Gene Therapy to Inhibit the Calcium Channel β Subunit: Physiological Consequences and Pathophysiological Effects in Models of Cardiac Hypertrophy

Eugenio Cingolani, Genaro A. Ramirez Correa, Eddy Kizana, Mitsushige Murata, Hee Cheol Cho, Eduardo Marbán

Abstract—Calcium cycling figures prominently in excitation-contraction coupling and in various signaling cascades involved in the development of left ventricular hypertrophy. We hypothesized that genetic suppression of the L-type calcium channel accessory β-subunit would modulate calcium current and suppress cardiac hypertrophy. A short hairpin RNA template sequence capable of mediating the knockdown of the L-type calcium channel accessory β-subunit gene was incorporated into a lentiviral vector (PPT.CG.H1.β2). Transduction of ventricular myocytes in vivo with the active short hairpin RNA partially inhibited the L-type calcium current. In neonatal rat cardiomyocytes, L-type calcium channel accessory β-subunit gene knockdown reduced calcium transient amplitude. Similarly, [3H]leucine incorporation was attenuated in PPT.CG.H1.β2-transduced neonatal rat cardiomyocytes compared with nonsilencing controls in a phenylephrine-induced hypertrophy model. In vivo gene transfer attenuated the hypertrophic response in an aortic-banded rat model of left ventricular hypertrophy, with reduced left ventricular wall thickness and heart weight/body weight ratios in PPT.CG.H1.β2-injected rats at four weeks post transduction. Fractional shortening was preserved in rats treated with PPT.CG.H1.β2. These findings indicate that knockdown of L-type calcium channel accessory β-subunit is capable of attenuating the hypertrophic response both in vitro and in vivo without compromising systolic performance. Suppression of the calcium channel β subunit may represent a novel and useful therapeutic strategy for left ventricular hypertrophy. (Circ Res. 2007;101:166-175.)

Key Words: hypertrophy ■ calcium ■ gene therapy

Calcium cycling in the heart is triggered by calcium influx through L-type calcium channels. Such calcium channels are present and functionally important not only in cardiac myocytes but also in diverse smooth muscles and in neurons. Enhancement of calcium-regulated signaling pathways contributes to the development of left ventricular hypertrophy (LVH). Thus, calcium channel inhibition represents a logical approach to treatment of LVH. Gene therapy allows for more directed and organ-specific delivery and thus may avoid undesired systemic effects, which may account in part for the limited clinical benefit observed with these agents.

L-type calcium channels are heteromultimers of various subunits. The accessory β subunit (LTCCβ) not only favors the trafficking of the calcium channel to the surface membrane, but also enhances the probability of channel opening resulting in increased calcium current. Interestingly, upregulation of a splice variant of the LTCCβ (β2) has been previously described in failing human cardiomyocytes. Whether or not β subunits are upregulated in hypertrophy, we hypothesized that suppression of LTCCβ might represent an attractive means to inhibit calcium influx, attenuate calcium-dependent signaling and ultimately prevent or treat LVH.

Using RNA interference technology to selectively modulate the expression of the gene of interest, we studied the physiological effects of LTCCβ modulation in native cardiac cells, and the effects in a cellular model of hypertrophy. To further investigate the effects of LTCCβ downregulation in cardiac hypertrophy, we performed in vivo gene transfer of an “advanced” generation lentiviral vector capable of permanently modulating the expression of LTCCβ. A rat pressure-overload LVH model was implemented to test the potential beneficial effect of modulating the expression of the L-type calcium channel accessory β subunit.

Materials and Methods

For a detailed Materials and Methods, please see the online data supplement available at http://circres.ahajournals.org.

Short Hairpin RNA Design and Vector Production

A series of short interference RNA duplexes (siRNAs) against the D2 conserved domain of LTCCβ were designed according to published algorithms and synthesized. The three most active sequences were designed into a short hairpin RNA (shRNA) oligonucleotide. These three active shRNAs and one NS-shRNA sequence were screened...
using heterologous transfection and flow cytometry analysis (FACS) as described in the next section. The entire shRNA expression cassette was incorporated into a lentiviral vector plasmid (provided by Dr Inder Verma, Salk Institute, San Diego, Calif) (Figure 1A). For the heterologous cotransfection experiments (see below) CMV-green fluorescent protein (GFP) was removed resulting in shRNA vectors PPT.H1.β2 and PPT.H1.NS. For viral vector production from these plasmids, the four-plasmid transient transfection of 293 cells was performed as previously described.11-13 For in vivo gene transfer efficiency experiments, the cDNA for nuclear-localized β-galactosidase (LacZnlS) was subcloned in place of the GFP gene and vector produced as described.

