Functional Redundancy of SWI/SNF Catalytic Subunits in Maintaining Vascular Endothelial Cells in the Adult Heart

Monte S. Willis, Jonathon W. Homeister, Gary B. Rosson, Yunus Annayev, Darcy Holley, Stephen P. Holly, Victoria J. Madden, Virginia Godfrey, Leslie V. Parise, Scott J. Bultman

Rationale: Mating type switching/sucrose non-fermenting (SWI/SNF) chromatin-remodeling complexes utilize either BRG1 or BRM as a catalytic subunit to alter nucleosome position and regulate gene expression. BRG1 is required for vascular endothelial cell (VEC) development and embryonic survival, whereas BRM is dispensable.

Objective: To circumvent embryonic lethality and study Brg1 function in adult tissues, we used conditional gene targeting. To evaluate possible Brg1-Brm redundancy, we analyzed Brg1 mutant mice on wild-type and Brm-deficient backgrounds.

Methods and Results: The inducible Mx1-Cre driver was used to mutate Brg1 in adult mice. These conditional-null mutants exhibited a tissue-specific phenotype and unanticipated functional compensation between Brg1 and Brm. Brg1 single mutants were healthy and had a normal lifespan, whereas Brg1/Brm double mutants exhibited cardiovascular defects and died within 1 month. BRG1 and BRM were required for the viability of VECs but not other cell types where both genes were also knocked out. The VEC phenotype was most evident in the heart, particularly in the microvasculature of the outer myocardium, and was recapitulated in primary cells ex vivo. VEC death resulted in vascular leakage, cardiac hemorrhage, secondary death of cardiomyocytes due to ischemia, and ventricular dissections.

Conclusions: BRG1-catalyzed SWI/SNF complexes are particularly important in cardiovascular tissues. However, in contrast to embryonic development, in which Brm does not compensate, Brg1 is required in adult VECs only when Brm is also mutated. These results demonstrate for the first time that Brm functionally compensates for Brg1 in vivo and that there are significant changes in the relative importance of BRG1- and BRM-catalyzed SWI/SNF complexes during the development of an essential cell lineage. (Circ Res. 2012;111:e111-e122.)

Key Words: BRG1  ■ BRM  ■ SWI/SNF  ■ cardiac vascular endothelial cell  ■ ventricular dissection  ■ conditional transgenic mouse  ■ endothelial dysfunction  ■ epigenetics  ■ genetics

The Saccharomyces cerevisiae SWI/SNF (mating type switching/sucrose non-fermenting) complex was the first chromatin-remodeling complex to be characterized and consists of 11 subunits with a total molecular mass of 1 to 2 MDa.1,2 SWI/SNF complexes have been evolutionarily conserved although mammals have approximately 21 subunits due to duplication and divergence events that occurred during vertebrate evolution.3 Rather than having a larger-sized complex consisting of 21 subunits, mammals have a number of 9- to 12-subunit complexes with different combinatorial assemblies (up to n=288 in theory).3,4 Subunit composition can change within a cell lineage as it differentiates and is known to vary among different tissues.4,5 However, despite this subunit diversity, all mammalian SWI/SNF complexes identified thus far contain either BRG1 or BRM (brahma-related gene 1 and brahma, also known as SMARCA4 and SMARCA2, respectively) as a catalytic subunit with DNA-dependent ATPase activity.3,4 Each complex also contains several core subunits (also known as BRG1/BRM-associated factors or BAFs) including BAF170, BAF155, and BAF47 (also known as SNF5 or SMARCB1).3,4 SWI/SNF-related complexes are recruited by sequence-specific transcription factors to the promoters of numerous target genes,6–8 where they slide or evict histone octamers in an ATP-dependent manner.9,10 This alters the number and position of nucleosomes near transcriptional start sites to regulate RNA Polymerase II occupancy and transcriptional initiation.10–12 The importance of SWI/SNF complexes in...
null mutations of Brg1, Baf250a/Arid1a, Baf155, and Baf47/Snf5 each confer peri-implantation lethality.4,13 This phenotype is consistent with the OCT4, SOX2, and Nanog pluripotency transcription factors recruiting a specific SWI/SNF subcomplex, called esBAF, to target genes in embryonic stem (ES) cells to facilitate their self-renewal and pluripotency.7,14–16 Null mutations of the BAF60c and Baf60c noncore subunits each result in midgestation lethality due to cardiac defects.17,18 Null mutations of the Baf180 and Baf60c noncore subunits each result in midgestation lethality due to cardiac defects.17,18 Baf250a/Arid1a null heterozygotes and Brg1 conditional mutants also exhibit cardiovascular defects that are lethal at midgestation.19–21 At a mechanistic level, Brg1 genetically interacts with Tbx5,21 and the BAF60c subunit facilitates a physical interaction between Brg1 and the cardiogenic transcription factors TBX5, GATA4, and Nkx2–5.17 Furthermore, a combination of BAF60c plus TBX5 and GATA4 can program noncardiac mesoderm into cardiomyocytes.22,23 Based on these studies, SWI/SNF complexes have emerged as key epigenetic regulators of cardiomyocyte development.22

Cardiomyocyte development is coordinated with vascular development, and Brg1 is also required for vasculogenesis, based on the midgestation lethality of Brg1Tie2-Cre conditional mutants in which Brg1 was mutated in vascular endothelial cells (VECs) during embryogenesis.24–26 The Brg1Tie2-Cre mutant phenotype was not exacerbated by Brm deficiency,25 which is surprising because Brm is expressed at high levels in the developing vasculature and encodes a protein that is 75% identical to Brg1 with similar or identical chromatin-remodeling properties on nucleosome arrays in cell-free systems.27,28 This finding suggests that VECs may rely on the PBAF class of complexes, which utilize only BRG1 as their catalytic subunit, rather than the BAF class that utilizes either BRG1 or BRM.3 This possibility has a precedent as the esBAF subcomplex utilizes BRG1 exclusively as a catalytic subunit.7,14 Alternatively, BRG1 vascular function may occur in the context of BAF complexes, but BRM cannot compensate either because it interacts with different transcription factors and is recruited to different target genes or it has different biochemical properties than BRG1 in vivo.8 Support for this possibility comes from the observation that the two catalytic subunits have antagonistic roles in the differentiation of certain cell lineages.29 Regardless of which possibility proves correct, BRG1 and BRM clearly have distinct functions. In fact, the function of BRM is different than all other SWI/SNF subunits that have been knocked out thus far because Brm–/– mice are viable and fertile instead of being embryonic lethal.4,13,30

Although BRG1-catalyzed SWI/SNF complexes are essential for cardiovascular development and other aspects of embryogenesis,4,13 it is not known whether they maintain cellular homeostasis after development is complete. To address this important issue, it was necessary to circumvent the requirement for Brg1 during embryogenesis; therefore we used the inducible Mx1-Cre driver to mutate Brg1 in several tissues of adult Brg1fl/fl mice. By analyzing these conditional mutants on both wild-type and Brm-deficient backgrounds, we discovered an unprecedented genetic interaction between Brg1 and Brm in cardiovascular homeostasis as described below.

