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Targeted Deletion of *Tsc1* Causes Fatal Cardiomyocyte Hyperplasia Independently of Afterload

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Abstract

Despite high expression levels, the role of *Tsc1* in cardiovascular tissue is ill defined. We launched this study to examine the role of *Tsc1* in cardiac physiology and pathology. Mice in which *Tsc1* was deleted in cardiac tissue and vascular smooth muscle (*Tsc1c/cSM22cre^{+/-}*), developed progressive cardiomegaly, hypertension and died early. Hearts of *Tsc1c/cSM22cre^{+/-}* mice displayed a progressive increase in cardiomyocyte number, and to a lesser extent, size between the ages of 1 and 6 weeks. In addition, compared to control hearts, proliferation markers (phospho-histone 3 and PCNA) were elevated in *Tsc1c/cSM22cre^{+/-}* cardiomyocytes at 0–4 weeks, suggesting that cardiomyocyte proliferation was the predominant mechanism underlying cardiomegaly in *Tsc1c/cSM22cre^{+/-}* mice. To examine the contribution of *Tsc1* deletion in peripheral vascular smooth muscle to the cardiac phenotype, *Tsc1c/cSM22cre^{+/-}* mice were treated with the antihypertensive, hydralazine. Prevention of hypertension had no effect on survival, cardiac size or cardiomyocyte number in these mice. We furthermore generated mice in which *Tsc1* was deleted only in vascular smooth muscle but not in cardiac tissue (*Tsc1c/cSMAcre-ERT2^{+/-}*). The *Tsc1c/cSMAcre-ERT2^{+/-}* mice also developed hypertension. However, their survival was normal and no cardiac abnormalities were observed. Our results suggest that loss of *Tsc1* in the heart causes cardiomegaly, which is driven by increased cardiomyocyte proliferation that also appears to confer relative resistance to afterload reduction. These findings support a critical role for the *Tsc1* gene as gatekeeper in the protection against uncontrolled cardiac growth.

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Keywords

Cardiomegaly; Cardiomyocyte Proliferation; Cardiac Hyperplasia; Tsc1; Hamartin; Smooth Muscle Cell

1. Introduction

TSC1 is believed to play an important role in cell growth and proliferation since its mutation can lead to tuberous sclerosis complex (TSC)¹, a syndrome characterized by multiple tumors of the brain, kidney, heart and skin [1]. An unusual and less well understood complication of TSC is vascular abnormalities, many of which are premature in onset presenting in infants and children, and are frequently lethal. Late-term fetuses and infants with TSC develop cardiac rhabdomyomas [2]. In addition, TSC infants and young children can develop aortic aneurysms and congenital defects of the heart, which are often lethal, such as tetralogy of fallot, transposition of the great arteries, or ventricular septal defects [3, 4]. Despite the association of such congenital abnormalities with TSC and the related high mortality, few studies have examined the underlying mechanism responsible for the pathogenesis of cardiovascular abnormalities in TSC.

Peripheral vascular smooth muscle explanted from mice heterozygous for *Tsc2* (another TSC-associated gene [5]), and rhabdomyomas from humans with TSC show a marked increase in phospho-S6 levels [6, 7], suggesting that activation of mammalian target of rapamycin (mTOR), the main downstream target of *Tsc1* and *Tsc2*, is critical to the molecular pathogenesis of cardiovascular anomalies in TSC. Mammalian target of rapamycin complex 1 (mTORC1) receives major regulatory input from hamartin and tuberin, the protein products of *TSC1* and *TSC2*, through their tumor suppressor function towards rheb (ras homolog enriched in brain). The active form of the GTPase Rheb (GTP-rheb) activates mTORC1 resulting in the downstream phosphorylation of p70 S6 kinase (S6K) and eukaryotic translation-initiation factor 4E-binding protein 1 (4E-BP1), which increases protein translation and cell growth [8, 9]. Loss of functional hamartin or tuberin disrupts the hamartin-tuberin complex and elevates GTP-rheb resulting in constitutive activation of mTORC1. This activation is part of an evolutionarily conserved pathway which leads to increased cell growth [10]. mTORC1 is also responsive to input from growth factors, AKT, and AMP-activated kinase (AMPK) [11], all of which play a role in the development of cardiomyopathy, such that mTORC1 acts as a central regulatory switch that functions in sensing and modulating protein synthesis, cell proliferation and size.

In this report we have studied the role of the Tsc1/Tsc2 complex in cardiovascular development. After demonstrating that *Tsc1* deletion in vascular smooth muscle and cardiac tissue (*Tsc1^c/cSM22^{cre}+/−*) is associated with cardiomegaly, hypertension, and early death at 6 weeks [12], we investigated the mechanism of increase in cardiac mass and whether it is related to the hypertensive phenotype. Hence, we temporally measured indices of cell

¹**Abbreviations:** TSC: tuberous sclerosis complex, mTOR: mammalian target of rapamycin, mTORC1: mTOR complex 1, Rheb: ras homolog enriched in brain, S6K: p70S6 kinase, 4E-BP1: eukaryotic translation-initiation factor 4E-binding protein 1, AMPK: AMP-activated kinase, SMA: smooth muscle α -actin, RVSP: right ventricular systolic pressure, LV: left ventricle, MLPA: Multiplex Ligation Probe Assay, PH3: phospho-histone H3.

growth and cell size in *Tsc1c/cSM22cre^{+/-}* mice, before and after treatment for systemic hypertension using hydralazine, a pure vasodilator. Furthermore we generated a mouse model in which *Tsc1* is knocked out specifically in vascular tissue only (*SMAcre-ER^{T2}*) without altering cardiomyocyte *Tsc1* expression, and examined cardiac size and survival in these mice. Our results suggest a role for *Tsc1* in regulating heart size by regulation of cardiomyocyte hyperplasia, and to a lesser extent cardiomyocyte hypertrophy, which are independent of *Tsc1* deletion in vascular tissue.