Figure 1. Vector-based expression of an shRNA attenuates LTCCβ gene expression and efficiently transduces NRCMs. A, Diagram of the vector PPT.CG.H1 and shRNA template. B, HEK293 cells (left panels) cotransfected with β2-GFP fusion and either PPT.H1.NS or PPT.H1.β2; FACS quantification of fluorescence reduction is shown on the right (*P <0.05). Calibration bar=10 μm. C, Transmitted light (left) and fluorescence microscopy images (right) of NRCMs transduced at an MOI of 50. GFP fluorescence indicates a transduction efficiency of ≈90%.
Cotransfection Heterologous Expression
To establish the knockdown efficacy of the PPT.CG.H1.β2 sequences, HEK293 cells were cotransfected with pLTCCβ-GFP and equimolar ratios of the vector plasmids PPT.H1.β2 or PPT.H1.NS. Twenty-four hours after transfection, β2-GFP expression was established by fluorescence microscopy and quantified by flow cytometric analysis. The most active shRNA against the LTCCβ (target DNA sequence: 5'-AAC AGG CTA CAG CAT GAA -3') was used for both in vitro and in vivo experiments.

Primary Culture of Neonatal Rat Cardiac Ventricular Myocytes
Neonatal rat cardiac myocytes (NRCMs) were isolated from 1 to 2 day-old rats and cultured as previously described.14,15 The cells were maintained at 37°C in the presence of 5% CO₂ in a humidified incubator.

Single Cell Electrophysiology
Myocytes were isolated from the left ventricle of adult rats, in a Langerdorff perfusion system as previously described.16 A xenon arc lamp was used to view GFP fluorescence and therefore recognize transduced cells. Membrane currents were recorded using the whole-cell patch clamp technique.

Calcium Transients
NRCMs were plated into 35 mm glass bottom dishes and analyzed 72 hours after transduction. Calcium transients (Rhod2 AM) were measured at 37°C during field stimulation at 1.5 Hz to ensure consistent diastolic intervals. Images were acquired on an inverted confocal laser-scanning microscope. Transduced cells, recognized by GFP fluorescence, were randomly selected for recording of calcium transients.

Cell Area and [3H]Leucine Incorporation
For cell area measurements, NRCMs were plated in 35 mm glass-bottom dishes. Cells were serum starved for 24 hours, after which they were incubated in phenylephrine (PE). Images were acquired and cell area measurements were performed offline using Image-J software. Transduced cells were recognized by GFP fluorescence. For [3H]leucine incorporation, 72 hours after transduction, cells were incubated for 24 hours with 2μCi/mL of [3H]leucine with and without PE. Cell lysates were solubilized and the incorporated radioactivity was determined by liquid scintillation counting.

In Vivo Cardiac Gene Transfer and Aortic Banding
Adult rats were assigned to receive PPT.CG.H1.B2, PPT.CG.H1.NS, or sham intervention. After baseline echocardiographic recordings, rats were anesthetized. Lentivirus vector was injected into the LV cavity while the aorta and pulmonary artery were cross-clamped for 50 seconds. After the aorto/pulmonary cross clamp was removed, the ascending aorta was banded with a 0.58 mm (internal diameter) tantalum clip as previously described.17 In sham-operated animals, ascending aorta was banded with a 0.58 mm (internal diameter) cavity while the aorta and pulmonary artery were cross-clamped for 50 seconds. In vivo transduction/aortic banding.

Primary Cardiac Gene Transfer and Aortic Banding
After baseline echocardiographic recordings, rats were anesthetized. Lentivirus vector was injected into the LV cavity while the aorta and pulmonary artery were cross-clamped for 50 seconds. In vivo transduction/aortic banding.

Quantitative Reverse-Transcription PCR
Four weeks after in vivo gene delivery, total RNA was isolated from rat hearts. For quantification of steady state mRNA levels the TaqMan gene expression assay was used. Primers and probes against the LTCCβ, β-myosin heavy chain (β-MHC), and atrial natriuretic peptide (ANP) were designed. Quantitative PCR was performed using a 7900HT Sequence Detection System (Applied Biosystems, Foster City, Calif).

Western Blots
For Ca, 1.2 expression, protein was isolated from tissue lysates as previously described.18 Samples were separated by 8% Tris-glycine SDS gel electrophoresis. Proteins were transferred to polyvinylidene sulfonyl fluoride membranes and blocked for 1 hour. A rabbit Ca,1.2 antibody (Alomone Labs) was incubated overnight at 4°C. Densitometry analysis was performed to assess α1C / tubulin ratios from sham, NS RNAi, and β RNAi groups.

Echocardiography
Rats were anesthetized and transthoracic 2-D and M-mode images were acquired using a high-resolution 25 MHz scan head attached to a rail system to assure standardization of imaging acquisition between animals. All images were recorded in a VisualSonics Vevo 660 high resolution rodent imaging system. Echocardiograms were performed at baseline, 2 and 4-week time points after vector transduction/aortic banding.

