Mouse Cardiac Surgery: Comprehensive Techniques for the Generation of Mouse Models of Human Diseases and Their Application for Genomic Studies.

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ABSTRACT

Mouse models mimicking human diseases are important tools in trying to understand the underlying mechanisms of many disease states. Several surgical models have been described that mimic human myocardial infarction and pressure-overload induced cardiac hypertrophy. However, there are very few detailed descriptions for performing these surgical techniques in mice. Consequently, the number of laboratories that are proficient in performing cardiac surgical procedures in mice has been limited. Microarray technologies measure the expression of thousands of genes simultaneously, allowing for the identification of genes and pathways that may potentially be involved in the disease process. The statistical analysis of microarray experiments is highly influenced by the amount of variability in the experiment. To keep the number of required independent biological replicates and the associated costs of the study to a minimum, it is critical to minimize experimental variability by optimizing the surgical procedures. The aim of this publication was to provide a detailed description of techniques required to perform mouse cardiac surgery, such that these models can be utilized for genomic studies. A description of three major surgical procedures has been provided: 1) aortic constriction, 2) pulmonary artery banding, 3) myocardial infarction (including ischemia-reperfusion). Emphasis has been placed on technical procedures with the inclusion of thorough descriptions of all equipment and devices employed in surgery, as well as the application of such techniques for expression profiling studies. The cardiac surgical techniques described, have been, and will continue to be important for elucidating the molecular mechanisms of cardiac hypertrophy and failure with high throughput technology.

Keywords: cardiac hypertrophy, heart failure, surgical procedures, gene expression profile.
INTRODUCTION

Despite major reductions in mortality rates for cardiovascular disease during the past 30 years, this disease continues to be the most common threat to life and health. The lifetime risk of developing coronary heart disease after the age of 40 is 49% in men and 32% in women. An estimated 8% of the United States population (approximately 20 million people) have some form of heart disease (1). In 1999, cardiovascular disease claimed 958,775 lives in the United States. More than 2,600 Americans die of cardiovascular disease each day, an average of 1 death every 33 seconds (2). By 2020 it is predicted that cardiovascular disease will annually claim 25 million deaths worldwide, and coronary heart disease will surpass infectious disease as the world’s number one cause of death and disability (3).

The substantial decline in the mortality rate due to cardiovascular disease was attributed in part, to the use of innovative preventive and therapeutic measures, which came about from extensive cardiovascular research. Historically, a large portion of cardiovascular research has been performed using laboratory animals (4-8). A vast number of animal models have been developed to imitate human diseases and investigate physiological and pathological processes. The tremendous achievements in molecular biology and genetics within the last decade have opened up new horizons for the management of heart disease. The demand for animal models has increased because a high proportion of genes are common in animals and humans (3).

In the last decade, animal surgery in cardiovascular research has largely shifted from the rat to the mouse. In the past, the major advantage of using the rat in surgery was due to its larger size (approximately 10 times bigger than the mouse). However, the
appearance of more sophisticated microdissecting microscopes and diligent microsurgical instruments has now made surgery in the mouse as feasible as in the rat (5). Mouse models have gained popularity for a number of reasons. These include small size, rapid gestation period (21 days), large litter size, and relatively low maintenance costs. Moreover, the mouse genome has been extensively characterized, and gene targeted “knockout” and transgenic over-expression experiments are performed using mice, rather than rats (5). It is now clear that similar genes and signaling pathways regulate the development of the heart and vasculature in mice and humans. Molecular genetics will most likely play a critical role in revolutionizing the ability to diagnose and treat patients with cardiovascular diseases (3).

To date, there are no schools or courses available for animal surgeons to attain and develop the necessary technical skills to perform surgical techniques in mice, and very few publications exist describing these techniques. Consequently, the number of laboratories that are proficient in performing cardiac surgical procedures in mice has been limited. To perform surgical procedures in mice, investigators are usually limited to brief descriptions in journal articles, and then they must work out the details of the surgical model by trial and error. Even for those with considerable surgical experience, this empirical approach is usually very time consuming, not always successful, and often involves the loss of a number of animals. Furthermore, the outcome of these interventions can be highly variable, resulting in the use of high numbers of animals to attain statistically significant results.

