter (a gift from Dr. Jeffery Robbins, University of Cincinnati) to generate the pMHC-lox vector. The cDNA fragment coding for the activated H-Ras-v12 mutant was then inserted at the EcoRV site behind the loxP/GFP fragment to generate the final construct pMHCloxRas. The transgenic mice were generated.

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**Fig. 1.** A: schematic structure of the \(\alpha\)-myosin heavy chain (MHC)-loxRas construct (top) and the Cre-mediated DNA recombination (bottom) between the two loxP sequences. GFP, green fluorescent protein; SV40, simian virus 40. B: picture of agarose gel electrophoresis showing PCR products from reactions using DNA samples prepared from tissues of two \(\alpha\)-MHC-loxRas (control) and two \(\alpha\)-MHC-loxRas/myosin light chain (MLC)-2c/Cre (Ras) double-transgenic mice. The 1.34-kb and 280-bp products indicated by arrows represent the transgene structures before and after Cre-mediated recombination, respectively. C: examples of immunoblots of GFP and Ras proteins expressed in ventricles of transgenic offspring from MLC-2v/Cre and MHC-loxRas mice. Arrows indicate the 21-kDa Ras protein (top) and the 27-kDa GFP protein (bottom).
by established intranuclear injection methods using a KpnI/SfiI fragment digested from the pMHC-floxRas vector (16). For PCR analysis, genomic DNA was prepared from the mouse tail and other tissues as previously described (36) using a 5′ primer (5′-cacctacggcaagctgaccc-3′) and a 3′ primer (3′-tgtaagctgctcgccg-5′) with an expected product of 601 bp in size. The recombination event between the two loxP sites was also detected by PCR using a 5′ primer for the α-MHC promoter sequence (5′-caggacttcacatagaagcc-3′) with an expected product of 1.34 kb long from the intact DNA structure and a 280-bp product from the DNA where the GFP sequences were deleted as a result of the recombination. Both the MLC-2v/Cre and the MHC-floxRas transgenic lines were established by breeding with Blackswiss wild-type mice.

Histological analysis. Tissues were excised and fixed in 4% paraformaldehyde PBS for >4 h, followed by dehydration, paraffin embedding, and sectioning at 1-μm thickness. Hematoxylin-eosin staining was applied according to established protocols, and trichrome staining was performed as described using a modified Masson’s method.

Hemodynamic measurements. Mice at 6 wk of age were anesthetized by an intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (5.0 mg/kg). Hemodynamic parameters, including the positive and negative first derivatives of left ventricular (LV) pressure (±dP/dt) and the time constant of relaxation (τ), were measured as previously described (19, 29). All double-transgenic animals (Ras) and single-transgenic controls were sex- and age-matched.

Calcium signaling in isolated myocytes. Mice (~6 wk of age) were euthanized by an intraperitoneal injection of pentobarbital (100 mg/kg). The heart was removed, and ventricular myocytes were dissociated as described previously (32). With the use of an Axopatch 200A amplifier (Axon Instruments), membrane currents were measured using the whole cell configuration (31, 32). Pipette solutions contained 130 mM CsCl, 20 mM triethylammonium chloride, 5 mM Mg-ATP, 10 mM HEPES, and 0.05 mM fluo-3 (potassium salt); pH 7.2 (with CsOH). Two extracellular solutions were used: solution 1 contained 140 mM NaCl, 5 mM KCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, 0.33 mM Na₂HPO₄, 5.5 mM glucose, and 5 mM HEPES; and solution 2 contained 140 mM NaCl, 5 mM CsCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, 0.33 mM Na₂HPO₄, 5.5 mM glucose, and 5 mM HEPES. After successful conversion to the whole cell mode of voltage clamp in solution 1, solution 2 was used to measure I_Ca at pH 7.4. The temperature was 34–37°C. Test depolarizations were applied after four 50-ms depolarizations to 0 mV at 1 Hz used to maintain SR Ca²⁺ load in the cells examined. A slow (500 ms) ramp depolarization from −80 to −40 mV was followed by a 50-ms period at −40 mV before 200-ms test depolarizations. All signals were analyzed using pCLAMP 6.01 (Axon Instruments) and Origin (versions 5 and 6) softwares.