Invasive Blood Pressure Measurement
Blood pressure was measured invasively four weeks after aortic banding in PPT.CG.H1.β2 and PPT.CG.H1.NS transduced animals. After rats were anesthetized, a fluid filled catheter was advanced until the aortic root (distal to the aortic band). Blood pressure was recorded and subsequently analyzed offline.

Statistics
Continuous variables are expressed as mean±standard error of the mean. Statistical analyses were performed using repeated measures ANOVA and Student paired t test, where appropriate. P<0.05 was considered to be indicative of statistical significance.

Results
In Vitro Effect of shRNA on LTCCβ Expression
The gene-silencing capacity of the shRNA was initially tested in a heterologous expression system. Twenty four hours after transfection with p.LTCCβ-GFP, ~85% of HEK293 cells were positive for GFP fluorescence. As evidenced by fluorescent microscopy, cotransfection of PPT.H1.β2 significantly decreased GFP expression relative to cells cotransfected with PPT.H1.NS (Figure 1B). To quantify the gene-silencing efficacy of our construct, cells were subjected to FACS analysis. In two separate experiments, PT.H1.β2 reduced the mean fluorescence intensity of pLTCCβ-GFP cotransfected cells by 64.6±7.5% compared with PPT.H1.NS cotransfected cells (Figure 1B).

Effect of LTCC β2 Gene Suppression on Adult Cell Electrophysiology
We have recently reported that the over expression of a small G-protein (Gem) in the heart has a dramatic effect on peak I_{Ca,L}.19 The Gem effects on I_{Ca,L} are because of binding and subsequent trapping of the endogenous β subunit.20 Accordingly we would predict that inhibition of β subunit expression will inhibit L-type calcium current (I_{Ca,L}). To test this prediction, we measured I_{Ca,L} in adult rat ventricular cardiomyocytes. Lentiviral vector was injected into the left ventricle of rat hearts, and 5 days later cells were isolated for patch clamp studies. Only GFP positive (transduced) cells were recorded. Genetic suppression of LTCCβ in transduced rat cells decreased I_{Ca,L} from a peak density of -9.3±1.52 pA/pF at 10 mV (n=11) in PPT.CG.H1.NS transduced (control, or NS RNAi) cells to -3.85±0.53 pA/pF at 10 mV (n=10) in PPT.CG.H1.B2 (β2 RNAi, Figure 2,A and B). LTCCβ gene knockdown was specific in that it did not affect another endogenous cation-selective inward current (the
Suppression of LTCC would be predicted to attenuate intracellular calcium cycling. For calcium transient recordings, NRCMs were transduced with either PPT.CMV.LacZnls or PPT.CG.H1.NS at a multiplicity of infection (MOI) of 50 to achieve a transduction efficiency greater than 90% by fluorescence microscopy (Figure 1C). Seventy-two hours later, calcium transient amplitude was reduced by 34% in β2 RNAi cells (n=101) compared with NS RNAi (n=102) controls (F/Fo=8.54±0.61 versus F/Fo=13.05±0.55, P<0.01) (Figure 3).

**Effect of LTCC β2 Gene Knockdown on Cardiac Hypertrophy In Vivo**

As a first step to investigate the role of posttranscriptional gene silencing of LTCC β2 on cardiac hypertrophy, we used the PE-induced NRCM hypertrophy model. Seventy-two hours after transduction (MOI of 50), cells were serum-starved for 24 hours and PE stimulation was initiated. After 48 hours, cell size was 54% smaller in β2 RNAi cells (n=53) compared with NS RNAi controls (n=56) (P<0.01) (Figure 3, D and E). Given that cultured cells can undergo changes in cell volume and area not necessarily related to the development of hypertrophy, we also measured [3H]leucine incorporation after 24 hours stimulation with PE. PE-stimulated [3H]leucine incorporation was suppressed in β2 RNAi cells (99.3±13% of control, n=9) compared with NS RNAi cells (173.6±18% of control, n=9) (P<0.05) (Figure 3F). No differences in beating frequencies were observed between groups (data not shown), therefore ruling out an indirect effect of beat frequency on hypertrophy.