Methods

An expanded Methods section is available in the Online Data Supplement. All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) review board at the University of North Carolina and were performed in accordance with federal guidelines. The Brg1 floxed and Δ floxed alleles and the Brm mutation were genotyped by PCR as previously described.30,31 To induce Mx1-Cre in vivo, mice were injected intraperitoneally with 300 mg of pl-pC (Sigma, St Louis, MO) dissolved in PBS in a volume of 0.2 cc. Mice were injected every other day for a total of 5 treatments. Control mice received the same injection regimen but with PBS only.

Histology was performed by fixing heart and other tissues in 4% paraformaldehyde, embedding in paraffin, and cutting 5-μm sections according to standard procedures. Sections were either stained with hematoxylin and eosin or processed for immunohistochemistry (IHC), using a BRG1 rabbit polyclonal antibody (Upstate/Millipore No. 04–1074) and a platelet endothelial cell adhesion molecule (PECAM)-1 antibody (Upstate/Millipore No. 04–1074) according to the manufacturer’s recommendations. X-Gal staining was performed using standard procedures on 200-μm vibratome slices of heart and other tissues from Rosa26 reporter (R26R, Rosa-los-stop-lox-LacZ) mice carrying the Mx1-Cre transgene.

For electron microscopy, after perfusion with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.15 mol/L sodium phosphate buffer (pH 7.4), heart and other tissues were fixed in the same solution overnight and then postfixed with 1% osmium tetroxide/0.15 mol/L sodium phosphate buffer. Samples were dehydrated with increasing concentrations of ethanol, infiltrated and embedded in LR white resin (Polysciences, Warrington, PA), and 70-nm ultrathin sections were cut with a diamond knife. Sections were mounted on 200-mesh copper grids and stained with 4% aqueous uranyl acetate and Reynolds lead citrate. Sections were observed with a LEO EM910 transmission electron microscope operating at 80 kV (LEO Electron Microscopy, Thornwood, NY) and photographed with a Gatan-Orius SC1000 CCD Digital Camera and Digital Micrograph 3.11.0 (Gatan, Pleasanton, CA).

Primary VECs were isolated and cultured as described with minor modifications.32 Mouse hearts were minced and digested with collagenase solution [0.2 mg/mL type II collagenase ( Worthington, Lakewood, NJ) in Hanks balanced salt solution (BioWhittaker/Lonza, Basel, Switzerland)] for 45 minutes at 37°C to generate single-cell

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**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>BAF</td>
<td>BRG1/BRM-associated factor</td>
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<tr>
<td>BRG1</td>
<td>brahma-related gene 1</td>
</tr>
<tr>
<td>BRM</td>
<td>brahma</td>
</tr>
<tr>
<td>fl</td>
<td>floxed (flanked by loxP sites)</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>LV</td>
<td>left ventricle</td>
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<tr>
<td>LV EDd</td>
<td>LV end-diastolic dimension</td>
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<tr>
<td>LV ESd</td>
<td>LV end-systolic dimension</td>
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<tr>
<td>LV EF</td>
<td>ejection fraction percentage</td>
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<tr>
<td>LV Vol d</td>
<td>LV volume in diastole</td>
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<tr>
<td>LV Vol s</td>
<td>LV volume in systole</td>
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<tr>
<td>Mx1</td>
<td>myxovirus resistance gene 1</td>
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<tr>
<td>PECAM-1</td>
<td>platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>pl-pC</td>
<td>polyniosine-polyctosine</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>mating type switching/sucrose non-fermenting</td>
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<tr>
<td>VEC</td>
<td>vascular endothelial cell</td>
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suspensions. Cells were filtered through cell strainers and washed in base media (DMEM containing 25 mmol/L HEPES, 20% FBS, 100 U/100 mg/mL penicillin-streptomycin, and 2 mmol/L glutamine). The samples were incubated with a biotinylated PECAM-1 antibody (BD Pharmingen, San Diego, CA) for 10 minutes. Streptavidin-coated magnetic beads (Miltenyi Biotech, Auburn, CA) were then added to the samples and incubated for 15 minutes at 4°C while rotating. The immunobead complex was then isolated by passing the samples through magnetic columns (Miltenyi Biotech), washing 3 times, and resuspending in complete culture media (basal media plus 100 mg/mL heparin (Sigma), 100 mg/mL endothelial cell growth stimulant (Biomedical Technologies, Stoughton, MA), and sodium pyruvate (Invitrogen, Carlsbad, CA)). Cells were grown in 10-cm culture dishes pretreated with 0.1% gelatin in complete culture media under standard conditions (5% CO₂ at 37°C), and cultures were refed every other day and could be passaged several times while retaining their morphological appearance and PECAM-1-positive status. To induce Cre expression and mutate Brg1, pl-pC (Sigma) was added to VEC cultures at a final concentration of 100 μg/mL for 3 successive days.

Transsthoracic echocardiography was performed on conscious mice using a VisualSonics Vevo 770 ultrasound biomicroscopy system (VisualSonics, Inc, Toronto, Ontario, Canada) with a 30-MHz 707B scan head as previously described. Two-dimensionally guided M-mode analysis of the left ventricle was performed in a genotype-blinded fashion in the plane of the parasternal long axis at the level of the papillary muscle. Epicardium and endocardium leading edges were used to measure the anterior and posterior wall thickness (IVSTD, IVSTS), posterior wall thickness (PWTD, PWTS), and left ventricular internal diameters (LVEDD, LVESD), posterior wall thickness (IVSTD, IVSTS), posterior wall thickness (PWTD, PWTS), and left ventricular internal diameters (LVEDD, LVESD), left ventricle (LV) volume in diastole (LV VolD) was calculated from the equation \( LV \text{ VolD} = (7/2.4 + LV \text{ ED}) \times LV \text{ ED} \times 1000 \), and LV volume in systole (LV VolS) was calculated from the equation \( LV \text{ VolS} = (7/2.4 + LV \text{ ES}) \times LV \text{ ES} \times 1000 \). Left ventricular systolic function was assessed by ejection fraction (EF), calculated from the equation \( EF\% = (LV \text{ VolD} - LV \text{ VolS}) \times LV \text{ VolD} \times 100 \), and fractional shortening (FS), calculated from the equation \( FS\% = (LV \text{ ED} - LV \text{ ES}) \times LV \text{ ED} \times 100 \). M-mode measurements represent 3 average consecutive cardiac cycles from each mouse.

Results

Functional Compensation of Brg1 and Brm

To investigate the role of Brg1 in adult tissues, we crossed previously characterized Mxl-Cre transgenic mice and Brg1 floxed mice to generate Brg1 conditional null homoyzogotes \((\text{Brg}1^{0/0}, \text{Mxl-Cre}^{+/-})\). Because Mxl-Cre is inducible, Cre is not expressed and the floxed-to-null event was induced by pI-pC as expected.