Confocal microscopy was performed as previously described (12, 32). Cells were loaded with the fluorescent Ca²⁺-sensitive indicator fluo-3 (potassium salt) through the patch pipette (50–100 μM) or were preloaded by exposure to 6 μM fluo-3 AM for 30 min. Fluorescence images were collected using a Bio-Rad MRC 600 (Cambridge, MA) laser scanning confocal microscope (LSCM) equipped with a Zeiss Neofluor ×63 oil immersion lens (numerical aperture =1.25). The LSCM was operated using an IBM personal computer (PC)-compatible computer running SOM and COMOS software (Bio-Rad). Images were analyzed on an IBM-compatible PC running IDL software (Research Systems; Boulder, CO). For analysis, images were normalized by dividing the fluorescence intensity of each pixel (F) by the average resting fluorescence intensity (F₀) at −80 mV. Data are

Fig. 2. A: survival curves of transgenic and wild-type (WT) animals. B: measurements of body weight (left; in g) and cardiac chamber weight versus body weight (right; in mg/g) in WT (n = 4), MHC-floxRas transgenic (n = 7), and MHC-floxRas/MLC-2v/Cre double-transgenic (Ras) mice (n = 7). Values are means ± SD. #P > 0.1 and *P < 0.001 by unpaired Student’s t-test (Ras vs. WT, Ras vs. MHC-floxRas). B. body weight; H, heart weight; LV, left ventricle; RV, right ventricle; LA, left atrium, RA, right atrium.
expressed as means ± SE and significance was tested using Student’s t-test.

**Immunofluorescence assay.** Whole hearts were perfusion fixed with 10% formalin and embedded in paraffin, and 6-μm-thick sections were placed on slides. The slides were deparaffinized and boiled in 0.1 M citric saline buffer in a microwave for 10 min. The sections were blocked by incubation in 1% BSA, 3% normal goat serum, and 0.3% Triton X-100 in PBS for 30 min at room temperature, followed by incubation overnight at 4°C with rabbit anti-dihydropyridine receptor (DHPR) antiserum (a gift from Dr. Marlene Hosey, Northwestern University, Chicago, IL; 1:50 dilution) or a monoclonal anti-desmin antibody (clone DE-U-10, ICN Biomedicals, 1:100 dilution), followed by a 2-h incubation at room temperature with a fluorescein-conjugated goat anti-rabbit secondary antibody. Images were captured on a Zeiss 410 LSCM.

**Protein and RNA analysis.** Protein extracts were prepared from mouse hearts as described previously (25). Western blot was performed using the following antibodies: anti-GFP (Clontech), anti-Ras (Upstate Biotechnology), anti-SERCA2a (a gift from Dr. M. P. Blaustein, University of Maryland, Baltimore, MD), anti-PLB (Affinity BioReagents), anti-phosphorylated PLB (p-Ser16 and p-Thr17), phospho-Proteins), anti-phosphorylated ERK (Thr202/Tyr204), anti-ERK, anti-Akt, anti-phosphorylated Akt (Ser473) (Santa Cruz Biotechnology), and anti-protein phosphatase 1-α (anti-PP1α) and anti-protein phosphatase 2A (anti-PP2A) catalytic subunits (Oxford Biomedical Research). The chemiluminescent autoradiograms were canned and quantified using NIH Image software (Scion Image). RNA samples were prepared from the same heart tissues using TRIzol reagent (Invitrogen) according to the manufacturer’s recommended protocol. The RNA dot-blot analysis was performed based on a published protocol (9) using a set of oligonucleotide probes kindly provided by Dr. Gerald Dorn II (University of Cincinnati). RNA Northern blots were performed as described using a mouse DHPR cDNA probe generated from RT-PCR using a pair of primers (5’-AACACGAG-GATGTACGTCC-3’ and 3’-CAATGCTTAGACGCCTC-5’). All hybridization signals were quantified using a PhosphorImager scanner (Molecular Dynamics) and were normalized against signals from GAPDH (dot blot) or β-actin (Northern blot). The levels of the targeting mRNAs in the double-transgenic hearts were calculated as the fold of induction or reduction relative to those from the control hearts.