**Lentiviral Vector Mediates Efficient In Vivo Gene Transfer and Knockdown of the LTCCβ In Vivo**

We and others have previously shown the efficacy of LV injection and aorto-pulmonary cross clamping for in vivo adenoviral vector-mediated gene delivery to the heart.21,22 Recently, advanced-generation lentiviral vectors have also been reported to efficiently transduce the rat heart by the same vector delivery technique.23 We therefore implemented a banding model of LVH in the rat and compared 3 groups: sham-operated (not-banded), β2 RNAi, and NS RNAi groups. The transduction efficiency achieved in the current study was ~50% as established by X-gal staining four weeks after injection of PPT.CMV.LacZnls (Figure 4, A and B). Four weeks after aortic banding, LTCCβ2 mRNA levels were increased by 2-fold in NS RNAi (n=7) animals compared with Sham / not banded animals (n=6). Importantly, the LTCCβ2 mRNA levels were reduced by 23% in β2 RNAi rats (n=7) compared with nonsilencing controls (n=7) (Figure 4C). To assess the extent of knockdown of the LTCCβ2 protein, Western blots were performed on tissue lysates and membrane preparations. Despite the use of commercial and a custom-made antibody, combined with sensitive detection systems, we were unable to achieve adequate signals from the endogenous expression of LTCCβ2 in adult rat heart. As a result, quantification of LTCCβ2 changes at the protein level was not possible. To rule out the possibility of an off-target effect of our RNAi, Ca,1.2 protein expression was measured from tissue lysates. No changes in Ca,1.2 expression were observed in Sham, NS RNAi, β2 RNAi groups (Figure 4D).

**In Vivo Effect of LTCC β2 Suppression on Cardiac Hypertrophy**

No significant differences in baseline characteristics were observed between the three study groups (Table). During follow up, β2 RNAi animals exhibited an attenuated hyper-
trophy response compared with NS RNAi controls. Four weeks after aortic banding, left ventricular wall thickness (LVWT) was 33% smaller in \( \beta_2 \) RNAi rats (n=6) compared with NS RNAi controls (n=7; Figure 5, A and B, and Table). Moreover, heart weight/body weight ratios (HW/BW), were also decreased (4.55±0.16 versus 5.64±0.22, \( P<0.05 \)) (Figure 5C and Table). Consistent with these findings, the mRNA levels of \( \beta \)-MHC and ANP were reduced by 26% and 52% respectively in \( \beta_2 \) RNAi rats (n=7) compared with nonsilencing controls (n=7) (Figure 6A and B). The systemic blood pressure (distal to the aortic banding) was comparable in the NS RNAi and \( \beta_2 \) RNAi groups (145/88 mm Hg versus 143/89 mm Hg respectively, n=5 in each group) (Figure 6C). Thus, differences in afterload do not confound the observed changes in the hypertrophic response.

**LTCC \( \beta_2 \) Knockdown Does Not Impair Systolic Performance**

Downregulation of LTCC\( \beta_2 \), by affecting calcium influx and release from intracellular stores, may impair excitation-
contraction coupling and systolic function. To exclude this possibility, animals were followed up to detect signs of congestive heart failure and cardiac function was studied noninvasively by high-resolution echocardiography. No signs of respiratory distress, fluid retention, or differences in body weight, ventricular heart rate were detected between groups (Figure 7, A and B and Table). Moreover, during follow-up, left ventricular diastolic diameter and shortening fraction were comparable in β2 RNAi and NS RNAi transduced rats (Figure 7, C and D and Table). This null effect may reflect attenuation of calcium cycling pathways ordinarily upregulated in cardiac hypertrophy.

Discussion

The fundamental role of calcium influx through the LTCC in normal excitation-contraction coupling, and the significance of calcium mishandling in heart disease, have recently been reviewed. The importance of the LTCC as a therapeutic target for LVH has been confirmed in many animal models that demonstrate reduction in hypertrophy by calcium channel blockers. However, there are only limited data on the effect of L-type calcium channel blockade on cardiac hypertrophy beyond blood pressure control. Clinically, calcium channel antagonists decrease blood pressure and induce regression of LVH, but prolongation of survival with these agents has not been demonstrated except in the ASCOT-
Figure 5. Attenuation of the hypertrophy response in rats with aortic banding. A, Representative short-axis and m-mode images 4 weeks after gene transfer and aortic banding showing reduced wall thickness in β2 RNAi injected rats. B, Mean LV wall thickness of NS RNAi (n=7), β2 RNAi (n=6) and Sham (n=4) injected rats was measured at baseline, 2, and 4 weeks after aortic banding. Attenuation of the hypertrophic response was evident at 2 weeks and persisted to the 4-week time point. C, Heart weight/body weight relationships was also measured in these rats at 4 weeks after aortic banding (*P <0.05, β2 RNAi vs NS RNAi).
BPLA trial. Blockade of noncardiac channels explains many of the undesired effects (e.g., systemic hypotension, constipation, edema) of these agents and could also account for the dubious benefit on cardiovascular mortality. We have previously shown that genetic calcium channel blockade can be achieved in the heart, by overexpressing the small G-protein, Gem, by adenoviral gene transfer. Here, by incorporating a shRNA expression cassette into an “advanced” generation lentiviral vector we were able to target the LTCC \(/H9252\) in a gene specific manner and achieve long-term modulation of cardiac calcium influx. Gene-silencing of LTCC\(/H9252\) in NRCMs decreased calcium transients by 34%, demonstrating the importance of the endogenous levels of LTCC\(\beta\) for the regulation of calcium handling. In a cellular model of hypertension, downregulation of LTCC\(\beta\) expression prevented an increase in relative cell size and abrogated PE-induced protein synthesis. These in vitro findings contribute to the body of evidence supporting the key role of