2. Materials and Methods

2.1. Animal Studies

2.1.1 Generation of *Tsc1c/cSM22cre^{+/-}* mice—The breeding strategy for mice where *Tsc1* is knocked down in cardiovascular tissue is described elsewhere [12]. In brief, mice with the desired genotype, *Tsc1c/cSM22cre^{+/-}* were bred using a conditional allele of *Tsc1* (*Tsc1^c*) [7] and a SM22 allele expressing cre recombinase (*SM22cre*) [13]. Littermates of target animals with the following genotypes were used as controls: *Tsc1w/wSM22cre^{+/-}*, *Tsc1w/wSM22cre^{-/-}*, *Tsc1c/wSM22cre^{+/-}*, *Tsc1c/wSM22cre^{-/-}*, and *Tsc1c/cSM22cre^{-/-}*. To avoid selection bias in the tested outcomes, all mice were randomly assigned for sacrifice at predetermined ages (1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks and 8 weeks of age). Mice with the target genotype, *Tsc1c/cSM22cre^{+/-}* were randomly assigned at 3 weeks of age to be treated with a primary vasodilator, hydralazine (2.5 mg/kg/day; a small subset of mice were also treated with 5 mg/kg/day) or vehicle control administered via oral gavage and were compared to sham treatments of littermate controls (Figure 1A). Treatments continued until mice were sacrificed at predetermined ages (3, 4, 5, 6 and 8 weeks). The outcomes tested pre- and post- treatments are outlined below and in the text. To ensure consistency and reproducibility of the phenotype in the colony and follow natural survival of *Tsc1c/cSM22cre^{+/-}* mice, animals with all genotypes were selected for continued observation only.

2.1.2 Generation of *Tsc1c/cSMAcre-ER^{T2}+/-* mice—We also generated mice with *Tsc1* knocked down in vascular smooth muscle tissue using the conditional *Tsc1^c* allele and a smooth muscle α -actin (SMA) allele expressing a modified cre recombinase (*SMAcre-ER^{T2}*), which remains inactive until administration of Tamoxifen [14]. Using mixed strain *Tsc1c/cSMAcre-ER^{T2}-/-* and FVB/N *Tsc1w/wSMAcre-ER^{T2}+/-* (from Professor Pierre Chambon, Inserm, Illkirch, France), we applied three different breeding strategies to generate mice with the desired genotype, *Tsc1c/cSMAcre-ER^{T2}+/-*. Littermates of target animals with the following genotypes were used as controls: *Tsc1w/wSMAcre-ER^{T2}+/-*, *Tsc1w/wSMAcre-ER^{T2}-/-*, *Tsc1c/wSMAcre-ER^{T2}+/-*, *Tsc1c/wSMAcre-ER^{T2}-/-*, and *Tsc1c/cSMAcre-ER^{T2}-/-*. Tamoxifen was injected intraperitoneally to *Tsc1c/cSMAcre-ER^{T2}+/-* and littermate controls beginning at 3 weeks of age for 28 consecutive days at a dose of 1.0 mg per day in 100 μ l of Sunflower Seed Oil (Sigma, St. Louis, MO).

2.1.3 Analysis of Recombination by Multiplex Ligation Probe Assay (MLPA)—

The extent of recombination at the *Tsc1* locus was determined by MLPA of DNA from the aortas of *Tsc1c/cSMAcre-ER^{T2}+/-* and *Tsc1c/wSMAcre-ER^{T2}+/-* mice after treatment with

Tamoxifen and sacrificed at ages of 2 months and 3 months. The procedure for MLPA has been previously described elsewhere [12].

PCR genotyping of the conditional, wild type, and null alleles of *Tsc1* was performed for all animals as previously described [12]. Gross and microscopic analyses described below were carried out by individuals blinded to the genotype and treatment. All animal experimentation and procedures were performed in accordance with the Guide for the Humane Use and Care of Laboratory Animals and were approved by the Tufts Animal Care and Use Committee.

2.2 Histology and Immunohistochemistry

2.2.1 Standard histology—At predetermined intervals 2–8 week old *Tsc1c/cSM22cre^{+/-}* and corresponding littermate control mice, were sacrificed, weights determined, tibial length measured, and heart and aorta were harvested. Hearts were sectioned axially at the level of the mitral valve, and corresponding sections were subsequently obtained for hematoxylin and eosin (H&E) staining, Masson Trichrome stain (Ventana, Tucson, AZ) and periodic acid Schiff stain (PAS) (Poly Scientific, Bayshore, NY) as previously described [12]. Cardiomyocyte analysis for cell size was performed at the mid-papillary level and was restricted to cardiomyocytes located in the middle third of the left ventricle (LV) wall. Cardiomyocytes located near the epicardium or the papillary muscles were not analyzed. Aortas were transversally cross-sectioned and stained with H&E for visualization of the smooth muscle layers.

2.2.2 Assessment of Cardiomyocyte Surface Area—The surface area of ventricular cardiomyocytes was measured in H&E sections of the left ventricle obtained from *Tsc1c/cSM22cre^{+/-}* mice and littermate controls using Image-Pro Plus software (Ver 7.0; MediaCybernetics, MD) [12]. Heart sections cut axially below the mitral valve, and corresponding sections were stained with H&E and cardiomyocytes located in the middle third of the LV wall were analyzed. Cardiomyocytes located near the epicardium or the papillary muscles were excluded. The cell membrane of individual cardiomyocytes that were sectioned axially and contained a centralized nucleus was outlined, and the number of pixels per cardiomyocyte determined. Cardiomyocytes (>50) from five separate regions were measured per sample. The surface area was calculated from the total pixel number per cardiomyocyte using a conversion factor and expressed as μm^2 .

2.2.3 Immunohistochemistry—Paraffin-embedded sections of the LV and aorta were processed as previously described [15]. In brief, slides were deparaffinized, and blocked with 1% BSA, then stained for TUNEL (Promega, Madison, WI), p62 (Sigma, St. Louis, MO), or appropriate control and then counterstained with hematoxylin (Dako, Carpinteria, CA). Labeled slides were examined using a Nikon Eclipse E400 microscope, and images were captured using Spot software v4.0.5. We defined staining based on % of nucleated cells stained positively as present (10–30%), moderate (31–50%), or strong (>50%).

2.3 Immunoblot Analysis

LV tissue extracts were processed and equal amounts of protein were analyzed by immunoblotting as previously described [16, 17]. Primary antibodies used were against:

GAPDH (Santa Cruz, CA), COXIV, hamartin, pS6 (Cell Signaling Technology, MA), p62 (Sigma, St. Louis, MO). Densitometric analysis was performed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA), and band intensity was expressed relative to GAPDH or COXIV control. Data representative of at least 3 immunoblots were expressed as fold-increase relative to control.