Microarray technologies measure the expression of thousands of genes simultaneously, allowing for the identification of genes and pathways that may potentially be involved in the disease process. The statistical analysis of microarray experiments is often confounded by the number of samples that are being studied and the amount of
variation within the experiment. Biological variation is influenced by genetic or environmental factors and is further enhanced in surgical models as compared to transgenic models due to the variability in the surgical procedure. To keep the number of required independent biological replicates and the associated costs of the study to a minimum, it is critical to minimize experimental variability by optimizing the surgical procedures.

The aim of this publication was to provide a comprehensive description of techniques required to perform mouse cardiac surgery, such that reproducible results are attained, allowing for the use of these models for high quality genomic studies. Specifically, a detailed description of the intubation of the mouse and three major surgical procedures used in cardiovascular research have been provided: (I) aortic constriction (left ventricular pressure-overload model), (II) pulmonary artery constriction (right ventricular pressure-overload model), and (III) myocardial infarction including ischemia-reperfusion. We guide the investigator step by step through the whole procedure from the initial handling of the mouse for anesthesia to its full recovery after surgery. These guidelines do not attempt to replace surgical training with a skilled animal surgeon, but to enable trained researchers to optimize their procedures in order to maximize their success and to minimize technical variability. All procedures follow the "Guide for the Care and Use of Laboratory Animals" of the Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council. Emphasis has been placed on technical procedures with the inclusion of thorough descriptions of all equipment and devices employed in surgery, as well as the application of such techniques for genomic studies.
MATERIALS AND METHODS

Animal Ethics.

All surgical procedures described in this publication were performed in accordance with the National Institute of Health standards and approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center.

Strain of mice.

Different strains of mice were utilized depending on genetic manipulations and conditions investigated. The most common strains used were FVB/N and C57BL/6. Surgical procedures were performed on transgenic, non-transgenic, knock-out and wild type mice. For experiments described in this publication adult male mice (11-12 weeks) were used. At this age, developmental growth of the heart is complete. The weight of mice at this age ranged between 25 and 30 g.

Anesthesia.

The surgical procedures described are relatively short, thus it is not necessary to withhold food and water from mice prior to surgery. Sodium Pentobarbital (70 mg/kg) was chosen as the anesthetic because it provides an adequate depth of anesthesia for 30-40 minutes. For longer procedures such as ischemia-reperfusion an additional dose of 30 mg/kg is necessary during the course of the surgery. The anesthetic is injected intraperitoneally with a short 27-Gauge ½ inch needle. The mouse should sit for 5-7 minutes in an empty cage while the anesthesia takes effect. It is important not to disturb the animal prematurely since this will agitate the mouse and affect the quality of the subsequent procedure.
The most useful indicator to ensure that an adequate depth of anesthesia has been attained is the toe pinch reflex. The toe of the hind limb should be pinched firmly between the operator’s fingernails. If the mouse attempts to withdraw its limb, then it is not sufficiently anesthetized and a top up dose should be given (approximately 10-20 % of the initial dose). When there is no response to the toe pinch, medium-deep anesthesia has been attained.