Figure 6. Effects on fetal gene program and systemic blood pressure. A, Changes on \(\beta\)-MHC in Sham (not banded) \((n=6)\), \(\beta2\) RNAi transduced rats \((n=7)\) compared with nonsilencing transduced controls \((n=7)\). B: Changes on ANP in Sham (not banded) \((n=6)\), \(\beta2\) RNAi transduced rats \((n=7)\) compared with nonsilencing transduced controls \((n=7)\). (#P < 0.05, NS RNAi vs Sham); (*) P < 0.05, \(\beta2\) RNAi vs NS RNAi). C: Systemic blood pressure 4 weeks after aortic banding \((n=5\) in each group).

Figure 7. No evidence of heart failure in rats with attenuated hypertrophy despite persistence of pressure overload. Body weight (A), heart rate (B), LV diastolic diameter (LVDD) (C), and mean LV shortening fraction (D) were comparable 4 weeks after aortic banding between sham operated \((n=4)\), NS RNAi \((n=7)\) and \(\beta2\) RNAi \((n=6)\) transduced rats.
calcium-regulated signaling pathways in the development of cardiac hypertrophy.\(^3\)

In pressure-overload conditions, enhanced calcium-regulated signaling also plays a central role in the development of LVH.\(^2,3\) Although initially considered a “compensatory” response projected to normalize wall stress and facilitate systolic performance,\(^3\) recent studies have challenged this premise.\(^30,32\) The inhibition of calcium-regulated signaling pathways has been shown to abolish pressure overload-induced LVH without compromising systolic function.\(^32\) Hence, the development of LVH is not essential for maintained systolic function in the presence of pressure overload. Conversely, suppression of hypertrophy need not undermine contractile performance. In our in vivo study system, we found an increase in: LTCC\(\beta\), \(\beta\)-MHC, and ANP mRNA levels 4 weeks after aortic banding. Interestingly, modulation of LTCC\(\beta\) attenuated the development of LVH as demonstrated by a relative decrease in LVWT and HW/BW. The reduced transcription of ANP and \(\beta\)-MHC in the \(\beta\) suppressive group found in our model is consistent with the changes found both in the echocardiographic and HW/BW measurements. The attenuated hypertrophic response in the setting of a “fixed” aortic constriction, together with the absence of a difference in systemic blood pressure between the nonsilencing and \(\beta\) suppressive groups, support a direct cardiac effect of LTCC\(\beta\) knockdown independent of changes in peripheral vascular resistance.

Although a depressed cardiac contractility might be expected from LTCC\(\beta\) gene silencing, no changes in systolic performance, as assessed by echocardiographic shortening fraction, were detected during follow-up. Moreover, no signs of impaired cardiac performance such as respiratory distress, or fluid retention were detected. In concordance with our findings, a reduction of \(I_{\text{calc}}\) with preserved cardiac contractility by endogenous \(\beta\) subunit trapping has previously been reported by Serikov et al in a transgenic mouse model.\(^33\) Our results may be explained by a partial reduction of LTCC\(\beta\) expression that is sufficient to prevent the activation of calcium-regulated signaling pathways and therefore the development of LVH, without impairing normal excitation-contraction coupling. An alternative explanation for the preservation of systolic function may relate to an antiapoptotic effect of LTCC\(\beta\) knockdown. Overexpression of LTCC\(\beta\) by adenoviral gene transfer has recently shown to induce calcium overload and apoptosis in adult feline cardiomyocytes.\(^34\) Although an antiapoptotic effect of LTCC\(\beta\) modulation could explain our findings further studies would need to be performed to test this hypothesis. A compensatory increase in sarcoplasmic reticulum calcium loading may also result in preserved contractility in our model.

The present strategy, associated with a vector capable of persistently modulating the expression of LTCC\(\beta\) may represent a novel and specific therapy for LVH and other cardiac diseases associated with calcium mishandling. Hypertrophic obstructive cardiomyopathy (HOCM), for example, is conventionally treated with pharmacological calcium channel blockers and in selected cases surgical, and more recently nonsurgical septal reduction techniques as a means of ameliorating LV outflow obstruction.\(^35–37\) However, the utility of these therapies is limited by side effects, including inflammation, fibrosis, and arrhythmogenesis. On this basis, focal modulation of LTCC\(\beta\) by a vector capable of chronically suppressing gene expression may represent an attractive alternative therapy in HOCM. Regional modification of endogenous LTCC\(\beta\) may improve outflow obstruction by nondestructively reducing septal hypertrophy without impairing global cardiac hemodynamics.

