Caveolin-3 Overexpression Attenuates Cardiac Hypertrophy via Inhibition of T-type Ca2+ Current Modulated by Protein Kinase Ca in Cardiomyocytes*

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Background: Ventricular remodeling altered caveolin-3 expression, and Ca2+ signaling is associated with cardiac hypertrophy. Cardiomyocyte-specific caveolin-3 overexpression prevented cardiac hypertrophy by inhibiting the T-type Ca2+ current and hyperactivation of calcineurin-dependent nuclear factor of activated T-cell signaling. Overexpression of Cav-3 is essential for protective Ca2+ signaling in pathological cardiac hypertrophy. Caveolin-3 overexpression in neonatal myocytes may be used as a therapeutic strategy for treatment of many cardiovascular diseases.

Pathological cardiac hypertrophy is characterized by subcellular remodeling of the ventricular myocyte with a reduction in the scaffold protein caveolin-3 (Cav-3), altered Ca2+ cycling, increased protein kinase C expression, and hyperactivation of calcineurin/nuclear factor of activated T-cell (NFAT) signaling. However, the precise role of Cav-3 in the regulation of local Ca2+ signaling in pathological cardiac hypertrophy is unclear. We used cardiac-specific Cav-3-overexpressing mice and in vitro and in vivo cardiac hypertrophy models to determine the essential requirement for Cav-3 expression in protection against pharmacologically and pressure overload-induced cardiac hypertrophy. Transverse aortic constriction and angiotensin-II (Ang-II) infusion in wild type (WT) mice resulted in cardiac hypertrophy characterized by significant reduction in fractional shortening, ejection fraction, and a reduced expression of Cav-3. In addition, association of PKCα and angiotensin-II receptor, type 1, with Cav-3 was disrupted in the hypertrophic ventricular myocytes. Whole cell patch clamp analysis demonstrated increased expression of T-type Ca2+ current (ICa,T) in hypertrophic ventricular myocytes. In contrast, the Cav-3-overexpressing mice demonstrated protection from transverse aortic constriction or Ang-II-induced pathological hypertrophy with inhibition of ICa,T and intact Cav-3-associated macromolecular signaling complexes. siRNA-mediated knockdown of Cav-3 in the neonatal cardiomyocytes resulted in enhanced Ang-II stimulation of ICa,T mediated by PKCα, which caused nuclear translocation of NFAT. Overexpression of Cav-3 in neonatal myocytes prevented a PKCα-mediated increase in ICa,T and nuclear translocation of NFAT. In conclusion, we show that stable Cav-3 expression is essential for protecting the signaling mechanisms in pharmacologically and pressure overload-induced cardiac hypertrophy.

Cardiac hypertrophy is a major predictor of many cardiovascular diseases, including arrhythmias, sudden death, and heart failure. Cardiac hypertrophy is an adaptive response of the heart during stress to preserve contractility and cardiac function. However, continued cardiac stress through either pressure or volume overload or neurohormonal stress leads to pathological hypertrophy and heart failure (1, 2), during which an alteration in cardiac myocyte Ca2+ handling is commonly observed (3–5). It is well established that an increase in cytosolic Ca2+ is responsible for activating calcineurin (Cn)2 and nuclear factor of activated T-cell (NFAT) signaling leading to the expression of genes involved in pathological cardiac hypertrophy. With the progression of cardiac hypertrophy, a structural remodeling of the ventricular myocytes results in T-tubule disruption at advanced heart failure (6). With myocyte remodeling during cardiac hypertrophy and heart failure (7, 8), it is likely that the micro-architecture of the sarcolemma and T-tubules, which are major determinants of the local control of Ca2+ in the heart, is altered.

The abbreviations used are: Cn, calcineurin; Cav-3, caveolin-3; TCC, T-type calcium channel; Ang-II, angiotensin II; Ica,T, T-type calcium channel current; Ica,T, T-type calcium channel current; NFAT, nuclear factor of activated T-cells; OE, overexpression; NMWM, neonatal mouse ventricular myocytes; TAC, transthoracic aortic constriction; pF, picofarad; TRPC, transient receptor potential; co-IP, co-immunoprecipitation; HW/BW, heart weight to body weight; AT1-R, angiotensin receptor type 1; eGFP, enhanced GFP; qPCR, quantitative PCR; AT1, angiotensin II receptor, type 1; TAC, transverse aortic constriction; LCC, L-type Ca2+ channel.
Caveolae Overexpression Attenuates Cardiac Hypertrophy

Caveolae are specialized microdomains in the sarcolemmal membrane of ventricular myocytes that serve to integrate sympathetic and parasympathetic inputs to the heart to precisely regulate cardiac function. Caveolae contain a variety of signaling proteins such as G-protein-coupled receptors, kinases, phosphatases, and ion channels, including the voltage-gated L-type and the T-type Ca\(^{2+}\) channels and other calcium cycling proteins (9–11). Caveolin-3 (Cav-3) is a muscle-specific scaffolding protein integral to caveolae in the cardiomyocyte and plays a significant role in the physiology of the heart (12). Reduced expression of Cav-3 and caveolae in cardiomyocytes is reported in cardiac diseases, including myocardial infarction and heart failure (13). In contrast, we have shown that overexpression of Cav-3 prevents ischemic injury (14) and cardiac hypertrophy (15). However, the precise role of Cav-3 in the regulation of local Ca\(^{2+}\) signaling and regulation of pathophysiology in cardiac hypertrophy is unclear.

In this study, we determined whether a loss of Cav-3 expression during pressure overload and angiotensin-II (Ang-II) treatment contributes to altered Ca\(^{2+}\)-induced Cn-NFAT signaling and the development of pathological cardiac hypertrophy. We demonstrated that a loss of Cav-3 expression after pressure overload or Ang-II treatment results in the disruption of caveola-associated macromolecular signaling proteins, increased stimulation of T-type Ca\(^{2+}\) channels (TTCC) current (\(I_{Ca,T}\)) mediated by PKC\(\alpha\), and the activation of Cn-NFAT signaling in cardiomyocytes. Additionally, Cav-3 overexpression in cardiomyocytes inhibits basal and Ang-II-stimulated \(I_{Ca,T}\) that is modulated by PKC\(\alpha\) and the activation of Cn-NFAT signaling. Using mice with cardiac-specific overexpression Cav-3 (Cav-3 OE) (14), generated using the \(\alpha\)-myosin heavy chain promoter system, we demonstrated that development of pressure overload-induced pathological cardiac hypertrophy \textit{in vivo} is prevented.