We administered either pl-pC or PBS to 3 categories of mice at 5 to 7 weeks of age and monitored their health and survival. The first category consisted of 40 control mice divided into 4 subcategories: 1, Brg1 \(^{0/0}\) mice lacking the Mxl-Cre transgene that were treated with pl-pC (n=10); 2, Brg1 \(^{0/0}\) mice lacking the Mxl-Cre transgene on a Brm\(^{-/-}\) background that were treated with pl-pC (n=10); 3, Brg1 conditional null homoyzogotes (Brg1\(^{0/0}\), Mxl-Cre\(^{-/-}\)) that were treated with PBS (n=10); 4, Brg1 conditional null homoyzogotes on a Brm\(^{-/-}\) background that were treated with pl-pC (n=10).

Figure 1. Rapid death of Brg1/Brm double mutants in the Mxl-Cre model after pl-pC treatment. A, Image of gel showing the Brg1 \(\Delta f1\) PCR product from the following tissues. Lanes 1 to 3, tail tissue from control mice that were constitutively \(\Delta f1\) (generated using a germline Cre driver) or \(\Delta f1\)++; lanes 4 to 7, liver (L) and heart (Ht) tissue from untreated (+) and pl-pC-treated (+) Brg1\(^{0/0}\), Mxl-Cre\(^{-/-}\) mice; lane 8, no template control (NTC). B, Kaplan-Meier survival curve of mice after administration of either pl-pC or PBS (denoted by asterisks on days 0, 2, 4, 6, and 8). Controls: Brg1\(^{0/0}\) mice lacking the Mxl-Cre transgene that were treated with pl-pC (n=10); Brg1\(^{0/0}\) mice lacking the Mxl-Cre transgene on a Brm\(^{-/-}\) background that were treated with pl-pC (n=10); Brg1 conditional null homoyzogotes (Brg1\(^{0/0}\), Mxl-Cre\(^{-/-}\)) that were treated with PBS (n=10); Brg1 conditional null homoyzogotes on a Brm\(^{-/-}\) background that were treated with pl-pC (n=10). Single mutants: Brg1 conditional null homoyzogotes on a wild-type (n=15) or Brm\(^{-/-}\) (n=10) background that were treated with pl-pC. Double mutants: Brg1 conditional null homoyzogotes on a Brm\(^{-/-}\) background that were treated with pl-pC (n=15).
were treated with pl-pC (n=15). These mice (herein referred to as Brg1\(^{Mx1-Cre}/Brm^{−/−}\) double mutants) either died or had to be euthanized within one month of pl-pC treatment due to their moribund condition (eg, hunched posture, lethargy, labored breathing, and reduced response to direct contact) (Figure 1B). This phenotype was 100% penetrant as all 15 Brg1\(^{Mx1-Cre}/Brm^{−/−}\) double mutants became moribund. These results indicate that the Brg1 and Brm catalytic subunits functionally compensate in the Mx1-Cre model.

**Brg1\(^{Mx1-Cre}/Brm^{−/−}\) Double-Mutant Mice Exhibit Cardiac Defects and Ventricular Dissections**

To begin to understand the cause of death, Brg1\(^{Mx1-Cre}/Brm^{−/−}\) double mutant mice were euthanized 20 to 30 days after pl-pC treatment, while they were moribund. Pleural effusions were present in nearly all mice, characteristically being a clear to blood-tinged fluid. Gross examination of these mice also revealed a kidney phenotype consistent with medullary congestion (Online Figure IA). Other organ systems, including the liver and gastrointestinal tract, were unremarkable on gross inspection and indistinguishable from control mice.

Since pleural effusions can be associated with cardiac defects such as heart failure, we first analyzed the hearts and lungs from Brg1\(^{Mx1-Cre}/Brm^{−/−}\) double mutants that had been treated 20 to 30 days earlier with pl-pC. Unlike control hearts (Figure 2A), numerous extravascular erythrocytes were identified within the myocardial interstitium of the atria and throughout the left and right ventricular myocardium from the apex to the base of double-mutant hearts (Figure 2B). This hemorrhage was particularly evident in the outer myocardium (representative sections shown in Figure 2B), whereas the endocardium and valve endothelium appeared normal at this level of magnification. Whereas the structural integrity of the aortic root, pulmonary artery, and coronary arteries remained intact (data not shown), we observed the subepicardial microvasculature as the primary source of the multifocal hemorrhages into the interstitium (Figure 2B).

**Figure 2. Histopathologic and 2D echocardiographic findings in Brg1/Brm double-mutant hearts.** Representative images of hematoxylin and eosin-stained heart sections from a control (A) and double-mutant (B) mice 25 days after pl-pC treatment. Animal numbers are indicated. The double-mutant hearts have diffuse and focal interstitial hemorrhage associated with myocyte necrosis (arrows) and calcification (arrowheads), particularly in the outer myocardium, which may result in myocardial dissection (C). Asterisk denotes artifactual disruption of ventricular wall due to fixative perfusion needle insertion. C, Images extracted from 2D video outlining the anterior wall (top) and posterior wall (bottom) cardiac dissections identified after pl-pC treatment (see Online Table I). The 2D video files are included (indicated in 2D image itself) in the Online Data Supplement. LA indicates left atria. Asterisk denotes area of dissection on day before death; 56% of mice had detectable dissections on mean day 38.8±9.9 (detailed in Online Table I).
and cardiomyocyte hypertrophy (Online Figure IIIA). Quantification of cardiomyocyte cross-sectional areas revealed a 1.6-fold increase in double mutants compared with controls (Online Figure IIIB). In contrast to the heart, we did not detect any histopathologic abnormalities or abnormal cell death in the lungs or kidneys from Brg1/Mx1-Cre/Brm/H11002/H11002 double-mutant mice (Online Figure I).

To more carefully define the cardiac phenotype and provide insight into the kinetics of disease progression, we conducted a longitudinal echocardiographic study. After pI-pC treatment, we performed daily transthoracic echocardiography on 9 conscious Brg1/Mx1-Cre/Brm/H11002/H11002 double mutants until each animal died or was euthanized due to humane endpoints. We also longitudinally analyzed 6 sibling-matched controls: 3 Brg1/Mx1-Cre/Brm/H11002/H11002 double mutants that were not treated with pI-pC and 3 Brg1/fl/fl, Brm/H11002/H11002 mice lacking the Mx1-Cre transgene that were treated with pI-pC. On the first day of echocardiography (on the day after the final pI-pC treatment corresponding to day 9 in Figure 1B), we did not detect any significant differences for 15 quantitative measurements, referred to as “baseline,” between the treated double mutants and the 2 control groups (Figure 3A and the Table).

On subsequent echocardiographic studies, we detected an increased thickness of the anterior or posterior left ventricular wall of treated double mutants between 7 and 21 days later (median of 14 days) (Online Figure IV and Online Table I). In contrast, ventricular wall measurements did not change over time in any of the control mice (Online Figure IV). The left ventricular wall of the treated Brg1/Mx1-Cre/Brm/H11002/H11002 double mutant mice became progressively thicker over time (Online Figure IV). On the final echocardiogram, acquired 1 day before their death [(ie, premortem) median of 25 days after first echocardiogram (Online Table I) or 34 days after their first pI-pC treatment], the left ventricular wall of the Brg1/Mx1-Cre/Brm/H11002/H11002 double mutant mice was nearly twice that of controls (Figure 3A and the Table). For example, the anterior wall thickness in diastole was 1.84 ± 0.11 mm in treated double mutants compared with 1.04 ± 0.04 mm and 1.06 ± 0.02 mm in the 2 control groups (Figure 3A and the Table). In addition to increased ventricular wall thickness, treated double mutants had a significant 2.4-fold increase in their left ventricular mass/body weight ratio and a 42% decrease in heart rate the day before their death (Figure 3A and the Table).