2.4 Hemodynamic Measurements

A non-invasive tail cuff pressure system (AD Instruments, PowerLab NIBP controller, Colorado Springs, Colorado) was used to measure systemic pressure (systolic blood pressure) as previously described [12]. Prior assessment in this cohort validated the accuracy of systolic pressure measurements, using this method, in mice as young as 3 weeks. Thus systemic pressure was measured using the same system in all mice 3–8 weeks of age. An average of three recordings per animal was measured in awake and restrained mice. Right ventricular systolic pressure (RVSP) was measured in anesthetized mice with a transthoracic needle stick, attached to a Statham pressure transducer as previously described [12].

2.5 Measurement of Vasoconstriction Response

A modification of the procedure we have previously described [12] was used in which aorta was dissected in full length. Next, the half near the aortic arch was fixed in 10% formalin, while the other half of the aorta was cut into two 3 mm rings. The rings were next suspended on tungsten wires in a bath of Earle's balanced salt solution at 37°C at a tension of 0.5 g. After 1 h the vasoconstrictive response to phenylephrine (10^{-5} M) was measured, and multiple measurements per ring were averaged per animal.

2.6 Murine Ventricular Cardiomyocyte Dissociation

Cardiomyocytes were extracted from *Tsc1c/cSM22cre^{+/-}* mice and their littermate controls as previously described [12, 18, 19]. In brief, ventricles were minced into 0.3mm thick sections and digested in 0.125% trypsin in Hank's Balanced Salt Solution (HBSS; Atlanta Biologicals, Lawrenceville, GA). Ventricular cells were then dissociated through repeated digestions with warm HBSS containing collagenase type II (1 mg/mL; Worthington Biochemical Corp., Freehold, NJ) at 37°C. Cell suspension was plated multiple times on 0.1% gelatin coated plates for 60 min each to enrich for ventricular cardiomyocytes, and then processed through a Beckman Coulter Z1 Coulter Particle Counter at 1:50 dilution (Beckman Coulter, Inc., Brea, CA).

2.7 Assessment of Proliferation

Tsc1c/cSM22cre^{+/-} and littermate control heart sections from date-of-birth (P0) and 4 week old animals were immunostained with anti-phospho-histone H3 (PH3) antibody, MYH7 antibody and Hoechst antibody and were visualized using co-immunofluorescence as previously described [20]. Using ImageJ software, PH3-positive cardiomyocyte nuclei and the total number of nuclei were counted in 20 randomly selected separate fields for each of the 3 hearts per group. The average number of PH3-positive cardiomyocyte nuclei per group was divided by total number of cardiomyocyte nuclei in order to determine the ratio of PH3-positive cardiomyocytes to total cardiomyocytes.

2.8 Statistical Analysis

Results are expressed as mean $\pm$ standard deviation. Non-parametric Kruskal-Wallis ANOVA with Dunn's multiple comparison test for follow-up analysis was performed using SigmaStat software.

3. Results

3.1 Hydralazine lowers systemic pressures in *Tsc1c/cSM22cre^{+/-}* mice

We had generated mice in which *Tsc1* expression is disrupted under the control of the SM22 promoter (*Tsc1c/cSM22cre^{+/-}* mice), which caused elevated systemic pressures and cardiac hypertrophy by 4 weeks of age in *Tsc1c/cSM22cre^{+/-}* mice [12]. Our previous study has linked this phenotype to constitutive activation of mTORC1 signaling during development in vascular and cardiac tissue [12]. In this report we further characterized the temporal development of the phenotype and the relation between cardiac and vascular manifestations. Elevated systemic pressures were observed by 3 weeks of age (*Tsc1c/cSM22cre^{+/-}* mice: 120.6 ± 4.6 versus controls: 98.6 ± 4.7 mmHg; $p < 0.01$) and steadily rose by 6 weeks of age (*Tsc1c/cSM22cre^{+/-}* mice, 130.6 ± 3.3 versus controls, 100.0 ± 2.7 mmHg; $p < 0.01$) (Figure 1B). Treatment with the antihypertensive, hydralazine beginning at 3 weeks of age, resulted in a significant reduction and normalization of systemic pressures in treated *Tsc1c/cSM22cre^{+/-}* mice compared to untreated *Tsc1c/cSM22cre^{+/-}* mice (Figure 1B) (At 6 weeks, untreated *Tsc1c/cSM22cre^{+/-}* mice: 130.6 ± 3.3 versus *Tsc1c/cSM22cre^{+/-}* mice receiving 2.5 mg/kg/day hydralazine: 105.2 ± 7.1 versus *Tsc1c/cSM22cre^{+/-}* mice receiving 5.0 mg/kg/day hydralazine: 103.9 ± 2.0 mmHg; $p < 0.01$). The response to hydralazine was acute and no significant differences in systemic pressures were observed at treatment doses of 2.5 versus 5 mg/kg/day. Both doses resulted in systolic pressures that were similar to those in control mice not on therapy (untreated control group systemic pressure: 100.0 ± 2.7 mmHg; $p > 0.05$). An effective response was maintained throughout the duration of the study. Thus, a satisfactory physiologic effect was achieved with hydralazine at a minimum dose of 2.5 mg/kg/day which was consequently utilized as the therapeutic dose for future experiments.

3.2 Hydralazine did not improve the survival, or alter the visible phenotype of *Tsc1c/cSM22cre^{+/-}* mice

Significant and early mortality of *Tsc1c/cSM22cre^{+/-}* mice was observed consistent with our earlier report [12]. In the cohort followed in this study, we noted a slight delay in the onset of death beginning at 3 weeks with no deaths observed prior to that age. This delay might reflect mild genetic drift that can occur in murine models [21] (Figure 1C). Hydralazine was not associated with a statistically significant survival benefit (survival at 5 weeks of vehicle: $73.5\% \pm 2.5\%$ versus hydralazine: $76.7\% \pm 2.0\%$ at 5 weeks; $p > 0.05$). Furthermore, no rescue of the visible phenotype was observed in the untreated animals in response to hydralazine. In fact, compared to the untreated group *Tsc1c/cSM22cre^{+/-}* mice treated with hydralazine appeared more sluggish and significantly more morbid for a longer period of time before death. No deaths, and none of these morbid features, were observed in sham-treated littermate control mice suggesting that the increased morbidity in hydralazine-treated mice was not procedurally related. Body weights in *Tsc1c/cSM22cre^{+/-}* mice were

paradoxically low, a feature observed in these animals in our prior studies, and were not improved by hydralazine therapy (untreated *Tsc1c/cSM22cre^{+/-}* mice: 16.02 ± 0.78 g versus hydralazine-treated *Tsc1c/cSM22cre^{+/-}* mice: 13.90 ± 0.5 g; $p > 0.05$) (Figure 1D). In fact, weight gain plateaued in mice treated with hydralazine and remained significantly lower when compared to littermate controls (hydralazine-treated *Tsc1c/cSM22cre^{+/-}* mice: 13.90 ± 0.5 g versus controls: 18.14 ± 0.34 g; $p < 0.01$) (Figure 1D).