Materials and Methods

Transverse Aortic Constriction (TAC) induced Pressure Overload Hypertrophy—TAC was performed in 12–16-week-old male mice to induce pressure overload as described earlier (16). Briefly, the mice were anesthetized with 2% isofluorane inhalation, and insertion was made to expose the aorta. A 27-gauge needle was placed on top of the aorta and ligated using 7-0 silk sutures, following which the needle was removed to produce refined stenosis of the vessel. The muscle cavity and skin were sutured, and the wound was closed with wound clip. Mice of the same genetic background received a sham operation in which a silk suture band was placed around the aorta but not ligated and was subsequently removed.

Ang-II Infusion Induced Cardiac Hypertrophy—Ang-II or saline was infused for 28 days using mini osmotic pumps (model 2002, ALZET Osmotic Pumps, Cupertino, CA). Osmotic pumps primed at constant rate of 0.5 \(\mu\)g/h, filled with 5 mg/ml Ang-II (Sigma) or isotonic saline, were inserted subcutaneously above the scapula under sterile conditions in anesthetized mice. For \textit{in vitro} Ang-II-induced cardiac hypertrophy, NMVM were isolated from 1- to 2-day-old pups and grown in culture treated with Ang-II (10\(\mu\)mol/liter) for 48 h.

Echocardiography Analysis—Noninvasive transthoracic echocardiography was performed using Visual Sonics Vevo 770 ultrasonograph. ECG was monitored continuously in anesthetized mice (1.5% isoflurane) maintained on a heated platform. After 4 weeks of saline or Ang-II infusion and sham or TAC surgery in mice, left ventricular wall thickness, chamber dimensions, and contractility were evaluated. The pressure gradients across the aortic constriction were measured to ensure similar pressure overload in the TAC mice.

Transmission Electron Microscopy—Rapidly excised mouse hearts were initially perfused with Tyrode’s solution (10 ml) in a Langendorff perfusion system followed by fixative (2.5% glutaraldehyde, 2.0% paraformaldehyde) in 0.1 mol/liter cacodylate buffer for 30 min. The left ventricle was dissected out, cut into 2 \times 2-mm blocks, immersed in the same fixative, and left overnight at 4 °C. The samples were rinsed in the same buffer, postfixed in 1% osmium tetroxide, dehydrated in a graded ethanol series, rinsed in propylene oxide, and embedded in Epon 812 substitute. After resin polymerization, the samples were then sliced into 70-nm sections with a Leica EM UC6 ultramicrotome and placed on 200 mesh transmission electron microscopy grids. The samples were post-stained in 8% uranyl acetate in 50% EtOH and Reynolds’s lead citrate, viewed on a Philips CM120 transmission electron microscope, and documented with a SIS MegaView III digital camera. A relative number of caveolae distributed in the myocyte sarcolemmal membranes was estimated by obtaining about 250 images from three preparations of WT or TAC samples. A threshold size for individual caveolae was set between 40 and 100 nm. The number of caveolae was counted as per unit length (\(\mu\)m) of myocyte sarcolemmal membranes using ImageJ software from a series of random EM micrographs. To confirm caveola vesicles from other regions, immunogold labeling using anti-Cav-3 antibody was performed. Data were analyzed by plotting frequency histograms of the number of caveolae per \(\mu\)m of sarcolemma for each observation.

Isolation of Mouse Ventricular Myocytes—Neonatal or adult mouse ventricular myocytes were enzymatically isolated as described previously (11). Rod-shaped myocytes with clear striations were randomly selected for electrophysiology studies. The neonatal myocytes were transfected by the electroporation method (11) by a Nucleofector device (Lonza, USA) using Ingenio electroporation reagent (catalog no. MIR 50115) from Mirus BioSciences, and cells were used for experiments 72–96 h after transfection.

siRNA-mediated Cav-3 Knockdown and shRNA-mediated PKC\(\alpha\) Knockdown—siRNA-mediated knockdown of Cav-3 in isolated neonatal mouse cardiomyocytes was archived by transfecting three pairs of pre-validated Cav-3–specific siRNAs (10 nmol/liter) as described earlier (11, 18). For shRNA-mediated knockdown of PKC\(\alpha\), the neonatal myocytes were transfected with 1 \(\mu\)g of plasmid containing an shRNA sequence specific for the PKC\(\alpha\) isoform (5′-GAAACAAAGGAAUGACUU-3′) (19), a kind gift from Dr. Scott Kaufmann (Mayo Clinic, Rochester, MN).

Quantitative Real Time PCR Analysis—MIQE guidelines were followed in designing qPCR experiments. Total RNA isolated from SHAM, TAC, saline, and Ang-II treated mouse left
ventricles using the GenElute Mammalian Total RNA Miniprep kit (Sigma). RNA quantity and quality were determined with UV spectrophotometry. First strand cDNA synthesis was performed with 1 μg of total RNA using iScript reverse transcription supermix for RT-qPCR (Bio-Rad). The levels of cDNA were analyzed by quantitative real time PCR using TaqMan gene expression master mix (Applied Biosystems). Probes and primers were designed for multiplex analysis (Integrated DNA Technologies). Primers and probes designed for analysis of the genes of interest are provided in Table 1. RT-qPCR was performed on CFX96™ real time systems (Bio Rad). For quantification of mRNA levels, the normalized cycle values were obtained by the subtraction of corresponding GAPDH (ΔC_T), and data are presented as fold change (for TAC or Ang-II treatment) with respect to expression in SHAM or saline-treated samples (ΔΔC_T).