In addition to the changes identified in wall thickness, a number of additional progressive cardiac defects were seen in
Treated Non-Tg and Untreated Tg Littermates

<table>
<thead>
<tr>
<th></th>
<th>Baseline Untreated Tg Controls (n=3)</th>
<th>Final Untreated Tg Controls (n=3)</th>
<th>Baseline Treated Non-Tg Controls (n=3)</th>
<th>Final Treated Non-Tg Controls (n=3)</th>
<th>Baseline Treated Double Mutants (n=9)</th>
<th>One-Day Premortem Treated Double Mutants (n=9)</th>
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<tr>
<td>Body weight, g</td>
<td>19.9 ± 1.2</td>
<td>25.5 ± 1.1†</td>
<td>20.4 ± 1.7</td>
<td>25.1 ± 1.2‡</td>
<td>18.1 ± 0.6</td>
<td>17.8 ± 0.6†</td>
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<td>HR, bpm</td>
<td>739 ± 5</td>
<td>747 ± 8</td>
<td>689 ± 17</td>
<td>757 ± 19</td>
<td>625 ± 41</td>
<td>438 ± 59§</td>
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<td>IVS d</td>
<td>0.96 ± 0.01</td>
<td>1.06 ± 0.02</td>
<td>0.94 ± 0.02</td>
<td>1.04 ± 0.01</td>
<td>1.04 ± 0.04</td>
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<td>2.52 ± 0.10</td>
<td>3.0 ± 0.19</td>
<td>2.71 ± 0.16</td>
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<td>2.45 ± 0.07</td>
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<td>0.91 ± 0.02</td>
<td>0.98 ± 0.02</td>
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<td>1.45 ± 0.03</td>
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<td>1.56 ± 0.05</td>
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<td>LV Vol d</td>
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<td>27.7 ± 3.8</td>
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<td>3.4 ± 0.5</td>
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<td>EF%</td>
<td>87.3 ± 1.1</td>
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<td>173.5 ± 15.9§</td>
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<td>LV mass/BW</td>
<td>3.88 ± 0.18</td>
<td>4.01 ± 0.02</td>
<td>3.83 ± 0.09</td>
<td>4.3 ± 0.2</td>
<td>4.6 ± 0.5</td>
<td>9.92 ± 0.64§</td>
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BW indicates body weight; LV, left ventricle; HR, heart rate; LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension; LV EF% = [(LV Vol d − LV Vol s)/LV Vol d] × 100%; LV Vol d, LV volume in diastole; LV Vol s, LV volume in systole. Baseline values acquired 1 day after the final pI-pC treatment; 1-day premortem values were acquired 1 day before the death of each treated double mutant; final values of controls acquired at the end of the experiment (1-day premortem of longest surviving treated double mutant); all values are mean ± SE, with significant differences indicated, based on a 1-way ANOVA followed by a Holm-Sidak pairwise comparison.

*P<0.001 versus all other groups.
†P<0.05 versus final untreated Tg, final treated non-Tg controls.
‡P<0.01 versus baseline.
§P<0.05 versus all other groups.
||P<0.05 versus final untreated Tg, final treated non-Tg controls, and baseline treated double mutants.

Cardiac Vascular Endothelial Cell Death Is the Primary Defect
to identify the primary cellular defect in the heart of Brg1Mx1-Cre/Brm−/− double mutants, it was necessary to determine where BRG1 is normally expressed in the heart.

Figure 4. Expression analysis of BRG1, Mx1-Cre, and PECAM-1 in control and Brg1/Brm double-mutant cardiac VECs. A, IHC demonstrating nuclear BRG1 staining in VECs (black arrow) and cardiomyocytes (white arrow) from a control mouse heart. B, X-Gal staining of a representative heart section from pl-pC-treated R26R mice carrying the Mx1-Cre transgene. Strong staining (blue) occurred in VECs such as those enclosed by dashed circle, but staining was absent in cardiomyocytes as exemplified by the arrows. C, No X-Gal staining occurred in heart sections from the same R26R mice carrying the Mx1-Cre transgene when they were not treated with pl-pC. D and E, IHC demonstrating PECAM-1 staining of VECs in heart sections from control (D) and Brg1/Brm double-mutant (E) mice. A continuous staining of VECs was observed within control vessels (D), whereas staining was discontinuous in double-mutant vessels (E).
and where Mx1-Cre inactivates the floxed allele. Therefore, we performed IHC and discovered that BRG1 is normally expressed in both VECs and cardiomyocytes throughout the heart in a nuclear distribution (Figure 4A). Because Mx1-Cre activity has not been characterized in the heart, we crossed the Mx1-Cre transgene onto the R26R background and performed X-Gal staining on heart sections from these mice. Strong nuclear Cre activity was induced by pI-pC in VECs throughout the heart but not in the cardiomyocytes (Figure 4B). No Cre activity was detected in either cell type in control mice that either lacked the Mx1-Cre transgene or that were not treated with pI-pC as expected (Figure 4C). Therefore, although BRG1 is expressed in both VECs and cardiomyocytes within the heart, it is only mutated in VECs suggesting this is the primary cell type responsible for the cardiovascular phenotype in double mutants.

We first investigated cardiac vascular endothelial cells by IHC using PECAM-1 (also known as CD31). A strong, continuous staining in vessels was identified in control mice (Figure 4D). In contrast, the cardiac vasculature of Brg1<sup>Mx1-Cre</sup> Brm<sup>−/−</sup> double mutants had a discontinuous staining in moribund mice at 20 to 30 days after pI-pC administration (Figure 4E). This result suggested that a subset of endothelial cells within double-mutant hearts either no longer expressed PECAM-1, or were dead/absent. To distinguish between these possibilities, we performed transmission electron microscopy at 25 days after pI-pC treatment. Two distinct phenotypes were seen in these cells. The first distinguishing phenotype of the pI-pC–treated double mutants was the presence of dying endothelial cells attached to live VECs in the capillaries (Figure 5A). Notably, the gap junctions were intact between the dying and live cells (Figure 5A and 5B), as they were between viable cells throughout the treated Brg1<sup>Mx1-Cre</sup> Brm<sup>−/−</sup> mutants and control mice. The second distinguishing feature of the pI-pC–treated double mutants is that they had variably thicker VECs with larger vesicles, compared with controls, including swollen mitochondria and rough endoplasmic reticulum (Figure 5C). Consistent with the histopathology seen in Figure 2, capillaries with breached endothelium were identified in areas of hemorrhage and clot (data not shown). Many of these microvascular remnants had immune cell infiltration, platelets, and proteinaceous material consistent with fibrin clot formation. As expected, there was a correlation between the presence of injured and necrotic cardiomyocytes and sites of hemorrhage.