We have demonstrated that LTCC\(\beta\) may be modulated by RNA interference following lentiviral vector-based shRNA expression both in vitro and in vivo. The physiological consequences of this were the reduction of the \(I_{\text{calc}}\), and an attenuated hypertrophic response in vitro and in vivo. The latter was associated with preserved cardiac systolic function. This study system represents a novel research tool for studying calcium-regulated signaling pathways. Furthermore, these findings advocate the LTCC\(\beta\) as a potentially novel therapeutic target for diverse cardiac diseases associated with calcium mishandling.

**Limitations**

The present study serves as a proof of concept of the role of RNA interference in modulating the expression of LTCC\(\beta\), regulating calcium influx and preventing LVH. Novel and less invasive gene delivery techniques will have to be developed to translate the present strategy to humans. In addition, further mechanistic studies are required to elucidate any potential antihypertrophic effects of LTCC\(\beta\) knockdown that are independent of the effects on the \(I_{\text{calc}}\).

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**Disclosures**

None.

**References**


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Gene therapy to inhibit the calcium channel β subunit: physiological consequences and pathophysiological effects in models of cardiac hypertrophy

Online Data Supplement

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Short title: Modulation of Ca channel β subunit prevents cardiac hypertrophy

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Methods.

*shRNA design and vector production.*

A series of 21 nucleotide short interference RNA duplexes (siRNAs) against the D2 conserved domain \(^1\) of LTCC\(\beta\) (NM_053851) were designed according to published algorithms \(^2\) and synthesized (Qiagen). A total of ten siRNA duplexes were screened by western blot analysis (data not shown). The three most active sequences and one scrambled, non-silencing (NS) sequence were designed into a short-hairpin RNA (shRNA) oligonucleotide template consisting of sense, hairpin loop, antisense and terminator sequences all of which were flanked by restriction enzyme sites to facilitate directional subcloning. These three active shRNAs and one NS-shRNA sequence were screened using heterologous transfection and flow cytometry analysis (FACS) as described in the next section. These oligonucleotides were subcloned into an shRNA expression cassette composed of an RNA polymerase III promoter (H1). The entire shRNA expression cassette was incorporated into the KpnI site of lentiviral vector plasmid pRRLsin18.cPPT.CMV.eGFP.Wpre (provided by Dr Inder Verma, Salk Institute) (Figure 1.A). The resulting vectors encoded eGFP under the transcriptional control of a CMV promoter and either shRNA against LTCC\(\beta\) (PPT.CG.H1.\(\beta\)2) or a non-silencing shRNA (PPT.CG.H1.NS) under the control of the H1 promoter. For the heterologous co-transfection experiments (see below) CMV-GFP was removed resulting in shRNA vectors PPT.H1.\(\beta\)2 and PPT.H1.NS. For viral vector production from these plasmids, the four-plasmid transient transfection of 293 cells was performed as previously described \(^3\)-\(^5\). Briefly, vector containing supernatant was collected 48-72 hours after transfection, 0.2um filter-purified and concentrated by ultracentrifugation (50,000g for 120 minutes at 10 °C). The viral pellet was resuspended in PBS. Transduction unit (TU) titre was assessed on HEK293 cells in the presence of Polybrene 8µg/mL (Sigma-Aldrich). Titers of 2-5 x 10\(^8\) TU/ml were routinely achieved. For *in vivo* gene transfer efficiency experiments, the cDNA for nuclear-localized \(\beta\)-galactosidase (*LacZnls*) was subcloned in place of the GFP gene and vector produced as described.
Co-transfection heterologous expression.

A full-length wild-type LTCCβ (NM_053851) fused to green fluorescent protein at its carboxyl terminus, (LTCCβ–GFP) was subcloned into an expression plasmid designated pLTCCβ-GFP. In order to establish the knockdown efficacy of the PPT.CG.H1.β2 sequences, HEK293 cells were co-transfected with pLTCCβ–GFP and equimolar ratios of the vector plasmids PPT.H1.β2 or PPT.H1.NS. Transfections were performed using Lipofectamine 2000 reagent as per manufacturers instructions (Invitrogen). Twenty-four hours after transfection, β2-GFP expression was established by fluorescence microscopy and quantified by flow cytometric analysis (Becton Dickinson). The most active shRNA resulted to be one designed against a region in the conserved (D2) domain of the LTCCβ (5’ – AAC ATG AGG CTA CAG CAT GAA –3’) and was the one used for both the in vitro and in vivo experiments.

Primary Culture of Neonatal Rat Cardiac Ventricular Myocytes.