Preparation of Caveolin-enriched Fractions—Caveolin-enriched membrane fractions from mouse ventricular myocytes from WT or Cav-3 OE following TAC or sham treatment were prepared by using a previously described method (18). Briefly, freshly isolated adult mouse myocytes (10 × 10^6 cells) were suspended in 2 ml of ice-cold 0.5 mol/liter sodium carbonate (pH 11.0) and homogenized sequentially by using a loose-fitting Dounce homogenizer (10 strokes), a Polytron tissue grinder (three 10-s bursts; Kinematica, Brinkmann Instruments, Westbury, NY), and a sonicator (three 20-s bursts; Branson Sonifier 250, Branson Ultrasonic, Danbury, CT). The homogenate was adjusted to 45% sucrose in MBS (25 mmol/liter Mes (pH 6.5), 0.15 mol/liter NaCl) and placed at the bottom of an ultracentrifuge tube. A 5–35% discontinuous sucrose gradient (in 0.15 mol/liter NaCl (TBS), and lysed in ice-cold solubilization buffer containing 25 mmol/liter Tris-HCl (pH 7.4), 150 mmol/liter NaCl (TBS), and 1% Triton X-100, 2 mmol/liter phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml benzamidine, 5 μg/ml leupeptin, and 5 μmol/liter pepstatin A. The lysate was centrifuged at 10,000 × g for 10 min to remove insol-

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### Table 1

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<th>Ref End</th>
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ub le debris, and the soluble supernatant was precleared by using protein G Dynabeads (Invitrogen), followed by incubation for 4 h at 4°C with anti-Cav-3 (2 μg) antibodies or control IgG in a total of 450 μl. 50 μl of a 1:1 slurry of protein G Dynabeads was added to the sample and further incubated for 1 h at 4°C. Beads were washed four times with solubilization buffer on a magnetic stand, and bound proteins were eluted with SDS-PAGE sample buffer by boiling for 5 min. Immune complexes were analyzed by SDS-PAGE (4–15% gradient gels, Bio-Rad) and Western blot by probing with antibodies to Cav-3, PKCα, angiotensin receptor type 1, NOS-3, and βAR.

Electrophysiology—Electrophysiological experiments were carried out using the whole cell patch clamp technique using Axopatch 200B amplifier (Axon Instruments, Foster City, CA) with pClamp version 10.2 software. The patch pipettes were pulled from thin-walled borosilicate glass capillaries (World Precision Instruments, Inc., Sarasota, FL) on Sutter P-87 micropipette puller (Sutter Instrument Co.) and polished using microforge MF900 (Narishige). All the experiments were carried out at room temperature with pipette resistance of 1.5–2.5 MΩ. Recordings were made from the freshly isolated healthy rod-shaped ventricular myocytes. The bath solution to holding potential of 50 mV, and a further 10-mV step depolarization was applied up to 70 mV for 200 ms (I_Ca,T). When this I_Ca,T is absent (absence of peak I_Ca,T at −30 mV) in the cells, the two I-V curves generated from holding potentials of −90 and −50 mV will overlap but may also exhibit a I_Ca,dif at, or membrane potentials positive to −10 mV. A small difference in the currents at potentials positive to −10 mV was not due to the presence of I_Ca,T but could be due to partial voltage-dependent inactivation of I_Ca,T, recorded with a holding potential of −50 mV. The current traces were corrected for linear capacitance and leak using −P/4 subtraction. The data were filtered at 5 kHz and digitized at 50 kHz and were analyzed using Microcal Origin software (Origin Lab Corp., North-ampton, MA). The data were analyzed using OriginPro.9.0.0 (OriginLab Corp.).

Statistics—Statistical significance was analyzed with Student’s paired t test. Average data are reported as mean ± S.E.

Results

Cav-3 and Caveola Expression Is Altered in Ventricular Myocytes in TAC or Ang-II Infusion-induced Cardiac Hypertrophy—Previous studies have indicated that the level of Cav-3 expression and the density of caveolae can change in various models of cardiac disease, including cardiac hypertrophy and heart failure (13, 20). We investigated for changes in the expression of Cav-3 in mouse models of pressure overload-induced cardiac hypertrophy. 12–16-Week-old C57BL6 mice were chronically treated with Ang-II via continuous infusion (via mini osmotic pumps; see under “Materials and Methods”) or TAC surgery. Cardiac function was measured by echocardiography before TAC, sham, or Ang-II or saline treatment and then after 4 weeks of treatment. Four weeks of TAC or Ang-II treatment resulted in the development of pathological cardiac hypertrophy as evidenced by significant changes in HW/BW, and reduced fractional shortening and ejection fraction in the WT mice compared with sham or saline-treated mice (Fig. 1, A–C). The RNA isolated from left ventricular myocytes showed a significant increase in atrial natriuretic peptide, B-type natriuretic peptide expression, and a significant reduction in the expression for Cav-3 and SERCA2a levels in TAC- and Ang-II-treated mice compared with sham or saline-treated mice (Fig. 2, A and B). The mRNA levels for Cav-1 were unchanged between the groups. We then estimated the expression of Cav-3 protein by semi-quantitative Western blot analysis in mouse ventricular myocytes. Cav-3 expression levels (Fig. 1, D and E) were significantly reduced (~50%) after TAC- or Ang-II-induced cardiac hypertrophy when compared with control hearts (sham or saline infusion). We then performed transmission electron microscopy analysis on the left ventricle tissue sections after 4 weeks of TAC or sham treatment. As shown in the representative electron micrograph (Fig. 1F), after 4 weeks of TAC, the number of caveolae was reduced significantly (64%) in the left ventricular myocytes compared with the sham mice (Fig. 1G).