Consistent with VECs being the primary cell type–affected, it was the cell type in which we first observed a cardiac phenotype in Brg1<sup>Mx1-Cre</sup> Brm<sup>−/−</sup> double mutants at
earlier time points after the induction of the Brg1 mutation. For example, when we analyzed Brg1\textsuperscript{Mx1-Cre}/Brm\textsuperscript{−/−} double mutants 14 days after the first dose of pl-pC, while they still appeared healthy and before any hemorrhage could be detected in hematoxylin and eosin–stained sections, the cardiomyocytes appeared normal, whereas the VECs were uniformly thickened with increased vacuolization (Figure 6B through 6D) compared with VECs found in control mice (Figure 6A). At this 14-day after the pl-pC time point, however, we did not identify any endothelial cells that were necrotic or absent. This phenotype was consistent with the thickened endothelial cells seen at the later time point (days 20–30) shown in Figure 5A and 5B. Since we were unable to identify any necrotic or missing endothelial cells at day 14 but found them readily at days 20 to 30, this supports a model in which mutation of Brg1 and Brm in the VEC is the primary event, which leads to VECs undergoing physical changes at or before day 14 and VEC cell death by day 25. Examples of vesicles of increased size (daggers) are found in most capillary sections. Data are from animals 2516 (A) and 2520 (B through D). All panels correspond to the epicardium.

To evaluate VEC survival/lethality in more detail, we used PECAM-1 magnetic bead sorting to isolate and culture primary VECs from the hearts of conditional mutant and control mice. These primary VECs had a characteristic cobblestone appearance and were PECAM-1–positive ex vivo, and their identity was confirmed by RT-PCR, since they were positive for the VEC markers VE-cadherin and FLK1/VEGFR2 (Online Figure VI). These same cells were negative for α-smooth muscle actin and FLT4/VEGFR3, which are pericyte and lymphatic endothelial cell markers, respectively (Online Figure VI). Cultures were established from Brg1\textsuperscript{fl/fl}; Mx1-Cre\textsuperscript{+/−}; Brm\textsuperscript{+/−} mice on a Brm\textsuperscript{−/−} background and nontransgenic controls (Brg1\textsuperscript{fl/fl} mice lacking the Mx1-Cre transgene on a Brm\textsuperscript{−/−} background). However, none of the mice used in these experiments were treated with pl-pC so the VECs were functional for Brg1 at the time of their derivation. Cells were then treated with pl-pC for 3 successive days, and cell number was monitored over a 2-week period. Relative to day 0 (immediately before the first pl-pC treatment), the number of VECs from Brg1\textsuperscript{fl/fl}; Mx1-Cre\textsuperscript{+/−}; Brm\textsuperscript{−/−} mice (double mutants) dropped to 70% at day 7 and then to 10% at day 14 (Figure 7A). In contrast, VECs from the same mice that were not treated with pl-pC ex vivo survived and proliferated increasing to 180% and 350% at days 7 and 14, respectively (Figure 7A). When a BRG1 cDNA expression vector was introduced into these cells, it rescued the drop in cell number after pl-pC–induced deletion of the endogenous Brg1 locus (Figure 7A). As a final control, the number of VECs from nontransgenic control mice increased to a similar extent regardless of whether or not they were treated with pl-pC as expected (Figure 7A). These results demonstrate that the VEC phenotype is cell autonomous and occurs under normoxic conditions.

After pl-pC treatment, the observed decrease in the number of double-mutant cells could be due to decreased cell proliferation and/or increased cell death. To distinguish between these possibilities, we performed BrdU incorporation, Annexin V, and propidium iodide assays. We did not observe a significant difference between double mutants and nontrans-
genetic controls for BrdU incorporation (Figure 7B) but did observe a significant difference for Annexin V and PI staining (Figure 7B). These findings indicate that the drop in cell number ex vivo arises because of increased apoptosis and cell death rather than decreased cell proliferation, and this is consistent with the cell death observed in vivo. Also similar to the in vivo phenotype, the cell death that occurred ex vivo resulted in permeability defects based on permeability assays that measured the movement of FITC-dextran macromolecules through a monolayer of VECs cultured on transwell inserts. The barrier function of double-mutant cells was intact 1 day after pl-pC treatment but was compromised 7 days after pl-pC treatment (Figure 7C). To determine whether this phenotype is associated with a perturbation in canonical SWI/SNF function, we analyzed the expression of genes that are expressed in endothelial cells and are regulated by BRG1-catalyzed SWI/SNF-related complexes. Accordingly, RT-qPCR showed a significant decrease in the mRNA levels of Cd44 and Pitx2 in double-mutant cells. For this and the other assays, the phenotypes observed in Brg1fl/fl; Mx1-Cre+/-; Brm-/- primary VECs were dependent on pl-pC treatment as expected (Figure 7A through 7D).

Consequences of Vascular Permeability Defects
The occurrence of vascular permeability defects in Brg1fl/fl; Mx1-Cre+/-; Brm-/- mutants might be expected to result in increased platelet activation and clot formation. Indeed, ELISA assays demonstrated that plasma samples from Brg1fl/fl; Mx1-Cre+/-; Brm-/- mutant mice 30 days after their first pl-pC treatment had elevated levels of platelet activation factor (PAF) and thrombin compared with controls (Online Figure VIIA and B). Platelets isolated from the double-mutant mice were less reactive to collagen in aggregation assays (Online Figure VIID), which is consistent with a previous exposure to collagen as a result of compromised vessels. Platelets are consumed during the clotting process so thrombocytopenia.
might also be expected, and complete blood counts showed that Brg1\textsuperscript{Mx1-Cre/Brm\textsuperscript{−/−}} mutant mice had lower numbers of platelets 30 days after the first pi-pC treatment compared with controls (Online Figure VII). Finally, one might also expect VEGF levels to be increased in an attempt to compensate for the loss of VECs, and ELISA assays demonstrated that this was the case (Online Figure VII). Taken together, these secondary effects are consistent with severe vascular permeability defects.

**Discussion**

Because SWI/SNF complexes are essential for embryonic development, we do not know whether they are required for cellular homeostasis in adults. To address this issue, we utilized the inducible Mx1-Cre driver to mutate the Brg1 catalytic subunit in various tissues of adult mice. These conditional mutants exhibited extensive cell death in cardiac tissue but not in other cell types that were also mutated such as the hematopoietic lineages and hepatocytes. The tissue specificity of this cardiovascular phenotype is consistent with previous studies demonstrating that BRG1-catalyzed SWI/SNF complexes play a particularly important role in cardiovascular development in utero,\textsuperscript{17–21,24–26} whereas our data clearly demonstrate that BRM does not compensate for BRG1 in either cardiomyocytes or VECs during embryonic development,\textsuperscript{20,21,25} whereas our data clearly demonstrate that BRM does not compensate in VECs of adults. This difference suggests that only BRG1-catalyzed complexes function in the establishment of VEC lineages during embryogenesis, whereas both BRG1- and BRM-catalyzed complexes function in the maintenance of differentiated VECs in the adult.