*Pre-surgical preparation.*

The animal’s chest is shaved with an animal hair clipper (Harvard Apparatus, size 40 clipper blade). The mouse is then positioned on an operating table for the subsequent intubation. A simple small styrofoam platform (a cover from a commercial freezer container) serves well as an operating table (a platform with ladder-shaped edges is optimal (Fig. 1A)). The mouse should be secured to the styrofoam platform by the tail with a piece of tape. 5-0 nylon silk is then placed behind the front upper incisors, pulled taut and fixed with tape to the lower step of the ladder-shaped platform (Fig. 1A). The nose of the mouse should be positioned in close proximity (5-10 mm) to the edge of the platform. The front legs should then be secured in place to the sides of the body with 5 mm wide strands of tape (it is important to embrace the left arm with a tape ring closed on the lower side of the strand and the right arm on the upper: refer to the arrows on Fig. 1A - such taping aids in the correct positioning of the mouse for the operation). It is important that the front limbs are not over-stretched as this can compromise respiration. The mouse is then ready for the intubation.
**Endotracheal intubation.**

There are numerous methods for performing endotracheal intubation described in the literature by both sharp and non-invasive approaches. The sharp method involves tracheotomy. We prefer not to use this method because it subjects the animal to an extra surgical invasion, is time consuming, and may cause complications in the post-operative period. The non-invasive method, described by Brown et al. (4), is similar to the one described here in principal but requires some extra equipment. The method described in this publication is simple, reliable and requires no more than proper restraint of the mouse as described above, curved forceps (used as a laryngoscope, Roboz Cat #RS-5228), and a light source for the trans-illumination of the neck. The intubation tube is made from a 20-Gauge i/v catheter (Fisher Cat #NC 9706397), cut at exactly 25 mm (1 inch) in length, attached to a connector (10 mm piece of PE-190 tubing; 1.19 mm ID, 1.70 mm OD; VWR Cat #63018-769, with one beveled end). The later is attached to an extender (60 mm piece of PE-240 tubing; 1.68 mm ID, 2.41 mm OD; VWR Cat #63018-827) used as a handle (Fig. 1B). It may be necessary to adjust the diameters of some pieces of tubing to ensure a tight fit. For instance, to attach the catheter and PE-190 tubing together, it is necessary to pull the end of the PE-190 tubing with forceps. This reduces the inner diameter of the PE-190 tubing and provides a tight fit with the catheter. The intubation tube construct is designed for multiple use (10-20 times).

The platform with the mouse should be brought to the very edge of the table with the head of the mouse directed towards the operator. The curved forceps (Roboz Cat #RS-5228) and intubation tube with the extender should be kept to the right of the mouse (for the right handed). Between surgeries the intubation tube is kept in alcohol for
disinfection. Before use the tube should be shaken vigorously to remove any remaining fluid within the tube, to avoid aspiration by the mouse. A drop of 1% lidocaine is put on the tip of the tube to numb the throat and reduce the gag reflex. A power light with flexible horns (MVI Cat #MVI-DGN) is used to illuminate the neck of the mouse. To enhance the view during the intubation and operation we use binocular lenses with 1.75 magnification (SCS Limited Cat #H10L1.75×). The operator should then kneel down so that the head of the mouse is at eye level. The tongue of the mouse is held with the curved forceps held in the operator’s right hand, it should be moved to the left, then using the left hand the tongue is held with thumb and index finger and moved up slightly. The forceps (still held in right hand) are put under the tongue to hold it firmly to the lower jaw (the forceps should be kept strictly horizontal with the ends turned up and opened 1-2 mm). The vocal cords and trachea (as a light hole closing like a valve) should then be visualized. The operator should carefully move the forceps to his/her left hand preserving the same position of the tongue. With the operator’s free right hand the catheter of the intubation tube is gently inserted into the trachea until the connector starts going into the oral cavity (Fig.1C). The extender is then gently detached using forceps and the connector should stay on the tube. Restraint and intubation of the mouse takes approximately 5 minutes.

*Ventilation.*

For artificial ventilation a mouse ventilator 687 series (Harvard Apparatus) is used. The tidal volume and ventilation rate are calculated by formulas provided by the company:

\[ V_t = 0.0062 \times M_b^{1.01} \]
Where $V_t$ is tidal volume and $M_b$ is animal mass in kg.

$$\text{Ventilation rate (breaths/minutes)} = 53.5 \times M_b^{-0.26}$$

A list of tidal volumes and ventilation rates for mice of particular body weights is shown in Table 1 (R Toubeau, Harvard Apparatus, personal communication). Periodic maintenance of the ventilator is important because the plunger in the pump gets worn resulting in a decreased tidal volume.