I_Ca,T Is Up-regulated in Left Ventricular Myocytes in Cardiac Hypertrophy—Previous studies have shown that I_Ca,T is expressed only during cardiac development and is undetectable in adult ventricular myocytes (21, 22). However, I_Ca,T was shown to be re-expressed in ventricular myocytes in diseased hearts, including pressure overload-induced cardiac hypertrophy (11, 23, 24) in cardiomyopathic hamster (25), and in post-infarction remodeled rat left ventricle (26). We measured the expression levels for the TTCC subunit isoforms, Ca3.1 and Ca3.2, in the ventricles by qPCR analysis. We noticed an increased mRNA expression for Ca3.1 and Ca3.2 subunits in the left ventricles from TAC- or Ang-II-treated mice compared with vehicle- or sham-treated mice, respectively (Fig. 2, A and B). The mRNA levels for Ca3.2 appeared to be significantly higher (p < 0.05) in the TAC ventricle compared with sham (Fig. 2A). In contrast, the mRNA levels for the Ca1.2 subunit of the LTCC did not change after TAC or Ang-II treatment compared with controls. We then investigated whether the I_Ca,T was detectable in the adult left ventricular myocytes after TAC or Ang-II infusion. I_Ca,T and L-type Ca2+ channel current (I_Ca,L) were measured using the whole cell patch clamp technique by applying a dual pulse protocol described previously by...
As shown in Fig. 3, a re-expression of $I_{\text{Ca, T}}$ (−1.6 ± 0.4 pA/pF) in ventricular myocytes after 4 weeks of TAC compared with negligible current (−0.02 ± 0.05 pA/pF) in ventricular myocytes from sham-treated mice (Fig. 3B) was observed. In the sham myocytes (Fig. 3C), there was no detectable $I_{\text{Ca, T}}$ at −30 mV. Similarly, Ang-II infusion resulted in re-expression of $I_{\text{Ca, T}}$ in ventricular myocytes (−0.84 ± 0.11 pA/pF) compared with saline-treated animals (−0.19 ± 0.34 pA/pF) (Fig. 3C). To confirm the expression of $I_{\text{Ca, T}}$ in the WT hypertrophied (TAC) cardiomyocytes, we first measured $I_{\text{Ca, T}}$ and then perfused cells with an $I_{\text{Ca, T}}$ inhibitor Ni$^{2+}$ (300 μM), which completely abolished the $I_{\text{Ca, T}}$ (Fig. 3E) but did not significantly impact the $I_{\text{Ca, L}}$ (data not shown). The inhibition of $I_{\text{Ca, T}}$ by Ni$^{2+}$ confirmed that the Ca$^{2+}$ current elicted at −30 mV is indeed $I_{\text{Ca, T}}$. These data confirm that $I_{\text{Ca, T}}$ is re-expressed in the ventricular myocytes during TAC- or Ang-II-induced cardiac hypertrophy. $I_{\text{Ca, L}}$ was not significantly different in the ventricular myocytes after TAC or Ang-II treatment compared with controls (Fig. 3D).

Cardiac-specific Cav-3 Overexpression Attenuates Cardiac Hypertrophy—Recently, we have demonstrated that the cardiac-specific overexpression of Cav-3 resulted in attenuation of
Caveolin-3 Overexpression Attenuates Cardiac Hypertrophy

Ang-II-induced cardiac hypertrophy. The data with TAC studies are in agreement with and confirm our previously published results (15).

Caveolin-3 Overexpression Inhibits $I_{Ca,T}$ in Cardiac Hypertrophy—A recent study suggested that a re-expression of the Cav3.2 TTCC current is responsible for the induction of pathological cardiac hypertrophy via calcineurin/NFAT hypertrophic signaling (27). We have shown that Cav-3 overexpression inhibits Cav3.2 ($\alpha_{1c}$) channel current but not the Cav3.1 ($\alpha_{1c}$) current in mouse neonatal cardiomyocytes (11). Therefore, we hypothesized that ventricular myocytes from Cav-3 OE mice will inhibit re-expression of $I_{Ca,T}$, specifically the $I_{Ca,v,2p}$, and attenuate pressure overload-induced pathological cardiac hypertrophy. We investigated the role of Cav-3 on $I_{Ca,T}$ inhibition in pathological hypertrophy using the Cav-3 OE or littermate WT control mice subjected to TAC or Ang-II infusion for 4 weeks. $I_{Ca,T}$ and $I_{Ca,L}$ were measured in adult ventricular myocytes (AVMs) from mice subjected to different treatment groups. Cell capacitance measured during voltage clamp measurement showed TAC or Ang-II infusion caused 27 and 34% increase, respectively, in the AVM size in the WT mice compared with saline-treated animals (Fig. 4E). Cell capacitance of the AVMs from Cav-3 OE mice was 50% greater than AVMs from WT saline-treated mice. TAC or Ang-II infusion did not significantly alter the cell capacitance in AVMs from Cav-3 OE mice (Fig. 4F). Peak $I_{Ca,T}$ measured at $-30 \text{ mV}$ normalized to cell capacitance and expressed as $\text{pA/pF}$ (Fig. 3C), was significantly increased in the AVMs from WT mice after either TAC or Ang-II infusion. $I_{Ca,T}$ expression was negligible in saline- and sham-treated WT AVMs (Fig. 3C). As shown in Fig. 4, the peak $I_{Ca,T}$ was completely inhibited in the AVMs from Cav-3 OE mice after TAC (Fig. 4, A and B) or Ang-II infusion (Fig. 4C), suggesting that cardiac myocyte-specific overexpression of Cav-3 inhibits the TAC- or Ang-II-induced increase in $I_{Ca,T}$ during pathological hypertrophy. The peak $I_{Ca,L}$ density elicited at 0 mV was not different in the AVMs from mice with all treatment groups (Fig. 4D). In addition, the activation and inactivation of $I_{Ca,L}$ were not different in the AVMs from mice with all treatment groups (data not shown). The $I_{Ca,L}$ data also confirmed our earlier demonstration that Cav-3 overexpression does not alter peak $I_{Ca,L}$ density in neonatal mouse ventricular myocytes (11).

Changes in Expression of Key Signaling Proteins in Ventricular Myocytes during Cardiac Hypertrophy—To examine whether the increase in $I_{Ca,T}$ expression in hypertrophic myocytes is associated with changes to the protein level for TTCC isoforms Ca3.1 and Ca3.2, and other key signaling proteins involved in cardiac hypertrophy, we performed semi-quantitative Western blot analysis on ventricular lysates prepared from WT and Cav-3 OE mice following TAC or sham treatments. As shown in Fig. 5, the expression levels for the Ca3.2 and PKCα proteins were significantly increased in WT TAC myocytes compared with sham mice. However, the expression levels of Ca3.2 and PKCα were normalized in the Cav-3 OE hearts following TAC and were not different compared with WT and Cav-3 OE sham hearts. The expression level of Ca3.1 and PKCβ1 was unchanged in all groups. Also, the Cav-3 overexpression in the hearts (Cav-3 OE mice) did not impact the expression profiles of any of the above proteins. However, the