The Mx1-Cre driver mutated Brg1 in VECs within the heart but not in cardiomyocytes. Consequently, VEC death was the primary defect, which resulted in vascular leakage, hemorrhage, ischemia, and cardiomyocyte death as a secondary effect. The VEC phenotype was most evident in the microvasculature, which is relatively fragile because it lacks the structural support that larger vessels receive from surrounding smooth muscle and adventitia. Furthermore, the hemorrhages were almost entirely confined to the heart, somewhat favoring the epicardium more than the endocardium, despite the fact that the Mx1-Cre driver targets VECs throughout the body. This organ and regional specificity may be attributed to the cardiac microvasculature being exposed to the physical forces associated with heartbeat compared with other organs. In support of this idea, we observed ultramicroscopic changes in double-mutant tongues, which are also subjected to physical forces in rodents due to their licking and grooming behavior (Online Figure VIII). Notably, the double mutants had a marked increase in the number and size of vesiculo-vacuolar organelles, which are associated with enhanced endothelial cell permeability (Online Figure VIII).\textsuperscript{41} We would expect to observe hemorrhages in other organs if the double mutants did not die of their cardiovascular defects and survived longer.

Vascular dissections have been studied most prominently in the aorta. When vascular dissections extend into the heart itself, there is a characteristic decrease in contractility, as seen in the present study. Recent studies have shown that an underlying cause of vascular dissections is the induction of apoptosis of smooth muscle cells in the aortic artery, which leads to the degradation of the media.\textsuperscript{42} Recent clinical and basic research demonstrates that there is an inflammatory component to these dissections, which may initiate apoptosis.\textsuperscript{43} The present study links endothelial cell death with cardiac dissections, which are generally extensions of aortic root dissections in humans. To our knowledge, Brg1 and Brm are the first genes shown to protect against cardiac dissections. The cardiac VEC death observed in vivo was recapitulated in primary cells ex vivo. This finding demonstrates that VEC death due to loss of Brg1/Brm is cell autonomous and occurs under normoxic conditions (ie, VEC death was not secondary to vascular leakage and ischemia).

In a previous study, we demonstrated that Brg1\textsuperscript{+/−} mice developed mammary tumors, whereas Brg1\textsuperscript{+/−}, Brm\textsuperscript{−/−} mice developed mammary and VEC (ie, hemangiosarcoma) tumors.\textsuperscript{44} This difference in tumor spectrum suggests that BRG1/BRM redundancy affects VEC homeostasis with respect to tumorigenesis in addition to cell viability. The finding that BRG1/BRM redundancy protects against oncogenic transformation instead of cell death in these mice is likely due to BRG1 dosage (+/− versus −/−) and the accumulation of mutations in other genes that protect against cell death. Because mutations of genes encoding SWI/SNF subunits are common in certain types of human primary tumors,\textsuperscript{45} it will be interesting to evaluate BRG1 and BRM for point mutations when human hemangiosarcomas undergo deep sequencing.

Finally, a negative result from this study has important mechanistic implications. Snf5\textsuperscript{Mx1-Cre} mutants exhibit a hematopoietic phenotype consistent with a hematopoietic stem cell defect,\textsuperscript{46} but Brg1\textsuperscript{Mx1-Cre/Brm\textsuperscript{−/−}} double mutants did not show this phenotype. Unlike the Snf5\textsuperscript{Mx1-Cre} mutants, we observed normal hematopoietic cellularity within bone marrow in hematoxylin and eosin-stained femur sections from Brg1\textsuperscript{Mx1-Cre/Brm\textsuperscript{−/−}} double mutants (Online Figure IX). We also performed flow cytometry and observed normal numbers of hematopoietic stem cells, common myeloid progenitors, and common lymphoid progenitors (data not shown). This finding suggests that hematopoietic stem cells require SWI/SNF complexes but in a noncanonical manner that is dependent on Snf5 but is independent of either catalytic subunit. It is surprising because SWI/SNF function is usually equated with the BRG1 or BRM DNA-dependent ATPase activity that remodels nucleosomes. Essentially all Snf5 copurifies with SWI/SNF complexes, and it is not required for complex stability.\textsuperscript{47,48} Furthermore, Snf5 protects against cancer in a BRG1-dependent manner,\textsuperscript{49,50} which suggests that the mechanism of SWI/SNF action is fundamentally different during early hematopoiesis. It is not clear how SNF5 and the other BAF subunits contribute to SWI/SNF function at the biochemical level in cancer or during development, but they probably do more than simply serve as scaffolding factors that stabilize complexes or enhance canonical catalytic activity as demonstrated previously.\textsuperscript{28} These subunits are known to participate in the recruitment and DNA binding of complexes to downstream target genes, which is usually viewed in an
ATPase-centric manner, but our results suggest that they also have crucial biochemical activities yet to be discovered. This idea is supported by a recent report of BAF250/ARID1 ubiquitination activity.51

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Disclosures

None.

References

Novelty and Significance

What Is Known?
- SWI/SNF chromatin-remodeling complexes are important for the development of cardiomyocytes and vascular endothelial cells during mammalian embryogenesis.
- The BRG1 catalytic subunit is required for vascular endothelial cell development, whereas the closely related alternative catalytic subunit, BRM, is dispensable.
- BRM does not compensate for BRG1, based on the observation that a Brg1 conditional mutation in mouse vascular endothelial cells is not exacerbated by Brm deficiency.

What New Information Does This Article Contribute?
- In contrast to developing vascular endothelial cells, genetic experiments showed that BRM does compensate for the loss of BRG1 in vascular endothelial cells of adult mice.
- The requirement for BRG1 and BRM in vascular endothelial cells of adult mice is most pronounced in the heart, particularly in the epicardium, which may be due to mechanical-physical stresses associated with heartbeat.
- Vascular endothelial cell death in the heart results in vascular leakage, ischemia, cardiomyocyte death, ventricular dissections, and lethality of Brg1/Brm double-mutant mice.

Based on gene-targeting experiments in mice, BRG1-catalyzed SWI/SNF complexes are required for many aspects of embryogenesis including cardiovascular development. However, because Brg1 mutants exhibit embryonic lethality, little is known about the role of BRG1 and SWI/SNF in the homeostasis of cells and tissues from adult mice. To circumvent this limitation, we used an inducible gene targeting approach to knockout Brg1 in various tissues of adult mice. These conditional mutant mice had a normal lifespan on a wild-type background but died within 1 month on a Brm-deficient background. Analysis of these double-mutant mice revealed a tissue-specific effect restricted to vascular endothelial cells, primarily within the heart, that was recapitulated using an ex vivo culture system. Vascular endothelial cell death in double mutants led to vascular leakage, ischemia, cardiomyocyte death, and ventricular dissections. These results demonstrate that Brm functionally compensates for Brg1 in vivo and that there are significant changes in the relative importance of BRG1- and BRM-catalyzed SWI/SNF complexes during the development of an essential cell lineage.
Functional Redundancy of SWI/SNF Catalytic Subunits in Maintaining Vascular Endothelial Cells in the Adult Heart
Monte S. Willis, Jonathon W. Homeister, Gary B. Rosson, Yunus Annayev, Darcy Holley, Stephen P. Holly, Victoria J. Madden, Virginia Godfrey, Leslie V. Parise and Scott J. Bultman

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**Supplemental Material**

Supplemental Methods, References for Supplemental Methods, Online Figures (n = 9), Online Table (n=1)

**Supplemental Methods**

*Mice, genotyping, and Mx1-Cre induction*

All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) review board at the University of North Carolina and were performed in accordance with federal guidelines. The *Brg1* floxed and Δfloxed alleles and the *Brm* mutation were genotyped by PCR as previously described.\(^1,2\) To induce *Mx1-Cre* in vivo, mice were injected intraperitoneally with 300 mg of pl-pC (Sigma, St Louis, MO, USA) dissolved in PBS in a volume of 0.1 cc. Mice were injected every other day for a total of 5 treatments. Control mice received the same injection regimen but with PBS only.