3.3 Cardiac mass is progressively elevated in *Tsc1c/c SM22cre^{+/-}* mice, and is not altered by hydralazine treatment

The most striking feature of SM22-driven *Tsc1* loss is the significant increase in heart size compared to mice where *Tsc1* is normally expressed. In prior studies we noted this difference began as early as E15 [12]. In this study, consistent with prior observations, cardiac mass remained significantly elevated in *Tsc1c/cSM22cre^{+/-}* mice at all ages tested, (Figure 2A). Heart weights almost doubled between 1 and 2 weeks of age and increased over 4 fold by 5 weeks of age (Figure 2B) (*Tsc1c/cSM22cre^{+/-}* mice untreated: 4.4-fold $\pm$ 0.8-fold vs. *Tsc1c/cSM22cre^{+/-}* mice, 2.5 mg/kg/day hydralazine: 3.9-fold $\pm$ 0.7-fold at 5 weeks; $p > 0.05$). All hearts continued to gain mass almost linearly with no heart weighing more than 300 mg prior to death in any of the *Tsc1c/cSM22cre^{+/-}* mice studied (Figure 2A). Since *Tsc1c/cSM22cre^{+/-}* have significantly reduced body weights compared to littermate controls, we chose to use the parameter of tibia length to adjust for size differences between individual mice. The latter parameter is more reliable for comparisons and it remains overall unchanged across all groups (Figure 2C). When expressed using tibia length as the denominator, the increase in cardiac mass in *Tsc1c/cSM22cre^{+/-}* mice compared to littermate controls remained consistent and significant (Figure 2D). Although hydralazine therapy in *Tsc1c/cSM22cre^{+/-}* mice appeared to retard the increase in cardiac mass, the differences between untreated and hydralazine-treated *Tsc1c/cSM22cre^{+/-}* cardiac mass never reached statistical significance (Figure 2A, D). Furthermore, the hearts of all treated mice reached the pre-morbid heart weight ~250 mg – 300 mg prior to the termination of the study. Grossly, hearts appeared larger in all *Tsc1c/cSM22cre^{+/-}* mice with no significant change in cardiac morphology in response to hydralazine (Figure 2E). Consistently, the left ventricular and septal walls were thicker in *Tsc1c/cSM22cre^{+/-}* mice than in control mice. Specifically, irregular thickening of the internal contours of the left ventricle cavity wall was observed. Rare incidental note was made of possible septal defects and occasional thinning of the posterior wall of the left ventricle (Figure 2E), however, no progressive dilatation of the left ventricle was observed to suggest a dilated cardiomyopathy and none of these features were reversed by treatment with hydralazine. These results suggest that loss of *Tsc1* is tightly associated with continued growth of the heart that remains unchecked until death. Furthermore, they also suggest that loss of *Tsc1* in the heart confers significant resistance to the treatment of systemic hypertension in this setting.

3.4 Cardiomyocyte hyperplasia in *Tsc1c/cSM22cre^{+/-}* mice

Our observations of increased heart weight and heart size in *Tsc1c/cSM22cre^{+/-}* mice suggested that the increased mass could be due to increased cardiomyocyte number. To investigate this as a possibility we subjected hearts to enzymatic digestion, removed fibroblasts by repeated plating and counted cells in a Coulter counter. As expected cell

counts in hearts of control mice increased by a small degree, but appeared to plateau after 4 weeks (Figure 3A). In contrast, cell counts in *Tsc1c/cSM22cre^{+/-}* mice increased over time in a linear fashion, which paralleled the observed increase in heart weight. Importantly, the increase in cell counts was not significantly affected by hydralazine treatment (untreated *Tsc1c/cSM22cre^{+/-}* mice, 6 weeks: $43.8 \pm 7.2 \times 10^5$ cells versus hydralazine-treated *Tsc1c/cSM22cre^{+/-}* mice, 6 weeks: $36.7 \pm 5.5 \times 10^5$ cells; versus littermate control mice, 6 weeks: $14.0 \pm 1.5 \times 10^5$ cells; $p > 0.05$) (Figure 3A). The change in cell number versus time is also reflected when it is expressed as fold change over week 1 such that it plateaus in control mice but keeps increasing along the life of *Tsc1c/cSM22cre^{+/-}* mice (Figure 3B). Next, we tested the relationship between cell number and heart weight. Plotting cell number/mg of heart tissue against age of mice we noted that the slope was nearly horizontal, regardless of the type of mouse group, suggesting a direct relationship between the variables of cell number and heart weight (Figure 3C). Also noteworthy was that while the slopes of these lines were similar for littermate controls and both groups of *Tsc1c/cSM22cre^{+/-}* mice (treated and untreated), there appeared to be consistently more cells per milligram of heart in *Tsc1c/cSM22cre^{+/-}* hearts than in littermate controls at each time point, regardless of treatment with hydralazine. To explore the correlation between hyperplasia and cardiac mass we plotted cell counts versus the corresponding weights of individual hearts from *Tsc1c/cSM22cre^{+/-}* untreated mice. As shown in Figure 3D, there is a strong correlation ($R^2=0.761$) between these two variables (Figure 3D). These results suggested that the increase in cardiac mass in *Tsc1c/cSM22cre^{+/-}* mice was driven by an increase in cell number.