Neonatal rat cardiac myocytes (NRCMs) were isolated from 1-2 day old Sprague-Dawley rats and cultured as previously described. Hearts were removed and ventricles minced in calcium- and bicarbonate-free Hanks’ buffer with HEPES. These tissue fragments were digested by stepwise trypsin dissociation. In order to diminish the amount of fibroblasts in the culture, the dissociated cells were pre-plated for 45 minutes. Non-adherent myocytes were plated at a density of 1300 cells/mm² in plating medium consisting of DMEM (Mediatech) supplemented with 5% FBS, penicillin (100 U/mL), streptomycin (100 mg/mL), and 2 µg/mL vitamin B₁₂. The cells were maintained at 37°C in the presence of 5% CO₂ in a humidified incubator. Bromodeoxyuridine (0.1 mmol/L) was added in the medium for the first 72 hours after isolation to inhibit fibroblast growth. For hypertrophy experiments cells were placed in serum-free DMEM containing 3.8 g/L glucose, vitamin B₁₂, transferrin, and insulin 24 hours before phenylephrine (PE) stimulation.
Single Cell Electrophysiology.

Myocytes were isolated from the left ventricle of adult Sprague-Dawley rats, using enzymatic digestions in a Langerdorff perfusion system as previously described. A xenon arc lamp was used to view green fluorescent protein (GFP) fluorescence at 488/530nm (excitation/emission) and therefore recognize transduced cells. Membrane currents were recorded using the whole-cell patch clamp technique with an Axopatch 200B amplifier (Axon Instruments). Borosilicate glass pipettes were pulled and fire-polished to final tip resistances of 1.5-2.5 MΩ when filled with recording solution.

Adult I_{Ca,l} recordings were performed at room temperature using the following external (in mmol/L): 140 NaCl, 5 KCl, 1 MgCl₂, 10 HEPES, 2 CaCl₂, and 10 glucose (pH 7.4 adjusted with NaOH), and pipette solutions (in mmol/L): 110 CsCl, 20 TEA, 10 HEPES, 5 BAPTA, 5 Mg-ATP, 1 MgCl₂, and 5 glucose (pH 7.2 adjusted with CsOH). For I_{Na} recordings, cell were perfused with a low-Na solution composed of (in mmol/L): 15 NaCl, 130 TEA-Cl 5 CsCl, 1 MgCl₂, 10 HEPES, and 10 glucose (pH 7.4 adjusted with CsOH). L-type calcium currents were elicited by 300ms-depolarizing steps from -40 to 60mV in 10mV increments. To inactivate Na current, a pre-pulse from -80mV to -40mV was used. Sodium currents were elicited by 300ms-depolarizing steps to potentials ranging from -70 to +50mV in 10mV increments.

Calcium Transients

NRCMs were plated into 35mm glass bottom dishes (MatTek Cultureware) and analysed 72 hours after transduction. Cells were loaded with Rhod2-AM (2µM) (Molecular Probes) for 18 minutes. Following this cells were washed with PBS and placed in phenol-free Modified Eagle medium (GIBCO/Invitrogen). Calcium transients were measured at 37°C during field stimulation at 1.5 Hz to ensure consistent diastolic intervals. Images were acquired on an inverted confocal laser-scanning microscope (Perkin Elmer/Nikon). Transduced cells, recognized by GFP fluorescence, were
randomly selected for recording of calcium transients. These were subsequently analyzed using image-J software (NIH).

**Cell area and [³H]leucine incorporation.**

For cell area measurements, NRCMs were plated at low density (100 cells/mm²) in 35mm glass-bottom dishes. Cells were serum starved for 24 hours, after which they were incubated in phenylephrine (PE), 10μM. Images were acquired on an inverted microscope (Nikon) and cell area measurements were performed offline using NIH’s Image-J software. Transduced cells were recognized by GFP fluorescence.

For [³H]leucine incorporation, cells were plated in 12 well plates at a density of 0.5 x 10⁶ cells/well. Seventy two hours after transduction cells were incubated for 24 hours with 2μCi/ml of [³H]-leucine (MP Biomedicals) with and without PE. After incubation, cells were washed with ice-cold PBS, and fixed with 10% trichloroacetic acid for 30 minutes. Cell lysates were then solubilized in 0.20 N NaOH and the incorporated radioactivity was determined by liquid scintillation counting.