**RESULTS**

**LoxP/Cre-mediated gene switch in ventricular muscle cells.** In the transgenic animals carrying the MHC-floxRas construct (Fig. 1A), significant GFP expression was detected in the ventricle, as expected by Western blot analysis (Fig. 1C) or directly under fluorescent microscopy (data not shown). After breeding between the MHC-floxRas and MLC-2v/Cre mice, specific recombination events occurred only in the ventricles of
the double-transgenic offspring as detected by the presence of a 280-bp PCR product (Fig. 1B). As a result, the GFP expression in the ventricle was significantly reduced as determined by Western blot in the double-transgenic animal hearts (Fig. 1C). Inversely, the expression of the Ras transgene was induced in the double-transgenic (Ras) hearts (4.08 ± 0.024, 0.73 ± 0.01 vs. Ras) compared with the wild type (0.906 ± 0.047, P < 0.001 vs. Ras; no significance vs. wild type).

Hypertrophic remodeling and downstream signaling in Ras transgenic hearts. All double-transgenic animals died between 6 and 8 wk, whereas littermates of the other genotypes had a life span over 1 yr (Fig. 2A). To characterize the cardiac pathology in these animals, 5- to 6-wk-old transgenic offspring were analyzed at histological, cellular, and molecular levels. Double-transgenic animals developed significant hypertrophy at this age as demonstrated by an increase in ventricular weight (Fig. 2B). Enlarged atria and the presence of thrombi in both atria (Fig. 3A) also indicated ventricular dysfunction as the transgene is targeted to only the ventricular myocytes. Cellular hypertrophy of ventricular myocytes was also confirmed by the cell capacitance measurements shown later. Associated with the development of hypertrophy, significant interstitial fibrosis was induced in the ventricle of double-transgenic heart as detected by trichrome staining (Fig. 3B). Lymphocyte infiltration and replacement fibrosis were not evident from histological analysis (data not shown), suggesting the absence of inflammatory events often associated with loss of cardiomyocyte viability. Although activated mutant H-Ras-v12 is a potent upstream activator of a downstream MAPK, ERK, in tissue culture cells (17), immunoblots using phosphorylated ERK antibody or direct kinase assay (data not shown) did not detect a significant induction in specific ERK activity in double-transgenic hearts versus controls (Fig. 4). However, total ERK protein was increased. Furthermore, the phosphorylation status of Akt, another related downstream signaling branch, was also not affected in the double-transgenic hearts versus controls. Thus the signaling pathway downstream of constitutively activated Ras has a very different character in vivo versus in vitro.

Cardiac function of transgenic mouse hearts as assessed by hemodynamic measurements. To further assess the cardiac function of the transgenic hearts, hemodynamic parameters were measured using previously established miniaturized techniques (27). As shown in Fig. 5, at the basal level, the double-transgenic mice (Ras) had similar mean heart rates compared with the control group.

Fig. 4. Downstream signaling activity of Ras in hearts. Representative immunoblots of protein samples prepared from double-transgenic (Ras) and WT (control) hearts are shown. Specific proteins were detected using anti-phosphorylated ERK (p-ERK), anti-ERK, anti-phosphorylated Akt (p-Akt), anti-Akt, and anti-Ras antibodies. Coomassie staining of protein gels showed equal loading of the protein samples.

Fig. 5. Hemodynamic analysis of cardiac function in transgenic hearts. The measurements of heart rate [HR; in beats/min (bpm); A], LV end-diastolic pressure (EDP, D), and both positive (dP/dt max, C) and negative LV pressure changes (dP/dt min, B) are presented as mean values from 7 MHC-floxRas (control) transgenic hearts and 7 double-transgenic (Ras) hearts. The error bars represent SDs. Significantly different (#P < 0.1, ##P < 0.05), control vs. double transgenic.
compared with the single-transgenic mice (control). There was a trend in the maximum LV systolic pressure to be lower in the Ras mice compared with the control mice (82.8 ± 17.3 vs. 115 ± 26 mmHg, respectively, \( P = 0.06 \)), but there were no significant differences in the responses to dobutamine, a β-adrenergic agonist (data not shown). However, the basal LV end-diastolic pressure was significantly elevated in the Ras hearts, the maximum LV dP/dt was reduced by 31.6% under basal conditions, and the minimum dP/dt was reduced by 51.3% (Fig. 5). These measurements indicated moderate depression of basal myocardial contractility but severe diastolic dysfunction evidenced by significantly elevated LV end-diastolic pressure and impaired relaxation (minimum dP/dt and \( \tau \)), resembling late-stage hypertrophic cardiomyopathy associated with overt heart failure. Upon stimulation with dobutamine at graded doses, the Ras hearts showed little response of contractile function, a subnormal increase in heart rate, and an impaired lusitropic response and LV diastolic pressure (minimum dP/dt and end-diastolic pressure) (Fig. 5). Similarly, the time constant of LV relaxation \( \tau \) was prolonged under basal conditions in the Ras mice versus controls (15.9 ± 4.0 vs. 10.6 ± 2.1 ms, \( P < 0.01 \)), and there was no shortening of \( \tau \) with β-adrenergic stimulation in Ras transgenic animals (data not shown).