To further examine increased cell number, we chose to look for markers of cell proliferation in the hearts of the *Tsc1c/cSM22cre^{+/-}* mice. We carried out co-immunofluorescence analysis for PH3, a marker of proliferating cells, along with MYH7, a marker of cardiomyocytes, and Hoechst nuclear stain. As shown in Figure 3E this analysis indicated that there are significantly more dividing cardiomyocytes in the hearts of *Tsc1c/cSM22cre^{+/-}* mice at P0 compared to littermate controls (*Tsc1c/cSM22cre^{+/-}*, P0: 2.83 ± 0.25 MYH7+, PH3+ nuclei per 1000 MYH7+ nuclei versus littermate control, P0: 1.15 ± 0.37 MYH7+, PH3+ nuclei per 1000 MYH7+ nuclei; $p = 0.026$) (Figure 3E). Interestingly, a notable amount of dividing cardiomyocytes were also observed in the hearts of 4 week old *Tsc1c/cSM22cre^{+/-}* mice, which is a highly unexpected observation in a mature heart and was not seen in littermate controls (Figure 3F). Although dividing cardiomyocytes were found in two-thirds of our *Tsc1c/cSM22cre^{+/-}* heart samples, our sample size was not large enough to reach statistical significance (*Tsc1c/cSM22cre^{+/-}*, 4 weeks: 1.59 ± 0.84 MYH7+, PH3+ nuclei per 1000 MYH7+ nuclei versus littermate control, 4 week: 0.20 ± 0.20 MYH7+, PH3+ nuclei per 1000 MYH7+ nuclei; $p = 0.354$, $n=3$). While these numbers are not significant, we believe that the presence of any dividing cardiomyocytes in the hearts of 4 week old *Tsc1c/cSM22cre^{+/-}* mice is substantial and supports our notion that some of the increase in cardiac mass is due to hyperplasia of cardiomyocytes caused by loss of *Tsc1*. Finally, to further confirm the effect on cell proliferation we carried out Western Blot analysis for the proliferative marker PCNA which showed that it was elevated in the hearts of *Tsc1c/cSM22cre^{+/-}* mice at the age of 4 weeks compared to littermate controls (Figure 3G).

3.5 Cardiomyocyte hypertrophy in *Tsc1c/cSM22cre^{+/-}* mice

Tsc1 as part of a complex with its sister protein *Tsc2*, exerts its biologic effect on mTOR to regulate cell size, a well-documented observation in both drosophila as well as in mammalian cells. We measured cardiomyocyte size in the hearts of *Tsc1c/cSM22cre^{+/-}* mice (untreated and treated with hydralazine), and compared them to control mice (Figure 3H). For the first 4 weeks of life cardiomyocyte size was not statistically different between any of the groups. Cell area steadily increased linearly in all three groups. However after 4 weeks, cardiomyocytes were significantly bigger in both groups of *Tsc1c/cSM22cre^{+/-}* mice compared to controls (*Tsc1c/cSM22cre^{+/-}* untreated, 6 weeks: $485.9 \pm 12.1 \mu\text{m}^2$ versus *Tsc1c/cSM22cre^{+/-}* mice, 2.5 mg/kg/day hydralazine, 6 weeks: $518.8 \pm 14.9 \mu\text{m}^2$ versus littermate control, 6 weeks: $380.8 \pm 8.5 \mu\text{m}^2$; $p < 0.002$). These results suggest that hypertrophy could also account for some of the increased cardiac mass observed in hearts lacking *Tsc1*.

3.6 Glycogen and collagen does not accumulate the hearts of *Tsc1c/cSM22cre^{+/-}* mice

In sarcomeric cardiomyopathies the accumulation of glycogen has been observed and thought to play a role in the development of cardiac arrhythmias, and glycogen deposits have been noted in cardiac rhabdomyomas in TSC patients. Additionally, in dilated cardiomyopathies deposition of collagen and the development of fibrosis interfering with cardiac function becomes evident as they progress. Hence, we tested for the accumulation of glycogen and collagen deposition using PAS and trichrome staining. Patchy PAS staining was rare and not different between the groups (Figure 4A). In addition, no obvious differences in trichrome staining were observed in the hearts of *Tsc1c/cSM22cre^{+/-}* mice compared to control mice up until the age of six weeks (Figure 4B). These results indicate that the deposition of glycogen and collagen was not very evident and it is unlikely that they contribute in a major way to the pathology observed in the hearts of *Tsc1c/cSM22cre^{+/-}* mice.

3.7 Autophagy is decreased while apoptosis is increased in the hearts of *Tsc1c/cSM22cre^{+/-}* mice

Recently autophagy has been reported as playing a role in the tumorigenesis of *Tsc2*-null xenograft tumors and spontaneous renal tumors in *Tsc2^{+/-}* mice [22]. Loss of *Tsc* complex function has also been shown to alter apoptosis in either direction [22]. We tested cardiac tissue for levels of p62, an autophagy marker, and for TUNEL staining to assess apoptosis in *Tsc1c/cSM22cre^{+/-}* mice and littermate controls. There was a marked increase in p62 staining by 3 weeks of age which was maintained for the duration of the study regardless of hydralazine therapy (Figure 4C, D). Similarly, a slight but notable increase in TUNEL staining was observed in the hearts of *Tsc1c/cSM22cre^{+/-}* mice (Figure 4E). Staining was more evident in older animals ~5 weeks of age and again was unresponsive to hydralazine therapy. These results suggest that in *Tsc1c/cSM22cre^{+/-}* mice decreased autophagy was a highly significant feature that occurred earlier in the development of cardiomyopathy, and was succeeded by an increase in apoptosis seen as a later phenomenon. Both of these processes could potentially contribute to the pathologic phenotype we observe in *Tsc1c/cSM22cre^{+/-}* mice.