TAC-induced cardiac hypertrophy via enhanced natriuretic peptide expression (15). Here, we investigated whether Cav-3 OE mice have attenuation of cardiac hypertrophy after Ang-II infusion. Male 12–16-week-old Cav-3 OE and WT mice were subjected to Ang-II or saline infusion or TAC or sham surgery for 4 weeks. As shown in Table 2, echocardiography revealed that WT mice had decreased ejection fraction and percentage fractional shortening after 4 weeks of TAC or continuous Ang-II infusion (Table 2), whereas Cav-3 OE mice subjected to TAC had no change in either measure of cardiac function compared with sham-treated mice. WT mice showed an increase in cardiac hypertrophy in response to TAC or Ang-II infusion with increased ventricular wall thickness and an increase in HW/BW ratio, but TAC or Ang-II infusion in Cav-3 OE mice did not show significant differences in these measures compared with sham or saline treatment, respectively (Table 2). The above data confirm that Cav-3 OE mice are protected from
**FIGURE 3.** $I_{Ca,T}$ is increased in the left ventricular myocytes from TAC- or Ang-II-infused hypertrophic mice. A, representative calcium current traces were measured using whole cell patch clamp technique in left ventricular myocytes from TAC or sham mice using a dual pulse voltage protocol (inset). $I_{Ca,T}$ is referred to as the difference between current recorded between step depolarization at holding potential $-90$ and $-50$ mV. B, mean current to voltage response of L-type ($E_L$) and T-type ($E_T$) current recorded from ventricular myocytes after 4 weeks of TAC or sham in WT mice. C, mean peak current densities of $I_{Ca,T}$ at $-30$ mV were significantly increased in ventricular myocytes from WT mice after 4 weeks of TAC or Ang-II infusion compared with sham or saline infusion, respectively. D, mean peak $I_{Ca,L}$ density at 0 mV was unchanged in the ventricular myocytes from WT mice after TAC or sham treatment and Ang-II infusion or saline treatment. $p < 0.001$, $n = 9$ cells from 5 animals in each group. E, representative $I_{Ca,T}$ traces from WT TAC myocytes perfused with 300 μM Ni$^{2+}$ (left) and mean peak $I_{Ca,T}$ at $-30$ mV (right). $n = 4$ from three animals. Data represent means ± S.E.

**TABLE 2**

Cardiac-specific Cav-3 overexpression attenuates TAC and Ang-II infusion-induced cardiac hypertrophy

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<td>LVID/d (mm)</td>
<td>4.38 ± 0.5</td>
<td>4.2 ± 0.9</td>
<td>3.27 ± 0.2</td>
<td>3.9 ± 0.8</td>
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* $p < 0.005$.
* $p < 0.05$, data ± S.E.
expression levels for AT-R were significantly reduced in WT and Cav-3 OE hearts following TAC in comparison with sham-treated hearts. These above data indicate that increased expression of the Cav3.2 and PKC/β/H9251 in cardiac hypertrophy could contribute to an increase in $I_{\text{Ca,L}}$ and altered Ca$^{2+}$/H11001 signaling.

Cav-3 Overexpression Prevents Disruption of Caveola-associated Macromolecular Signaling Complex—We investigated the impact of reduced Cav-3 and caveola expression on caveola-localized signaling proteins in pathological hypertrophy. Myocyte lysates from mice after 4 weeks of sham or TAC treatment were separated by SDS-PAGE and Western blot analysis by probing with specific antibodies to Ca$_{3.1}$, Ca$_{3.2}$, PKCα, PKCβ/H9251, NFATc3, AT1 receptor, and GAPDH. Representative immunoblots indicated for respective proteins and GAPDH signals as loading control are shown on the left. The bar plots on the right show semi-quantitative densitometry analysis for indicated protein expression normalized to GAPDH signals. The expression levels for Ca$_{3.2}$ and PKCα were significantly increased in WT TAC hearts compared with WT sham hearts. The AT1 receptor levels were significantly reduced in WT and Cav-3 OE TAC hearts compared with respective sham hearts. (Note that same immunoblot membrane was used to probe for Ca$_{3.2}$ and AT1-R and also for PKCβ and NFATc3). Data represents mean ± S.E. n = 4 experiments, *, p < 0.05.

NOS3, PKCα, and AT1 receptors co-precipitated with Cav-3 from WT or Cav-3 OE myocytes following sham treatment. In contrast, PKCα and AT1 receptor did not co-IP with anti-Cav-3 antibody from Cav-3 OE myocytes. We then tested whether the overexpression of Cav-3 in the ventricular myocytes from Cav-3 OE mice...
prevented TAC-induced myocyte remodeling and disruption of caveola-localized signaling proteins. As shown in Fig. 6A, PKCα, AT1 receptor, and NOS3 co-IPed with anti-Cav-3 antibody from Cav-3 OE mice subjected to TAC or sham myocytes. To further confirm these above results, we also performed sucrose density membrane fractionation on the WT and Cav-3 OE subjected to TAC or sham surgery and isolated caveola-enriched membrane fractions. As described under “Materials and Methods,” equal volumes of the sucrose density gradient membrane fractions were loaded onto SDS-polyacrylamide gels and analyzed by Western blot with probing antibodies to PKCα, AT1-R, and Cav-3. Representative immunoblots show NOS3, PKCα, and AT1-R but not the β1-adrenergic receptor (β1-AR) co-immunoprecipitated with anti-Cav-3 antibody from WT sham lysates, whereas control IgG does not immunoprecipitate the proteins (A). However, PKCα and AT1-R did not co-IP with anti-Cav-3 from WT TAC myocyte lysates, whereas NOS3 co-immunoprecipitated with Cav-3. In contrast to WT TAC, the NOS3, AT1-R, and PKCα co-immunoprecipitated with anti-Cav-3 from ventricular myocyte lysates from Cav-3 OE mice subjected to TAC. B, representative Western blot analysis performed on caveola-enriched membrane fractions prepared using ventricular myocytes from WT sham, WT TAC, and Cav-3 OE TAC hearts. Precipitated proteins from gradient membrane fractions analyzed Western blot by probing with antibodies to PKCα, AT1-R, and Cav-3. C, respective plots show relative distribution for PKCα (●), AT1-R (○), and Cav-3 (▲) and protein recovery in each of the gradient fractions as indicated ( ■). Results are representative of data from two separate experiments.