**Calcium signaling in the isolated cardiomyocytes.** To investigate the cellular physiology of the transgenic hearts, intracellular calcium dynamics and membrane calcium currents were analyzed in Ras-expressing transgenic hearts using patch-clamp and line-scanning confocal microscopy as previously described (12, 37) (Fig. 6). Myocytes from the MLC-2v/Cre mice were used as controls because GFP expressed in the α-MHC-floxRas transgenic myocytes would interfere with the fluorescent recording from the calcium-sensitive indicator indo-3 for calcium measurement. Confirming observations made in the intact heart by histology, cellular hypertrophy in Ras-expressing myocytes was indicated by an increase in cell capacitance [Ras myocytes \( (n = 26) \) 234.94 ± 22.09 pF vs. control myocytes \( (n = 36) \): 164.82 ± 10.04 pF, \( P < 0.001 \)], whereas no significant differences were detected in the membrane \( I_{Ca} \) density \( (I_{Ca}/C) \) at potentials positive to −40 mV between the Ras and control myocytes. However, calcium concentration \( ([Ca^{2+}]) \) transients in the Ras-expressing myocytes were lower at broad membrane potentials compared with control cells.

**Expression of cardiac calcium handling proteins.** In previous studies (14, 15) in neonatal myocytes, Ras activation was associated with a reduction in cardiac L-type calcium channels (DHPR) and SERCA2a expression and abnormal sarcomere