3.8 Vascular smooth muscle specific *Tsc1* knockout mice exhibit systemic hypertension and increased vasoconstrictive response

Vascular abnormalities were observed in *Tsc1c/cSM22cre^{+/-}* mice in accordance with the vascular smooth muscle knockdown of *Tsc1* exhibited in those mice; these included systemic hypertension and an increased vasoconstrictive response [12]. To test if the observed vascular pathology was a direct result of *Tsc1* knockdown in the vascular smooth muscle we generated a model in which *Tsc1* is knocked out specifically in vascular tissue. To achieve this we used the conditional *Tsc1^c* allele and a SMA allele expressing a modified cre recombinase (*SMAcre-ER^{T2}*), which remains inactive until administration of Tamoxifen [14]. SMA is expressed transiently in the myocardium and skeletal muscle during fetal development, but its expression is restricted to vascular smooth muscle in the adult mouse [23]. Using an inducible cre recombinase provided us with temporal specificity such that administration of Tamoxifen to adult mice allowed us to limit the recombination of *Tsc1* to the vasculature of the mice with our target genotype: *Tsc1c/cSMAcre-ER^{T2+/-}*. After treatment with Tamoxifen, *Tsc1c/cSMAcre-ER^{T2+/-}* mice had a survival rate of greater than 90% through 6 months of age and showed no visible phenotype aside from an increased susceptibility to rectal prolapse. Analysis of genetic recombination of *Tsc1* in DNA from aortic smooth muscle by MLPA consistently showed 62–65% recombination of the conditional allele after Tamoxifen treatment in both *Tsc1c/cSMAcre-ER^{T2+/-}* and *Tsc1c/wSMAcre-ER^{T2+/-}* mice at ages of 2 months and 3 months (Figure 5A). On the other hand *Tsc1c/cSMAcre-ER^{T2-/-}* and *Tsc1c/wSMAcre-ER^{T2-/-}* mice injected with Tamoxifen did not show any recombination of their conditional alleles as expected. Immunoblots of aorta homogenates confirmed the knock down of hamartin (*Tsc1*) in *Tsc1c/cSMAcre-ER^{T2+/-}* mice treated with Tamoxifen. Furthermore, there was significant elevation of pS6 levels, confirming physiologic activation of mTOR in the vasculature, an expected effect of hamartin knockdown (Figure 5B). The vascular knockdown of hamartin was moderate at 2 months of age (or 1 week after completion of Tamoxifen treatment) but became more significant by 3 months of age, likely due to reduction in the expression of the protein over time. In these *Tsc1c/cSMAcre-ER^{T2+/-}* mice we observed an onset of statistically significant systemic hypertension measured by tail cuff as mean systolic pressure between the ages of 2–3 months (littermate control, 3 months: 126.1 ± 2.9 versus *Tsc1c/cSMAcre-ER^{T2+/-}*, Tamoxifen treated, 3 months: 162.1 ± 3.0 mmHg; $p < 0.01$).

Significantly the increase in systolic pressure coincided with the disappearance of hamartin (Figure 5C). The magnitude of the elevation in mean systolic pressure compared to littermate controls is almost exactly the same as that observed in *Tsc1c/cSM22cre^{+/-}* mice (*Tsc1c/cSMAcre-ER^{T2+/-}*, 3 months: 36.0 versus *Tsc1c/cSM22cre^{+/-}*, 4 weeks: 35.9 mmHg) [12]. Similarly to what was previously observed in *Tsc1c/cSM22cre^{+/-}* mice, there was a lack of excessive muscularization of aortic smooth muscle but a significantly greater vasoconstrictive force generated by aortas lacking *Tsc1* upon treatment with phenylephrine (littermate control, 3 months: 0.193 ± 0.019 g versus *Tsc1c/cSMAcre-ER^{T2+/-}*, Tamoxifen treated, 3 months: 0.281 ± 0.018 g; $p < 0.02$) (Figure 5D). Immunoblot analysis of the cardiac tissue from *Tsc1c/cSMAcre-ER^{T2+/-}* mice after treatment of Tamoxifen showed no detectable decrease in hamartin levels, confirming the vascular specificity of the knockdown (Figure 5E). No differences were observed in right ventricular systolic pressure (RVSP)

between mice lacking vascular *Tsc1* and littermate controls (Figure 5F). Examination of heart weights (normalized to body weight) revealed no cardiomegaly in *Tsc1c/cSMAcre-ERT2^{+/-}* mice (Figure 5F). More importantly, these results suggest that the pressure overload observed in *Tsc1c/cSMAcre-ERT2^{+/-}* mice is not sufficient to cause the type of cardiac phenotype seen in the *Tsc1c/cSM22cre^{+/-}* animals. This finding lends further support to the notion that the increased heart weight observed in *Tsc1c/cSM22cre^{+/-}* was a phenomenon driven by the loss of *Tsc1* in the heart.

4. Discussion

Our results describe phenotypic changes in the hearts of mice that do not express *Tsc1*. Specifically, *Tsc1* deletion driven by the SM22 promoter leads to cardiomegaly and increased mortality that are independent of the effects of *Tsc1* knockdown in vascular cells. Furthermore, the increased cardiac mass appears to be predominantly due to increased cardiomyocyte proliferation. These findings suggest that *Tsc1* is independently responsible for controlling cardiac size, and contributes to a maladaptive response of the heart to hypertensive stress.

Previously, we described the role of *Tsc1/2* in cardiovascular tissue using a SM22-driven *Tsc1* conditional knockout approach [12]. In mice with the target genotype (*Tsc1c/cSM22cre^{+/-}*), *Tsc1* was efficiently knocked down ~90% in cardiomyocytes. As a major regulator of mTORC1, knockdown of *Tsc1* resulted in the expected downstream constitutive activation of mTORC1 S6 kinase and S6. Significant cardiac enlargement began as early as E15 and was associated with early death. Interestingly, as we described in [12], administration of the mTORC1 inhibitor, rapamycin, could reverse this phenotype in the *Tsc1c/cSM22cre^{+/-}* animals without altering hamartin expression, but through altering mTORC1 signaling which is downstream of hamartin. This reversal was observed regardless of the stage of disease severity suggesting that mTORC1 is critical for both the induction and the evolution of the observed cardiac pathology.