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FIGURE 7. siRNA-mediated knockdown of Cav-3 expression increases Ang-II stimulation of I_{Ca,T} in neonatal mouse ventricular myocytes. NMVMs were transfected with either eGFP alone, Cav-3 siRNA + eGFP, scrambled Cav-3 siRNA + eGFP, and Cav-3 cDNA + eGFP. I_{Ca,T} was measured using a dual pulse protocol as described under "Materials and Methods." A, representative peak I_{Ca,T} traces (at −30 mV) recorded from NMVMs that were transfected as indicated and treated with vehicle (control) or Ang-II (10 μmol/liter for 48 h). B, mean peak I_{Ca,T} in NMVMs. Ang-II treatment caused significant increase in the I_{Ca,T} (*, p < 0.05). Cav-3 siRNA caused a further robust increase in the Ang-II stimulation of peak I_{Ca,T} (#, p < 0.005). Scrambled Cav-3 siRNA, used as control, also significantly increased I_{Ca,T} when compared with vehicle (&, p < 0.005). Basal and Ang-II stimulation of I_{Ca,T} was significantly inhibited in NMVMs transfected with Cav-3 cDNA compared with control treatment (*, p < 0.05). Data represent means ± S.E.; n = 5–7 cells from three separate transfections. C, representative Western blots show protein expression for Cav-3 and GAPDH in NMVMs. D, semi-quantitative densitometry analysis for Cav-3 expression normalized to GAPDH signals. siRNA-mediated knockdown of Cav-3 caused a significant reduction in the expression of Cav-3 in the NMVMs compared with control scrambled siRNA-transfected cells (p < 0.001). The NMVMs transfected with Cav-3 cDNA showed significantly higher Cav-3 expression compared with eGFP (p < 0.005). Data represent means ± S.E., n = 6.

ated cardiac hypertrophy, we used cultured NMVMs, which are known to endogenously express the I_{Ca,T} (11, 31, 32). Cultured NMVMs were treated with Ang-II (10 μmol/liter) or vehicle for 48 h, and I_{Ca,T} was measured. As shown in Fig. 7, A and B, Ang-II treatment caused a significant increase (38%) in the peak I_{Ca,T} (−8.7 ± 0.8 pA/pF) compared with control (−6.3 ± 1.1 pA/pF) NMVMs. In separate experiments using NMVMs, we performed siRNA-mediated knockdown of Cav-3 using specific siRNA oligonucleotides to Cav-3 or overexpression of Cav-3 using cDNA of Cav-3 as described previously (11). siRNA-mediated knockdown of Cav-3 or overexpression of Cav-3 was confirmed by Western blot analysis (Fig. 7, C and D).

As shown in Fig. 7, A and B, siRNA-mediated knockdown of Cav-3 further enhanced (112%) Ang-II stimulation of peak I_{Ca,T} (−18.6 ± 7 pA/pF) compared with scrambled Cav-3 siRNA (−7 ± 0.8 pA/pF) or vehicle-treated NMVMs (−3.9 ± 0.8 pA/pF; 372%). In contrast, Cav-3 overexpression inhibited the basal peak I_{Ca,T} and abolished the Ang-II stimulation of peak I_{Ca,T} (−2 ± 0.7 pA/pF). Previous studies have demonstrated that PKCα activates and regulates the Ca_{2.3} channel current (33, 34). Increased PKCα expression and signaling were reported in cardiac hypertrophy and heart failure (35, 36). Chronic activation of the renin angiotensin system is known to induce cardiac hypertrophy, and Ang-II stimulation of cardiomyocytes causes increase in I_{Ca,T} in a PKC-dependent fashion (37, 38). We rationalized that with reduced expression of Cav-3, PKCα may couple to the Ca_{2.3} channels resulting in an enhanced regulation of the I_{Ca,T} in the myocytes. To test this, we performed knockdown of the PKCα using specific shRNA. The NMVMs were co-transfected with eGFP and either Cav-3 siRNA + shRNA PKCα, scrambled Cav-3 siRNA + shRNA PKCα, scrambled Cav-3 siRNA + empty vector, Cav-3 + shRNA PKCα, or Cav-3 + empty vector. Knockdown of the PKCα or Cav-3 or overexpression of Cav-3 was confirmed by semi-quantitative Western blot analysis as shown in Fig. 8, C and D. The transfected myocytes were treated with vehicle and Ang-II (10 μmol/liter) for 48 h, and I_{Ca,T} was measured in single cells expressing GFP. The knockdown of PKCα completely abolished the Ang-II stimulation of peak I_{Ca,T} (−1 ± 0.37 pA/pF) compared with Ang-II stimulation of peak I_{Ca,T} (−21.8 ± 4.6 pA/pF) (Fig. 8, A and B). Interestingly, knockdown of PKCα did not impact the peak I_{Ca,T} in the vehicle-treated control NMVMs (4.6 ± 1 pA/pF) compared with vehicle-treated NMVMs transfected with GFP alone (−6.3 ± 1.1 pA/pF) or scrambled Cav-3 siRNA (−6.3 ± 3.7 pA/pF), indicating that PKCα did not regulate the basal I_{Ca,T} currents in the NMVMs. These data clearly suggest that the Ang-II stimulation of the I_{Ca,T} is specifically mediated by PKCα in the NMVMs. Moreover, in NMVMs co-transfected with shRNA to PKCα and Cav-3 cDNA, the basal (vehicle-treated) and Ang-II-stimulated I_{Ca,T}
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FIGURE 8. Caveolin-3 overexpression prevents PKCα-mediated Ang-II stimulation of I_{Ca,L} in neonatal mouse ventricular myocytes. NMVMs were transfected with either Cav-3 siRNA or scrambled (scr) sequence of Cav-3 siRNA as control, or shRNA to PKCα or cDNA plasmid of Cav-3. I_{Ca,L} was measured in the NMVMs transfected as indicated. A, representative peak I_{Ca,L} traces from vehicle control or Ang-II-treated (10 μmol/liter for 48 h) NMVMs transfected as indicated on top. B, mean peak I_{Ca,L} measured at −30 mV in NMVMs with vehicle or Ang-II treatment. siRNA-mediated Cav-3 knockdown resulted in significant increase in the Ang-II stimulation of I_{Ca,L} compared with control. shRNA-mediated knockdown of PKCα and Cav-3 overexpression completely inhibited the basal and Ang-II stimulation of I_{Ca,L} (p < 0.005, data ± S.E., n = 6–8 cells from three separate experiments). C, representative Western blots show protein expression for PKCα and GAPDH in transfected NMVMs. D, semi-quantitative densitometry analysis for PKCα expression normalized to GAPDH signals. shRNA-mediated knockdown of PKCα caused a significant reduction in the expression of PKCα in the NMVMs compared with control vector-transfected cells (p < 0.001). Data represent means ± S.E., n = 6.