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**Fig. 6.** Calcium signaling in transgenic mouse heart cells. **A:** with the use of single heart cells from MLC-2v/Cre (Cre) mice, depolarizations from −80 to −40 mV by slow (500 ms) ramp preceded each step depolarization to a test potential (200-ms duration) over the range of −30 to +70 mV (0 mM shown; top). Step depolarization from −40 to the test potential elicited the L-type \( Ca^{2+} \) channel current \( (I_{Ca}, \text{bottom}) \) and a intracellular \( Ca^{2+} \) concentration \( ([Ca^{2+}]) \) transient (middle two panels). A confocal line-scan image of \( [Ca^{2+}] \) was obtained in fluo-3-loaded cells (see MATERIALS AND METHODS) and the time course of the \( [Ca^{2+}] \) transient is plotted as the fluorescence intensity of each pixel \( (F) \) divided by the average resting fluorescence intensity \( (F_0) \). **B:** traces similar to those shown in A but in a RAS-expressing double-transgenic mouse (Ras) heart cell. **C:** voltage dependence of \( I_{Ca} \) density (in pA/pF). • Data from MLC-2v/Cre cells \( (n = 25) \); ○, data from RAS-expressing myocytes \( (n = 17) \). Values are means ± SE. At potentials positive to −40 mV, no significant difference in the two data sets was identified \( (P < 0.05) \). **D:** voltage dependence of the \( [Ca^{2+}] \) transient plotted as \( F/F_0 \). *Significant differences \( (P < 0.05) \) between Cre \( (n = 10) \) and Ras data \( (n = 17) \). Clear cellular hypertrophy in Ras cells (compared with Cre cells) is indicated by the measured cell capacitance for cells from Ras (234.94 ± 22.09 pF, \( n = 26 \)) compared with Cre (164.81 ± 10.04 pF, \( n = 35 \)) animals \( (P < 0.001) \). The temperature was 37°C for all experiments.
organization. At the mRNA level, as determined by Northern blot or dot-blot analysis, DHPR, SERCA, and PLB expression were indeed significantly lower in Ras transgenic hearts versus controls (DHPR 55.0 ± 5.6%, SERCA 69.2 ± 18.3%, and PLB 71.5 ± 17.7%, respectively; Fig. 7). In contrast, induction was observed in hypertrophic marker genes, such as atrial natriuretic factor (11.6 ± 1.9-fold vs. controls) and β-MHC (5.7 ± 2.4-fold vs. controls) (Fig. 7). At the protein level, as determined by immunohistochemistry, however, the staining intensity and distribution pattern of DHPR was indistinguishable between Ras and control samples (Fig. 8), confirming the membrane current measurements shown earlier (Fig. 6). The expression and cellular distribution of desmin, an intermediate filament protein that is associated with sarcomere structure in cardiac cells, remained unchanged in Ras hearts compared with controls (Fig. 8). These data suggest that Ras-induced hypertrophy is not associated with significant changes in DHPR expression or with gross sarcomere disorganization at the cellular level. The total protein level of SERCA2a was modestly reduced to 79.6 ± 12.5% of controls (P = 0.06; Fig. 9, A and B). PLB protein was not changed in Ras transgenic hearts versus controls (Fig. 9C), but its phosphorylation status at Ser16, a protein kinase A (PKA)-dependent site, was reduced to a mostly undetectable level compared with controls (Fig. 9C). Loss of PLB phosphorylation at the Ser16 site in Ras transgenic hearts was correlated with a significant increase in PP1α but not PP2A (Fig. 9D). These in vivo analyses in Ras-activated transgenic hearts suggest that decreased SERCA expression and hypophosphorylation of PLB may contribute to the impaired SR calcium uptake that leads to diastolic dysfunction in the HCM heart.

**DISCUSSION**

We report here the application of the loxP/Cre-mediated gene switch system to achieve conditional gene expression in ventricular muscle cells in an attempt to fully assess the in vivo role of the Ras signaling pathway in the development of HCM. Massive cardiac hypertrophy occurred in the double-transgenic offspring from MLC-2v/Cre and α-MHC-floxRas mice when the expression of the Ras mutant was switched on. Direct hemodynamic measurements revealed a significant decrease in contractility and diastolic dysfunction associated with interstitial fibrosis in the ventricles and a blunted response to a

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**Fig. 7.** mRNA levels of cardiac genes in transgenic hearts. Total RNA samples were prepared from ventricles of transgenic animals. The relative mRNA levels were determined by the dot-blot method using the GAPDH signal as an internal control for normalization and using the mean values from the 4 MHC-floxRas (control) samples as 1 arbitrary unit compared with the values from the 4 double-transgenic (Ras) samples. SERCA2a, sarco(endo)plasmic reticulum Ca2+-ATPase 2a; PLB, phospholamban; DHPR, dihydropyridine receptor; ANF, atrial natriuretic factor. Values are means ± SD. #P < 0.01 vs. control.

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**Fig. 8.** Immunohistochemistry for DHPR and sarcomere structure. Representative immunofluorescent images of sections were prepared from α-MHC-floxRas (control) and double-transgenic (Ras) hearts stained with anti-DHPR antibodies or anti-desmin antibodies. Images were recorded digitally from a Zeiss confocal microscope using identical exposure parameters.
β-adrenergic agonist. At the cellular level, contractile dysfunction of Ras transgenic myocytes was associated with a depressed intracellular calcium transient but with no changes in I_{Ca} or gross sarcomere integrity. Although SERCA and PLB showed lower expression at the mRNA level in the Ras transgenic heart, only SERCA2a showed a modest decrease at the protein level. In comparison, Ser^{16} phosphorylation of PLB was significantly diminished in Ras transgenic hearts compared with controls, suggesting that loss of SERCA activity due to enhanced inhibition by PLB plays an important role in the contractile dysfunction in the Ras-induced hypertrophic heart.