In the current study, we focused on the mechanism responsible for the striking increase in cardiac mass in *Tsc1c/cSM22cre^{+/-}* mice. Mechanisms such as deposition of collagen, glycogen and increased cell size have all been shown to contribute to the development of cardiomyopathy. For example remodeling and fibrosis characterized by accumulation of collagen were reported to play a role in pathogenesis of dilated cardiomyopathy (for review see [24]). Furthermore, glycogen deposition occurred in cardiac rhabdomyomas and sarcomeric cardiomyopathy, while hypertrophy followed by fibrosis predominated in cardiomyopathy due to increased afterload [7, 25, 26]. In this study, we explored several of these mechanisms including the novel possibility of cardiomyocyte proliferation. No significant differences in Trichrome or PAS staining between the *Tsc1c/cSM22cre^{+/-}* mice and littermate controls were observed, eliminating fibrosis and glycogen accumulation as potential etiologies. In contrast, we identified significant increases in markers of cardiac cell proliferation as well as increased cell size in the hearts of *Tsc1c/cSM22cre^{+/-}* mice. Increased cardiomyocyte proliferation in *Tsc1c/cSM22cre^{+/-}* mice was concluded from cell counts of plated cardiomyocytes, as well as from quantifying proliferation markers. Specifically, we observed significant PH3-labeling, which is typically only detected in

proliferating cells, in MYH7-positive cardiomyocytes in heart sections of *Tsc1c/cSM22cre^{+/-}* mice. It should also be noted that at 4 weeks some of the mice in our cohort begin to die. Considering that cardiomyocytes positive for PH3 are not usually observed in adult hearts, it is possible that mice which do have them are less likely to survive up to 4 weeks of age, which might explain why PH3 positive cardiomyocytes were not identified in every *Tsc1c/cSM22cre^{+/-}* 4 week heart sample. Another proliferation marker, PCNA, was also elevated in heart homogenates. Moreover, at 6 weeks of age, a three-fold increase in cell number (Figure 3A) paralleled a similar increase in cardiac mass (Figure 2D). Although cell area was significantly larger in *Tsc1c/cSM22cre^{+/-}* mice, the magnitude of effect (1.28-fold) was not as great (Figure 3H). Furthermore, unlike cardiomyocyte counts which correlated strongly with cardiac mass in *Tsc1c/cSM22cre^{+/-}* mice (Figure 3D), cell size correlated poorly with the latter. These results support the concept that deletion of *Tsc1* in cardiomyocytes drives cell proliferation (and to a lesser extent cell size) that is unchecked, and that likely leads to increased cardiac mass and eventual death.

Typically the heart stops growing soon after birth. The adult heart often responds to stress by cell hypertrophy. However, our results suggest that the heart is capable of growth when *Tsc1* function is lost. In support of this concept, it has been reported that adult mammalian hearts have a reservoir of progenitor cells (or stem cells) capable of growth when necessary [27]. Furthermore, recent findings on the plasticity and de-differentiation of cardiomyocytes challenge the notion that they are terminally differentiated [28, 29]. This knowledge, together with the relatively high prevalence of resident fibroblasts in the adult heart, suggest that there are many processes that could contribute to increased cardiac mass in a way that is more important than previously recognized [30]. We postulate that our results reflect cardiomyocyte de-differentiation or lack of differentiation based on earlier reports implicating *Tsc2* (the sister protein of *Tsc1*) in embryonic cardiomyocyte differentiation [31].

One limitation of our murine model was the possible contribution of increased after-load to the observed cardiac phenotype because *Tsc1* was knocked down in both the heart and vascular tissue. Thus, the independent effects of mTORC1 activation due to *Tsc1* deletion in the heart alone could not be determined. To address this variable, we reduced afterload by treating the hypertension using the smooth muscle relaxant hydralazine. The advantage of this approach is that the contribution of increased afterload to the cardiac phenotype could be significantly mitigated by hydralazine's direct effect on peripheral vascular smooth muscle which occurs in the absence any known biologic effect on cardiac tissue. As our results show, while hydralazine was very effective in ameliorating the systemic hypertension, it did not affect the observed cardiomegaly, nor did it improve survival of the *Tsc1c/cSM22cre^{+/-}* mice. Although the effect of hypertension in the first 3 weeks of life cannot be determined, our findings suggested that, when *Tsc1* function is lost in the heart, the expected response to increased afterload is absent.

In an attempt to consolidate this observation, we sought to knockdown *Tsc1* specifically in vascular smooth muscle cells without affecting its expression in cardiac tissue. We successfully generated a vascular smooth muscle specific knockout of *Tsc1* in mice using a conditional knockout approach with a Tamoxifen-inducible SMA-driven cre recombinase

[14]. The advantage of this inducible model is that it allows tissue specificity as well as temporal specificity. The latter is an important issue because although SMA is specifically expressed in smooth muscle cells, it could be expressed by other cell types during development. Hence, we needed to activate the SMA promoter-driven Cre in adult mice, which was accomplished by treatment of these mice with Tamoxifen. After treatment with Tamoxifen, effective vascular smooth muscle-specific recombination of *Tsc1* and subsequent reduction of hamartin levels in this model were found. The vascular knockdown of *Tsc1* caused systemic hypertension and an increased vasoconstrictive response, which mirrored the vascular pathology seen in *Tsc1c/cSM22cre^{+/-}* mice [12]. Significantly, these *Tsc1c/cSMAcre-ERT2^{+/-}* mice showed no increased morbidity, no cardiomegaly as a result of afterload, and a survival rate of greater than 90% through 6 months of age despite the vascular phenotype. Absence of detectable cardiac hypertrophy in response to afterload alone, might be related to the duration of the hypertension in these mice, or to its constrictive rather than vascular remodeling nature. These results lend further support to the suggestion that the systemic hypertension observed in the SM22 model is a result of a vasoconstrictive phenomenon caused by lack of *Tsc1* in vascular smooth muscle and does not substantially contribute to the cardiac pathology and mortality in the *Tsc1c/cSM22cre^{+/-}* mice. In regards to what causes the increased mortality in these mice, we are investigating several explanations. We suspect that the observed hyperplasia and evident cardiomyocyte proliferation might yield less mature cardiomyocytes. We speculate and will test the possibility that these cells disrupt normal heart electrophysiology, and trigger arrhythmias that cause the premature death observed which occurs suddenly.

As to what processes might have contributed to the observed hyperplasia, further studies are needed. Observed decreased autophagy followed by increased apoptosis in our mouse model could reflect de-differentiation of cardiac cells that needs to be explored. We postulate that the increased cardiac mass observed in our model is maladaptive as it is associated with decreased survival and is massive (over 300% increase in mass) compared to physiological hypertrophy (usually 10–20%). Since SM22 is more active in cardiomyocytes during early development than in adult cells, our findings suggest that modulation of TSC/mTOR signaling during these critical stages might have devastating consequences on cardiac function later in life. In summary, these data support a critical role for the *Tsc1* gene as gatekeeper for mTORC1 in the protection against uncontrolled cardiac growth, and in the modulation of resistance to the hypertrophic response of the heart to after-load.