**Histology and immunohistochemistry**

Following perfusion with 4% paraformaldehyde according to standard procedures, heart and other tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and 5-µm sections were cut according to standard procedures. Sections were either stained with H&E or processed for IHC using a BRG1 rabbit polyclonal antibody (Upstate/Millipore #07-478, Temecula, CA, USA), a PECAM-1 antibody (Upstate/Millipore #04-1074, Temecula, CA, USA), or for TUNEL assays (Chemicon/Millipore, Temecula, CA, USA) according to the manufacturer’s recommendations. The TUNEL positive control was heart sections treated with DNase I. X-Gal staining was performed following standard procedures on 200-µm vibratome slices of heart and other tissues from Rosa26 reporter (R26R, *Rosa-lox-stop-lox-LacZ*) mice carrying the *Mx1-Cre* transgene.

**Transmission electron microscopy**

Following perfusion with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.15 M sodium phosphate buffer (pH 7.4), heart and other tissues were fixed in the same solution overnight and then post-fixed with 1% osmium tetroxide/0.15M sodium phosphate buffer. Samples were dehydrated with increasing concentrations of ethanol, infiltrated and embedded in Polybed 812 epoxy resin (Polysciences, Warrington, PA, USA). One micron sections were prepared to select representative areas by light microscopy, and 70 nm ultrathin sections were cut with a diamond knife. Sections were mounted on 200 mesh copper grids and staining with 4% aqueous uranyl acetate and Reynolds’ lead citrate. Sections were observed with a LEO EM910 transmission electron microscope operating at 80 kV (LEO Electron Microscopy, Thornwood, NY, USA) and photographed with a Gatan Orius SC1000 CCD Digital Camera and Digital Micrograph 3.11.0 (Gatan, Pleasanton, CA, USA).

**Isolation and culture of primary vascular endothelial cells**

Primary VECs were isolated and cultured as described with minor modifications.\(^3\) Briefly, mouse hearts were minced and digested with collagenase solution [0.2 mg/mL type I collagenase (Worthington, Lakewood, NJ, USA) in Hank’s Balanced Salt solution (Biowhittaker/Lonza, Basel, Switzerland)] for 45 min at 37°C to generate single-cell suspensions. Cells were filtered through a 70-micron cell strainer and washed in base media (DMEM containing 25 mM HEPES, 20% FBS, 100U/100mg/mL penicillin-streptomycin, and 2 mM glutamine). The samples were
incubated with a biotinylated PECAM-1 antibody (BD Pharmingen, San Diego, CA, USA) for 30 min at 4°C while rotating. Streptavidin-coated magnetic beads (Miltenyi Biotech, Auburn, CA, USA) were then added to the samples and incubated for 15 min at room temperature while rotating. With the tubes placed on a magnetic separator, the immunobeads were washed 5 times by aspirating the supernatant and adding fresh base media. The immunobead-bound cells were then resuspended in complete culture media [basal media plus 100 mg/mL heparin (Sigma), 100 mg/mL endothelial cell growth stimulant (Biomedical Technologies, Stoughton, MA, USA), and sodium pyruvate (Invitrogen, Carlsbad, CA, USA)]. To minimize the number of contaminating fibroblasts, two 30 min pre-plating steps were performed to allow the quickly adhering fibroblasts to attach, followed by the transfer of the non-adhered VECs to T75 culture flasks where they were grown in complete culture media under standard conditions (5% CO₂ at 37°C). Cultures were re-fed every other day and could be passaged up to 4 times while retaining their morphological appearance and PECAM-1-positive status. To induce Cre expression and mutate Brg1, 100 mg/mL of pl-pC (Sigma) was added to VEC cultures at the second or third passage for 3 successive days.

ELISAs and other assays
Several assays were performed using a plate reader. For ELISAs, blood was drawn in the presence of sodium citrate (1:10 v/v) and plasma was prepared and analyzed for several factors using the following kits: platelet activating factor (PAF) (Eiab #E0526m), thrombin (Eiab #E1878m), and vascular endothelial growth factor (VEGF-A) (R&D Systems #MMV00). Apoptosis and necrosis was determined in cardiac VEC cultures using annexin V-FITC and propidium iodide (PI) kit (BD #600300). Cell proliferation of these cells was assessed using a BrdU kit (Millipore/Chemicon #7250). Permeability assays were performed by growing these cells on transwell inserts and incubating them in the presence of FITC-dextran for 15 min as described in Bubik et al. (2012). J. Molecular and Cellular Cardiology 52, 196-205.

RT-PCR
RNA was prepared using Trizol reagent (Invitrogen) and reverse transcribed using random hexamers and SuperScript RT II RT (Invitrogen) according to standard procedures. For end-stage PCR, the following primers were used: VE-cadherin, 5'-tcaacgcatcgtgccccagagat-3' and 5'-cagatattttacaacgactgt-3'; VEGFR2/FLK1, 5'-gccccgtctggttctcactac-3' and 5'caacagcatgccccattctg-3'; a smooth muscle actin, 5'-cagcatggatgcatacactac-3' and actctagcttgagctcagctg-3'. For qPCR, reactions were amplified using SYBR Green (ABI) or TaqMan assays on an ABI7300 instrument. The following primers were used for qPCR: Pitx2, 5'-cgtgtaatgctccttcacaa-3' and 5'-ctggcccttatctttcttat-3' from Curtis and Griffin (2012) Mol Cell Biol PMID: 22290435; Cd44, 5'-catccacagcaagacagtc-3' and 5'-ttgtgctccaccttcttgct-3') from Cheng et al. (2006) Genes Dev. PMID: 16818603; Gapdh, TaqMan assay (#4331182). Relative expression levels were normalized to Gapdh and determined using the ΔΔAct method.

Echocardiography
Trans-thoracic echocardiography was performed on conscious mice using a VisualSonics Vevo 770 ultrasound biomicroscopy system (VisualSonics, Inc., Toronto, Ontario) with a 30-MHz 707B scan head as previously described. Two-dimensional guided M-mode analysis of the left ventricle was performed in a genotype-blinded fashion in the plane of the parasternal long-axis at the level of the papillary muscle. Epi- and endo- cardium leading edges were used to measure the anterior and posterior wall thickness (IVSTD, IVSTS), posterior wall thickness (PWTD, PWTS), and left ventricular internal diameters (LVEDD, LVESD). LV volume in diastole (LV VolD) was calculated from the equation LV VolD = (7/2.4 + LVEDD) x LVEDD³ x 1000, and LV volume in systole (LV VolS) was calculated from the
equation LV VolS = (7/2.4 + LVESD) x LVESD³ x 1000. Left ventricular systolic function was assessed by ejection fraction (EF), calculated from the equation EF % = (LV VolD-LV VolS)/LV VolD x 100, and fractional shortening (FS), calculated from the equation FS % = (LVEDD-LVESD)/LVEDD x 100. M-mode measurements represent 3 average consecutive cardiac cycles from each mouse.