A modified Y-shaped connector (Fisher cat #15-320-10A) is used to attach the mouse to the ventilator (Fig. 1D). The lower edge of the connector has been cut off to reduce the dead space. A short 5 mm piece of PE-240 tubing is inserted into that end to be used as an adapter. The connector is attached to the ventilator with regular i/v infusion system tubing. The Y-shaped connector is attached to the styrofoam platform with a piece of tape (5 mm in width). All pieces of tape holding the mouse in position, except those on the upper legs should be removed and the mouse positioned to the proximity of the Y-shaped connector. The mouse is positioned and secured on its right side with the chest rotated and exposed to the operator at a 45° angle to the plane of the table (Fig. 2). The connector of the intubation tube (beveled) should then be inserted into the adapter of the Y-shaped connector (the operator should be careful while making this connection so as not to leave a large dead space or on the other hand, not to block an airway). The operator should now visually confirm rhythmic movements of the chest synchronized with the ventilator. The ventilator is set up to 133 breaths/minutes and a tidal volume of 0.2 ml (accounts for system dead space) for a 30 g mouse (Table 1). 100% oxygen (2L/min) is loosely connected (via i/v catheter) to the inflow of the ventilator to provide a supplement to room
air. After intubating the mouse, the hind legs and tail should be fixed to the platform with strands of tape 5 mm in width and secured with wider pieces of tape (Fig. 2). All the steps described above are used for all of the open chest surgeries. At this point the operator can proceed with the surgery itself.

If an accidental extubation should occur during surgery, the first and most critical step (for the survival of the mouse) is to close the chest immediately by placing a suture on the skin. After that the mouse should be re-intubated. Attempting to re-intubate the mouse with the chest open will usually result in loss of the animal.

General considerations for surgery and tools.

Procedures involving the measurement of physiological parameters such as blood pressure require body temperature to be strictly maintained between 36-37 °C. By contrast, the majority of surgeries described here, can be performed at room temperature (20-25 °C). The body temperature of the mouse may fall to 33-34 °C after anesthesia, but this is not a problem for short surgical procedures (10-15 minutes). For longer procedures (i.e. ischemia-reperfusion model) the body temperature should be strictly monitored.

For the operations described six basic surgical tools are used: (I) Curved forceps (Roboz Cat #RS-5228) used for the intubation, (II) Straight forceps (Roboz Cat #RS-5130), (III) Curved forceps (Roboz Cat #RS-5101) used for the operations, (IV) Scissors with large rings (Roboz Cat #RS-5850), (V) Chest retractor (Roboz Cat #RS-6510): the two proximal pairs of teeth (closest to the junction) should be removed with the other two pairs left in place, and (VI) Needle holder (Roboz Cat #RS-6410). Occasionally some other tools are used, these are described further in the text. The only optics necessary for the surgical
procedures described is a binocular lens (SCS Limited Cat #H10L1.75×) with a 1.75 magnification. For illumination a power light with flexible horns (MVI Cat #MVI-DGN) is used.

As routinely practiced in small animal surgery, the operations are carried out under aseptic conditions. Surgical tools are autoclaved once a day prior to surgery. The same instruments are used for 5-10 mice, however between operations they are sterilized in a hot bead sterilizer (Harvard Apparatus Cat #HA61-0183). 6-0 nylon is used for the chest cavity and skin closure, 6.0 vicryl (absorbable suture) for suturing the muscles, and 7-0 silk for manipulations on the heart.