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Manuscript Highlights

- Tsc1 disruption in cardiomyocytes and vascular smooth muscle cells causes hypertension and cardiomegaly.
- Cardiomegaly due to Tsc1 disruption is associated with cardiomyocyte proliferation.
- Fatal cardiomegaly is not affected by antihypertensive treatment.
- Inducible Tsc1 disruption in vascular smooth muscle cells produces hypertension but not cardiomegaly.
- Tsc1 plays an important role in cardiomyocyte development and proliferation.

Figure 1. Effects of hydralazine treatment on systemic pressures and phenotype of *Tsc1^{fl/w}/cSM22^{Cre}^{+/+}* mice (*c/c+*). **(A)** Experimental Design. **(B)** Systemic blood pressures as measured by tail cuff systolic pressure (*n* = 7, 3–6 weeks; *n* = 4, 8 weeks; * *p* < 0.05). **(C)** Kaplan-Meier curves showing survival of *c/c+* mice with or without 2.5 mg/kg hydralazine treatment compared to littermate controls of mixed genotypes (*n* = 30, each group). **(D)** Body weight from 1 week through 8 weeks (*n* = 6; * *p* < 0.05).

Figure 2.

Cardiac weights and morphology in *Tsc1c/cSM22cre^{+/-}* mice (c/c+) with or without hydralazine treatment. **(A)** Heart weights at 1 week through 8 weeks (n = 6, * p < 0.02, c/c+ untreated or c/c+ 2.5 mg/kg hydralazine versus littermate control). **(B)** Heart weight data represented as fold change over 1 week weight of each group. **(C)** Tibia lengths at 1 week through 6 weeks (n = 6). **(D)** Heart weight normalized to tibia length at 1 week through 8 weeks (n = 6, * p < 0.02, c/c+ untreated or c/c+ 2.5 mg/kg hydralazine versus littermate control). **(E)** H&E stained axial sections showing variable pathologies in hearts from c/c+ mice, both with or without hydralazine treatment.

Figure 3.

Analysis of cell proliferation and size in *Tsc1/cSM22cre^{+/-}* (c/c+) mouse hearts. (A) Ventricular cell count from hearts of 1–8 week old animals (n = 6, * p < 0.02, c/c+ untreated or c/c+ 2.5 mg/kg hydralazine versus littermate control). (B) Ventricular cell count data expressed as fold change over 1 week count. (C) Cell count per milligram of heart weight (n = 6). (D) Linear regression and Coefficient of Determination ($R^2 = 0.761$) of cell count versus corresponding heart weight for 36 individual hearts from untreated c/c+ mice. (E) Co-immunofluorescence for MYH7 (cardiomyocyte marker), PH3 (proliferation marker), and Hoechst nuclear marker on heart sections from mice at P0. Graph represents dividing cardiomyocytes (MYH7+, PH3+) per 1000 total cardiomyocytes (MYH7+) (n = 3; p = 0.026). (F) Co-immunofluorescence on heart sections from 4 week old mice (n = 3; p = 0.354). (G) Representative western blot and semi-quantitative densitometric analysis (n = 6) for proliferation marker PCNA in homogenates from 4 week old mouse hearts compared to GAPDH (loading control). (H) Ventricular cell size as measured from H&E stained axial sections of hearts from 1 week through 8 week old mice.

A.

		1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	8 weeks
Periodic Acid-Schiff	littermate control	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	c/c+ untreated	Neg	Neg	Neg	Neg	Neg	Neg	N/A
	c/c+ 2.5 mg/kg Hydralazine	N/A	N/A	N/A	Neg	Neg	Neg	Neg

B.

		1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	8 weeks
Masson's Trichrome	littermate control	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	c/c+ untreated	Neg	Neg	Neg	Neg	Neg	Neg	N/A
	c/c+ 2.5 mg/kg Hydralazine	N/A	N/A	N/A	Neg	Neg	Neg	Trace Pos

Figure 4.

Analysis of glycogen accumulation, fibrosis, autophagy and apoptosis. **(A)** Periodic Acid-Schiff (PAS) staining of heart sections from *Tsc1c/cSM22cre^{+/-}* mice with or without hydralazine treatment at 2, 5, or 8 weeks of age. **(B)** Masson's trichrome staining of heart sections from *Tsc1c/cSM22cre^{+/-}* mice with or without hydralazine treatment at 2, 5, or 8 weeks of age. **(C)** Analysis of autophagy by p62 immunohistochemistry (IHC) of heart sections from mice at 2, 5, or 8 weeks of age. **(D)** Representative western blot for hamartin, p62, and GAPDH (loading control) accompanied by semi-quantitative densitometric analysis for p62 levels (n = 6). **(E)** Analysis of apoptosis by TUNEL staining, Representative pictures of TUNEL stained heart sections from mice at 2, 5, or 8 weeks of age Scale bar = 50 μ m at 20x magnification; + = staining present, ++ = moderate staining, +++ = strong staining).

A. **B.**

C. **D.**

E. **F.** **Figure 5.**

Phenotypic characterization of *Tsc1*c/*cSMA*cre-ER^{T2}^{+/-} mice. (A) MLPA analysis of genetic recombination of *Tsc1* in 2 month and 3 month old mice of genotypes *Tsc1*c/*wSMA*:cre-ER^{T2}^{+/-} and *Tsc1*c/*cSMA*:cre-ER^{T2}^{+/-} after treatment with Tamoxifen (n = 5). (B) Representative western blot for hamartin, pS6, and COXIV (loading control) accompanied by semi-quantitative densitometric analysis for hamartin and pS6 levels (n = 6). (C) Systemic blood pressures as measured by tail cuff systolic pressure (n = 7, * p < 0.01). (D) Visualization of aortic smooth muscle layers by H&E stain (scale bar = 100 μm at 10x magnification). Vasoconstrictive response as measured after treatment of aortas with 10⁻⁵ M phenylephrine in a vessel bath (n = 9; * p = 0.003). (E) Representative western blot for hamartin and COXIV (loading control) accompanied by semi-quantitative densitometric analysis for hamartin levels (n = 6). (F) Heart weight normalized to body weight (n = 6) and right ventricular systolic pressure (n = 6).