References for supplemental methods

5. Willis MS, Ike C, Li L, Wang DZ, Glass DJ, Patterson C. Muscle ring finger 1, but not muscle ring finger 2, regulates cardiac hypertrophy in vivo. Circ Res. 2007;100:456-459
Online Figure I. H&E-stained analysis of kidney and lung tissue from control and Brg1/Brm double-mutant mice 25 days after the first pl-pC treatment. (A) Brg1/Brm treated double-mutant kidneys had vascular congestion of the outer renal medulla, but no significant histopathologic abnormalities. Shown are sections from control 09-160-2 and double mutant 2236. (B) No histopathologic pulmonary abnormalities were detected in Brg1/Brm treated double-mutant lungs. Shown are sections from control 09-160-1 and double mutant 09-155-1.
Online Figure II. H&E and Masson’s Trichrome analysis of hearts from control mice and Brg1/Brm double-mutant mice. H&E (top row) and Masson’s Trichrome (bottom row) stained sections of control and Brg1/Brm double-mutant hearts 25 days after the first pl-pC treatment (right two columns). Control hearts did not have any evidence of myocyte necrosis, inflammation, or collagen infiltration. Brg1/Brm double-mutant hearts have ongoing myocyte necrosis and loss associated with a mixed inflammatory cell infiltrate (light blue, top row third column), myocyte calcification (dark blue, top row fourth column), and early interstitial fibrosis (light blue, bottom row, right 2 columns) throughout the heart but concentrated in the outer left ventricular myocardium.
Online Figure III. *Brg1/Brm* double-mutant mice exhibit cardiomyocyte hypertrophy 25 days after first pl-pC treatment compared to parallel control groups. (A) Mason Trichrome stained heart sections from control (top panels) and *Brg1/Brm* double-mutant mice 25 days after the first pl-pC treatment (bottom panels) showing cardiomyocyte hypertrophy in double mutants. (B) Quantification of cross-sectional area of cardiomyocytes from Masson’s Trichrome stained sections. Cross-sectional areas of controls (untreated Tg and non-Tg pl-pC treated) and double mutants were analyzed using the scales from Aperio scanned slides (picture is 100 μm x 100 μm). 250 measurements from 5 sections of 2 mice per group were analyzed and statistically analyzed using a Student’s t-test (*p<0.001 vs. control group).
A. Individual Brg1/Brm Mice

Online Figure IV. Serial echocardiographic analysis of the left ventricle in individual mice over time. (A) Data for individual Brg1/Brm double-mutant mice (animal numbers are shown), which died between day 13 and 92 after pl-pC treatment. Arrows indicate when first changes were seen in anterior or posterior wall thickness. The presence of cardiac dissections and time between dissection and death are detailed in Online Table I. (B) Parallel analysis of the treated non-Tg and untreated Tg control groups.
Online Figure V. Transient left ventricular posterior wall stunning seen in a *Brg1/Brm* double-mutant mouse (2332) at day 10 on M-mode images. The stunning gradually improved and resolved by day 18, when increased anterior and posterior wall thickness increased. A more comprehensive representation of this phenotype can be seen in 2D images (AVI files numbered 1-10.MyoStunning). *First day stunning was identified. Arrows indicates posterior wall.
Online Figure VI. RT-PCR of cell-specific markers in mouse cardiac VEC cultures. **Top,** PECAM-1 immunofluorescence of cultured cardiac VECs. **Bottom,** RT-PCR was performed on mouse embryos (E11.5 as a positive control), mouse cardiac VECs, and no template (as a negative control) for VE-Cadherin, VEGFR2/FLK1, α-smooth muscle actin (α-SMA), and VEGFR3/FLT4 as shown. The lane furthest to the left is a molecular-weight ladder, and fragment sizes are indicated in bp. Sizes of RT-PCR products are indicated at the top.
Online Figure VII. Consequences of vascular permeability defect in Brg1/Brm double-mutant mice 30 days after first pl-pC treatment. (A-C) Levels of platelet activation factor (PAF) (A), thrombin (B), and vascular endothelial growth factor A (VEGF) (C) in plasma from control and double-mutant mice as detected by ELISA assays. Data are presented as mean ± SE from 3 independent experiments. (D) Aggregation response to 1 µg/mL collagen for control and double mutant platelets (n=5 for both genotypes). Double mutant platelets were also treated with 10 µg/mL collagen at 5.5 min after start of the assay to show viability. (E) Platelet (PLT) cell number based on complete blood counts.
Online Figure VIII. *Brg1/Brm* double-mutant mice exhibit VEC changes in the tongue 30 days after first pl-pC treatment compared to parallel control groups. TEM analysis of representative capillary beds from *Brg1/Brm* double-mutant tongues (B) show an increase in the number and size of cytoplasmic vesicles as compared to the controls (A). Vesicles in the control (C) are uniform and normal in size (50-60 nm) while many in the mutant VECs (D) show pronounced dilation, some greater than 200 nm (*). Necrotic endothelial cells were not found in mutant tongue VECs as they were in day 25 mutant cardiac VECs, however the vesicular changes are consistent. Data from animals 3006 (A,C) and 2967 (B,D).
Online Figure IX. Hematopoiesis is not perturbed in *Brg1/Brm* double-mutant mice following pl-pC treatment. H&E-stained femur sections from *Brg1/Brm* double mutants 25 days after the first pl-pC treatment (A) and controls (B). The maturation of the erythroid, granulocytic, and megakaryocytic lineages appears intact and properly distributed, and the overall cellularity does not differ between the groups. The cortical bone is of uniform thickness, with morphologically normal appearing ostoblasts rimming the intramedullary surface. The H&E-stained that are shown are from formalin fixed, paraffin embedded, and decalcified femurs. 600X magnification.
Supplemental Table 1. Description of the natural history of the cardiac defects identified after inducible knock-out of Brg1/Brm double-mutant mice. Associated 2D AVI movies available in supplemental data (right column).

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<th>Myocardial Stunning?</th>
<th>Day of Significant Increase in Anterior/Posterior Wall Thickness Increases N=9</th>
<th>Dissection Present?</th>
<th>Day of Dissection detected by Echo Mean N=9</th>
<th>Length of time Cardiac Dissection Present before death Mean N=9</th>
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<td>Mean 15.2±4.3 (Median 14 Days) Range 7-21 Days</td>
<td>Mean 15.2±4.3 (Median 14 Days) Range 7-21 Days</td>
<td>56% (5 of 9) had dissections Mean 34.4±8.4 (Median 22.0 Days) Range 20-56 Days</td>
<td>Days Dissection to Death Mean 4.6±0.6 (Median 5 Days) Range 5-7 Days</td>
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