Before surgery the operating field is disinfected with betadine solution and 70% alcohol three times in alternance. To prevent contamination of the surgical site during the operation, a large transparent sterile plastic drape should be placed over the mouse, leaving only the operating field exposed. A set of sterile gloves is used for each individual mouse. For hemostasis it is usually sufficient to use a piece of gauze or cotton applicators, though in some instances cautery is necessary, as indicated in the text.

Operation I: Ascending Aortic Banding (Left Ventricular Pressure-Overload Model).

Aortic banding is a very popular method to induce left ventricular hypertrophy in mice and mimics human aortic stenosis with development of pressure-overload induced left ventricular hypertrophy. The aortic band can be placed on either the ascending, transverse or descending portion of the aorta depending on the study design. In this publication we describe constriction of the ascending aorta because it provides a more direct and rapid source of pressure-overload on the left ventricle with a significant degree of hypertrophy.
after 48 hours (9). The method has been described (with some modifications) in a number of scientific papers (7, 8, 10), but technical details were very limited.

The landmark for the incision is the level of the left axillary (“arm pit”, with paw extended at 90°). Before the incision, a local anesthetic (0.1 ml of 0.1% lidocaine) is injected subcutaneously. A transverse 5 mm incision of the skin is made with scissors 2 mm away from the left sternal border, 1-2 mm higher than the level of the armpit (Fig. 2A). The operator should try to visualize the superficial thoracic vein that runs under the skin at the lateral corner of the incision. Both layers of thoracic muscles should be cut, taking caution to avoid the vein. Damaging it is not life threatening but can result in profuse bleeding. Cotton applicators are sufficient to stop the bleeding. Through the thin and semi-transparent chest wall the ribs and inflating lung should be visible. The chest cavity is opened with scissors by a small incision (5 mm in length) at the level of the second intercostal space (in this space the very tip of the lung is visible) 2-3 mm from the left sternal border. It is important not to approach the sternal border any closer than this, because the internal thoracic artery (running along the sternal border of the inner surface of the thoracic cavity) can be easily damaged and intense bleeding may occur. If the artery is damaged cautery may be used to stop the bleeding. While opening the chest wall, extreme care should be taken not to damage the underlying lung. The chest retractor is gently inserted to spread the wound 4-5 mm in width, taking care to avoid the lungs getting caught in the teeth of the retractor. The view will be obstructed by the thymus and fat, which should be pulled away with forceps to the left arm of the retractor. With two forceps the pericardial sac should be gently pulled apart and attached to both arms of the retractor. Note that the pericardial sac and thymus are intimately connected to the left superior vena
cava which runs immediately next to the left side of the heart (mice and rats have two superior venae cavae in contrast to humans). While being mobilized the pericardial sac should not be pulled apart with too much force since it may rupture the wall of the left superior vena cava. The great vessels and upper part of the left atrial appendage can then be visualized. With the curved forceps the ascending portion of the aorta is bluntly dissected from the pulmonary trunk on the right (extreme caution should be taken, since bleeding from any of the great vessels is fatal). From the left side, the curved forceps are placed under the ascending aorta. The tips of the forceps should appear on the right side between the aorta and the pulmonary trunk (a slight poking movement is required to go through the connective tissue between the aorta and pulmonary trunk). 7-0 silk is grasped by forceps, moved underneath the aorta, and a loose double knot is made (7-10 mm diameter loop). For aortic constriction of 11-12 week mice we use a 25-Gauge needle with O.D. 0.51 mm. The size of the needle depends on the amount of stenosis and degree of hypertrophy/failure desired. The needle should be blunted and bent to make an L-shape in advance. To hold the needle in position a “needle holder” construct was made (Fig. 3A). The needle is attached to a 1 ml syringe which is held on a retort stand by means of two rods (Harvard Apparatus Cat #AH 50-4415 and #AH 50-4407), connected by a 2-ball joint connector (Harvard Apparatus Cat #AH 50-4431). The needle is delivered through the loose double knot from the left side and placed directly above and parallel to the aorta. The loop is then tied around the aorta and needle, and secured with the second knot (this should be done very quickly to minimize ischemia and a build-up of pressure) (Fig.4). The needle is immediately removed to provide a lumen with a stenotic aorta. Two more knots are made to secure the tie. The chest retractor is then removed and the thymus is moved back to its normal position. Since
the lungs were partially compressed by the retractor, they should be re-inflated by shutting off the outflow on the ventilator for 1-2 seconds using a finger. This step is important, since collapse of the lungs will result in respiratory distress and poor recovery after surgery. The chest cavity is closed by bringing together the second and third ribs with one 6-0 nylon suture (taking care not to suture the lungs). While making a knot, slight pressure is applied on the chest with the needle holder to reduce the volume of free air in the chest cavity. All layers of muscle and skin are closed with 6-0 continuous absorbable and nylon sutures, respectively. With practice, the whole procedure takes approximately 15 minutes. An important control for aortic banding as well as the subsequent operations described below is the sham surgery. For the sham operation the mice undergo a similar procedure but the intervention stops when the curved forceps are moved underneath the ascending aorta without placing a ligature. The lungs are then re-inflated and the chest is closed as described above.