**In Vivo Cardiac Gene transfer and Aortic Banding.**

Adult (240-260g) Sprague-Dawley rats, were randomly assigned to receive `PPT.CG.H1.B2`, `PPT.CG.H1.NS`, or sham intervention. After baseline echocardiographic recordings, rats were anesthetized with isoflurane, intubated and placed on a volume-cycled mechanical ventilator. Body temperature was monitored and kept constant at 37° C. throughout the procedure. After dissection of the aorta and pulmonary artery, lentivirus vector (200ul = 10 x10⁸ TU/heart) was injected into the LV cavity through a 28 G needle syringe while the aorta and pulmonary artery were cross-clamped for 50 seconds. Fifteen minutes after the aorto/pulmonary cross clamp was released, the ascending aorta was banded with a 0.58 mm (internal diameter) tantalum clip as previously described. In sham operated animals, 150ul of normal saline was injected.
into the LV cavity while the aorta and pulmonary artery were cross-clamped for 50 seconds. After cross-clamping was released the chest was closed, and no aortic banding was performed. *In vivo* transduction efficiency was assessed by X-Gal staining of *PPT.CMV-LacZnls* injected animals. After 4 weeks of gene delivery, hearts were extracted and whole heart fixation and X-Gal staining was performed by retrograde perfusion as previously described. Paraffin-embedded tissue sections (15µm) were deparaffinated, stained with Hoecht® nuclear staining and mounted. Same field (20X) images were acquired by fluorescence and light microscopy and subsequently analyzed using Image J software.

*Quantitative Reverse-Transcription PCR (QPCR).*

Four weeks after in vivo gene delivery, total RNA was isolated from rat hearts using RNeasy midi Kit (Qiagen) per manufacturer instructions. For quantification of steady state mRNA levels the TaqMan® gene expression assay (Applied Biosystems) was used. Primers and probes against the LTCCβ, β-myosin heavy chain (β-MHC), and atrial natriuretic peptide (ANP) were designed using the Primer-express software (Applied Biosystems). Single-step QPCR was performed using a 7900HT Sequence Detection System (Applied Biosystems) under the following conditions: A total of 20ng of RNA was used in each QPCR reaction using the 18S ribosomal subunit as an internal control with each sample in triplicate. QPCR was performed in a 96-well optical plate with an initial RT step for 38 minutes at 48ºC, followed by 10 minutes of RT inactivation before 40 cycles of PCR at 95ºC /60ºC. Results were analyzed according to the relative standard curve method using the SDS 2.1 software (Applied Biosystems).

*Western Blots*

For Ca, 1.2 expression, protein was isolated from tissue lysates using a modified RIPA buffer as previously described. Samples (50 µg total protein / lane) were separated by 8% Tris-glycine SDS gel electrophoresis (Invitrogen) under reducing conditions. Proteins were transferred to polyvinylidene sulfonyl fluoride membranes (BioRad) and
blocked for 1 hour with 10% non-fat milk (BioRad) and 0.05% Tween20 in Tris-buffered saline (TBS-T). A rabbit Ca\textsubscript{v1.2} antibody (dilution 1:200, ACC-003, Alomone Labs.) was incubated overnight at 4 °C. Following washes with TBS-T membranes were incubated with a HRP-conjugated anti-rabbit antibody (dilution 1:20,000, Pierce) and bands were detected by Super Signal West Dura (Pierce). Membranes were stripped, and probed with a tubulin antibody (Abcam, 1:5,000) as a loading control. Densitometry analysis was performed to assess \( \alpha_{1C} / \) tubulin ratios from sham, NS RNAi, and \( \beta \) RNAi groups.

**Echocardiography.**

Rats were anesthetized with ketamine HCL (50mg/kg IP) and xylazine (20mg/kg IP), shaved over the praecordium and placed on a rodent-handling platform (VisualSonics) that allowed electrocardiography (ECG) monitoring and body temperature to be kept constant at 37°C. Transthoracic 2-D and M-mode images were acquired on the para-sternal long-axis and short-axis at the midpapillary level using a high-resolution 25 MHZ scan head (RMV-710, VisualSonics) attached to a rail system to assure standardization of imaging acquisition between animals. All images were recorded in a VisualSonics Vevo 660 high resolution rodent imaging system, and subsequently analyzed. Echocardiograms were performed at baseline, 2 and 4-week time points after vector transduction/aortic banding. LV wall thickness and LV end diastolic (LVDD) and end systolic (LVSD) diameters were measured offline from M-mode recordings. Fractional shortening (%) was calculated as \( 100 \times (LVDD-LVSD)/LVDD \).

**Invasive Blood Pressure Measurement**

Blood pressure was measured invasively four weeks after aortic banding in PPT.CG.H1.\( \beta \)2 and PPT.CG.H1.NS transduced animals. After rats were anesthetized with isoflurane, intubated and placed on a volume-cycled mechanical ventilator the right carotid artery was dissected and a fluid filled catheter was advanced until the aortic root (distal to the aortic band). Blood pressure was recorded using a pressure transducer.
attached to a digital acquisition system (Biopac Instruments) and subsequently analyzed offline.

Statistics.

Continuous variables are expressed as mean ± standard error of the mean. Statistical analyses were performed using repeated measures ANOVA and Student paired t test, where appropriate. p<0.05 was considered to be indicative of statistical significance.