Discussion

This study investigated whether and how a loss of Cav-3 in pressure overload-induced cardiac hypertrophy impacts myocyte Ca^{2+} signaling and leads to pathological cardiac hypertrophy.
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**FIGURE 9.** Overexpression of Cav-3 inhibits Ang-II-induced NFATc3-GFP translocation to nucleus. Representative images of NMVMs showing localization of NFATc3-GFP following vehicle (control) or Ang-II treatment. Freshly isolated NMVMs were transfected with Cav-3 siRNA or Cav-3 cDNA plasmid, grown in culture for 12 h, and then infected with NFATc3-GFP adenovirus. 24 h after infection, the cells were treated with vehicle (control) or Ang-II (10 nmol/liter), and the culture media were supplemented with 4 mmol/liter CaCl2. GFP-tagged NFAT-C3-infected NMVMs were co-stained with nuclear stain DAPI (blue) and m-Cherry as transfection control. 24 h of Ang-II treatment caused a nuclear translocation of NFATc3-GFP compared with control. siRNA-mediated Cav-3 knockdown caused nuclear translocation of NFATc3-GFP in nearly all of NMVMs. In contrast, the NMVMs overexpressing Cav-3 nuclear translocation of NFATc3-GFP was completely inhibited. Scale bar, 50 µm. Data are representative of four separate experiments. N.S., not significant.

The results presented here highlight several novel and important findings. We show reduced Cav-3 expression and abundance of caveolae and a simultaneous increase in the Ca2+ current in the ventricular myocytes in cardiac hypertrophy. Reduced Cav-3 expression resulted in dissociation of the AT1 receptor and PKCα from Cav-3 in the hypertrophic ventricular myocytes. In NMVMs, siRNA-mediated knockdown of Cav-3 results in increased Ang-II stimulation of I_{Ca,T} mediated by PKCα and caused calcineurin-dependent NFAT translocation into the nucleus. In contrast, Cav-3 overexpression inhibited the PKCα-mediated Ang-II stimulation of the I_{Ca,T} and prevented NFATc3 translocation into the nucleus. In addition, mice with cardiac-specific Cav-3 overexpression had reduced expression of I_{Ca,T} and prevented disruption of the Cav-3-associated macromolecular signaling complexes after exposure to cardiac hypertrophic stimuli. Taken together, our data demonstrate that Cav-3 overexpression protects against pressure overload-induced cardiac hypertrophy via inhibition of I_{Ca,T} and suppression of the Ca2+−dependent hypertrophic calcineurin-NFAT signaling pathway.

Previous work (15) and current investigations demonstrate that caveola and Cav-3 expression is essential to cardiac protection (anti-hypertrophic signaling). The reduced Cav-3 expression in the cardiomyocytes during cardiac hypertrophy (Fig. 1) is consistent with previously published results (13, 20). Besides a variety of signaling proteins, Cav-3 associates and localizes the Ca3.2 channels, AT1 receptor, and the PKC isoforms into caveolae and provides local regulation of Ca2+ signaling in the cardiomyocytes (9). During pathological remodeling of myocardium, the structural integrity of myocytes is altered, resulting in changes in the distribution of the ion channels and associated signaling proteins, which causes a loss of protein-protein interaction (41). A reduction in Cav-3 expression and reduced abundance of caveolae in cardiomyocytes in cardiac hypertrophy could lead to altered subcellular localization and changes in composition of caveola-associated macromolecular signaling proteins. Previous studies have demonstrated that caveolar localization of key signaling proteins, including soluble guanylyl cyclase and cGMP-dependent protein kinase, is disrupted during pressure (30) or volume overload (29)-induced cardiac hypertrophy. The latter study also showed that caveolar localization protected soluble guanylyl cyclase against oxidation. It was shown that Cav-3 knockdown prevented the redistribution of 5-HT2A receptors into caveolar domains (20). Similarly, our data show that Cav-3, AT-1 receptor, and PKCα were associated and formed a macromolecular signaling complex in normal cardiomyocytes, which was disrupted by a loss of Cav-3 and caveola expression in the hypertrophic ventricular myocytes (Fig. 6). The loss of Cav-3 expression and combined with an up-regulation of the PKCa and dissociation of PKCα from Cav-3 augmented enhanced coupling of PKCα with the Ca3.2 channels resulting in increased Ang-II stimulation of the I_{Ca,T}. In contrast, the overexpression of Cav-3 reversed these effects. Therefore, we propose that caveolae provide a safety mechanism against activation of hypertrophic signaling. Re-expression of fetal I_{Ca,T} in the ventricular myocytes during pathological hypertrophy is well established (22, 23, 42). Studies have demonstrated the expression of the Ca3.2 (α1H) channel current responsible for the development of cardiac hypertrophy (27, 44) and the expression of the Ca3.1 (α1C) channels is attributed to anti-hypertrophic effect and cardioprotective function (45). It was reported that in pathological hypertrophy the Ca2+ influx via the re-expressed Ca3.2 channel initiates the binding of calcineurin to the C terminus of Ca3.2 leading to activation of NFAT (46). Furthermore, treatment with TTCC blockers could prevent the development of cardiac hypertrophy and heart failure (27, 47, 48). We have recently demonstrated that both the cardiac TTCC isoforms, Ca3.1 and Ca3.2 subunits, are associated with Cav-3. However, Cav-3 specifically inhibits the Ca3.2 current but not the Ca3.1 cur-
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A, during pressure overload-induced cardiac hypertrophy, a reduced expression of Cav-3 and caveola leads to disruption of caveola-localized and Cav-3-associated signaling proteins, including the AT1-R and PKCa. As a result, an increased Ang-II stimulation and PKCa-mediated activation of the $I_{Ca,T}$ lead to an increase in the local intracellular Ca$^{2+}$ levels. This enhanced Ca$^{2+}$ then activates the calmodulin-sensitive calcineurin, which then dephosphorylates NFATc3 triggering a hypertrophic response. However, in model B Cav-3 overexpression inhibits $I_{Ca,T}$ and prevents up-regulation of local Ca$^{2+}$ levels and prevents activation of downstream calcineurin/NFATc3 signaling. Cav-3 overexpression also causes caveola formation, which may stabilize the Cav-3-associated macromolecular signaling proteins and therefore protects against pressure overload-induced cardiac hypertrophy.