Our in vivo characterization indicated that the Ras-expressing transgenic mice developed a severe HCM phenotype that resembled many clinical features of human HCM at an advanced stage. In a previous in vivo study (19) with a MLC-2v/Ras model, the transgene expression level was very low, detectable only by the sensitive RT-PCR method. A mild hypertrophic phenotype was observed only in homozygous animals after selective breeding. The effect on physiological function of the transgene was limited to impairment of relaxation, whereas the systolic function as assessed by maximum dP/dt and echocardiography was normal, and the response to β-adrenergic agonist stimulation was also preserved (13, 19). Therefore, a significant correlation between the expression level of the Ras and the severity of the HCM phenotype can be established in transgenic animals. The relevance of this observation is supported by a recent clinical study (20) that also
reported a significant correlation between the expression level of the c-H-Ras gene and the severity of the cardiomyocyte hypertrophy in patients with HCM. All these findings further implicate the Ras-mediated signaling pathway in the induction and development of concentric cardiac hypertrophy and diastolic dysfunction in HCM.

In our single-cell studies, defects in calcium dynamics were observed in Ras myocytes with suppressed intracellular calcium transients and unchanged membrane peak calcium currents. This observation is consistent with other studies (10–12) in a number of disease models, where defects in excitation-contraction coupling have been associated with reduced calcium transients rather than reduced membrane calcium currents. Although we could not perform Western blot analysis using the currently available anti-DHPR antibodies, immunohistochemistry did not observe any significant changes in either the expression level or cellular distribution pattern in the Ras transgenic heart (Fig. 8). On the other hand, the diastolic dysfunction seen in the Ras transgenic heart could be attributed to the defects in calcium uptake during diastole as a result of diminished SERCA2a activity due to hypophosphorylation of PLB at the Ser16 site (Fig. 9C). The loss of PLB phosphorylation at this PKA-dependent site raises the possibility of an impaired β-adrenergic signaling pathway in the Ras transgenic heart, which is also supported by the lack of lusitropic response at the whole heart level.

Our observations, along with the fact that the gross sarcomere organization in Ras myocytes is largely preserved (Fig. 8), contradict the results from earlier reports (14, 15) that showed reduced ICa and sarcomere disorganization in Ras-activated myocytes. The discrepancies between these studies may be due to the difference in experimental models (in vivo transgenic mouse hearts vs. cultured rat neonatal cardiomyocytes) or in the methods of analysis (transgenic vs. electroporation, direct mRNA measurement vs. promoter/reporter gene assay). Although Ho et al. suggest that a downstream signaling molecule of Ras, including Raf/MEK/ERK, is responsible for the observed changes in calcium cycling defects and sarcomere disorganization (14, 15), specific activation of ERK in vivo using MEK1 only results in a mild form of cardiac hypertrophy but with enhanced basal contractility and an absence of apparent cardiomyopathy (5). Our results from Fig. 4 showing a lack of ERK and phosphatidylinositol 3-kinase/Akt activation in Ras transgenic hearts at 5–6 wk of age further supports the notion that a Ras-Raf-MEK1-ERK pathway may mediate the development of compensatory hypertrophy, whereas other branches of Ras downstream signaling cascades may be responsible for the pathological remodeling and calcium cycling defects. The attenuated phosphorylation of PLB by PKA and the blunted response to a β-adrenergic agonist observed in the Ras transgenic animals are likely a result of a desensitized β-adrenergic signaling pathway (21). However, it is unclear whether desensitization of β-signaling in the Ras transgenic heart is a direct result of Ras activation or an indirect consequence of chronic elevation of catecholamine associated with Ras-induced hypertrophy and ventricular remodeling. Earlier studies have implicated PP1 as one of the major protein phosphatases that can contribute to PLB hypophosphorylation and its inhibitory function to SERCA2a in the failing heart (2, 3, 18, 23, 38). Our finding that PP1 but not PP2A expression is significantly induced in the Ras transgenic heart indicates an exciting possibility: that changes in specific protein phosphatase activity are part of Ras-induced downstream signaling events that may play an important role in the manifestation of cellular pathology. Clearly, further studies in this transgenic model of the Ras-induced hypertrophic cardiomyocyte are necessary to establish a specific causal relationship between Ras signaling and different aspects of pathological remodeling, including hypertrophy and SR calcium dysfunction.

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