FIGURE 10. Proposed model of Cav-3-mediated cardiac protection during cardiac hypertrophy. A, during pressure overload-induced cardiac hypertrophy, a reduced expression of Cav-3 and caveola leads to disruption of caveola-localized and Cav-3-associated signaling proteins, including the AT1-R and PKCa. As a result, an increased Ang-II stimulation and PKCa-mediated activation of the $I_{Ca,T}$ lead to an increase in the local intracellular Ca$^{2+}$ levels. This enhanced Ca$^{2+}$ then activates the calmodulin-sensitive calcineurin, which then dephosphorylates NFATc3 triggering a hypertrophic response. However, in model B Cav-3 overexpression inhibits $I_{Ca,T}$ and prevents up-regulation of local Ca$^{2+}$ levels and prevents activation of downstream calcineurin/NFATc3 signaling. Cav-3 overexpression also causes caveola formation, which may stabilize the Cav-3-associated macromolecular signaling proteins and therefore protects against pressure overload-induced cardiac hypertrophy.

suggested a role for the LTCC current in the pathological hypertrophy (51, 52). A recent report indicates that caveola-localized LTCC can activate the calcineurin/NFAT-mediated hypertrophic signaling in cardiomyocytes (53). Subsequently, it was shown that Ca$^{2+}$ influx through LTCCs primarily activates the Cn-NFAT signaling, and Ca$^{2+}$ entry through transient receptor potential (TRP) channels also participated in this process (54). An earlier report indicated that TRP channels as necessary mediators of pathological cardiac hypertrophy through a calcineurin-NFAT signaling pathway (55). Although the TRP3 channel has been shown to localize to caveolae in the arterial smooth muscle cells (17), it is not known whether the TRP channels are associated with caveolar signaling proteins in the ventricular myocytes. We did not examine the role of Cav-3 in

rents (11). These above reports, including ours, suggest a likely scenario of Ca$_{3.1}$ and Ca$_{3.2}$ channels activating different signaling pathways within the same caveola via specific coupling mechanisms with different signaling proteins. In this study, we show an increase in the mRNA level for Ca$_{3.1}$ (α$_{1C}$) and Ca$_{3.2}$ (α$_{1H}$) mRNA (Fig. 2) and protein (Fig. 5) and an increase in the $I_{Ca,L}$ in cardiac hypertrophy. We could not specifically measure the contribution of $I_{Ca_{3.2}}$ versus $I_{Ca_{3.1}}$ in the cardiomyocytes during hypertrophy due to nonavailability of specific inhibitors for these TTCC isoforms. Interestingly, we did not observe any changes to the $I_{Ca,L}$ density in cardiomyocytes in cardiac hypertrophy in the WT or the Cav-3 OE mice (Figs. 3D and 4D). Some studies reported a reduction or no change or an increase in the $I_{Ca,L}$ density in hypertrophy (49, 50), whereas other reports
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Our data clearly suggest that cardiac myocyte caveolae localize essential signals that regulate the Ca\(^{2+}\) influx-mediated hypertrophic signaling. Likely differences between our observations of unchanged I\(_{\text{Ca,L}}\) density with other studies could be due to differences in the models of pathological cardiac hypertrophy (early stage) versus heart failure (43). Future studies should investigate a clear role for the Ca\(_{1.2}\) channels, including the expression of auxiliary subunits.

Cav-3 overexpression in ventricular myocytes in cardiac hypertrophy impacts the Cav-3-mediated compartmentalized regulation of local signaling. A loss of Cav-3 inhibition of the I\(_{\text{Ca,T}}\), specifically the I\(_{\text{Ca,v3.2}}\), results in increased local intracellular Ca\(^{2+}\) levels that activate calmodulin-dependent calcineurin, which then dephosphorylates the NFAT and triggers hypertrophic signaling complexes and prevents increased coupling of PKC\(\alpha\) with the Cav3.2 channels (Fig. 10B). We conclude that Cav-3 overexpression in ventricular myocytes is essential for promoting the protective signaling during pressure overload-induced cardiac hypertrophy and thus could be used as therapeutic strategy for treatment of such disease.

Conclusion

We demonstrate that the loss of Cav-3 and caveola expression in ventricular myocytes in cardiac hypertrophy impacts the Cav-3-mediated compartmentalized regulation of local signaling. A loss of Cav-3 inhibition of the I\(_{\text{Ca,T}}\) specifically and inducible expression of an atrial natriuretic factor transgene decreases natriuretic peptide expression and signaling. Circulation 118, 1979–1988.

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Conclusion

We demonstrate that the loss of Cav-3 and caveola expression in ventricular myocytes in cardiac hypertrophy impacts the Cav-3-mediated compartmentalized regulation of local signaling. A loss of Cav-3 inhibition of the I\(_{\text{Ca,T}}\), specifically the I\(_{\text{Ca,v3.2}}\), results in increased local intracellular Ca\(^{2+}\) levels that activate calmodulin-dependent calcineurin, which then dephosphorylates the NFAT and triggers hypertrophic signaling complexes and prevents increased coupling of PKC\(\alpha\) with the Cav3.2 channels (Fig. 10B). We conclude that Cav-3 overexpression in ventricular myocytes is essential for promoting the protective signaling during pressure overload-induced cardiac hypertrophy and thus could be used as therapeutic strategy for treatment of such disease.

Acknowledgments—We thank Dr. Steven Houser, Temple University, for generously providing adenovirus NFATc3-GFP and Dr. Scott Kaufmann, Mayo Clinic, Rochester, MN, for providing PKC\(\alpha\) shRNA plasmid.

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