The subsequent post-operative care is identical for all the operations and has been described in detail in a later section (see Post-Operative Care).

Operation II. Pulmonary Artery Banding (Right Ventricular Pressure Overload Model).

The pulmonary artery banding model mimics pulmonary coarctation in humans. Pulmonary arterial stenosis is usually a congenital syndrome. It can be presented as an isolated form but is commonly associated with other cardiovascular abnormalities such as tetralogy of Fallot. It also may be seen as a sequela of congenital rubella, Williams’, Noonan’s or Alagille’s syndrome (1).
In cardiovascular research pulmonary artery banding has been used as a model of right ventricle hypertrophy and failure (11). In small animals, the pulmonary banding model has not been as widely used in research as the aortic banding model. This may be explained by the much lower prevalence of right ventricular hypertrophy and failure compared to the left in humans (12), as well as certain difficulties in performing the surgery itself. To our knowledge, there is only one report in the literature describing the technique of pulmonary artery banding in mice. This method was described in 1993 by Rockman et al. (11), but it lacked sufficient technical details to be reproduced by other investigators.

The major challenges which arise during this surgery are due to 1) the extremely thin fragile walls of the pulmonary trunk and 2) the inability of the right ventricle to withstand stress while the pulmonary artery is being manipulated. The blood pressure in the right ventricle is very low compared to that in the left. Thus, any dissection underneath the pulmonary trunk using forceps leads to blockage of blood flow to the lungs, and this results in immediate respiratory and cardiac distress. Consequently, recovery after surgery is very poor and the animal is invariably lost. To overcome this complication special techniques are required (see below).

In theory, pulmonary artery banding is technically similar to aortic banding. However, some unique tools and devices are employed to aid in the success of this surgery. The access to the great vessels is obtained via the second intercostal space in the same manner as described for aortic banding. After mobilization of the pericardium, the pulmonary trunk (partially covered by the left atrium) should be visualized. The position of the atrium creates an additional complication, as its location and fragile nature makes it very
vulnerable during the dissection. The pulmonary trunk should be bluntly dissected with curved forceps from the aorta (on the left) and left atrium (on the right). Great care must be taken while performing this dissection. The dissection should be relatively superficial and no attempt should be made to go underneath the pulmonary trunk at this stage. To create a tunnel underneath the pulmonary trunk an L-shaped 28 gauge blunted needle is used (an insulin syringe with the needle attached serves as a convenient tool). The needle should be placed from the side of the pulmonary trunk closest to the left atrium, and gently pushed underneath the pulmonary trunk so that the end of the needle appears between the pulmonary and aortic trunks. A poking movement is required to rupture the connective tissue between the trunks. The shape of the needle allows a tunnel to be created underneath the pulmonary trunk without compromising the pulmonary blood flow, which as noted above, would not be possible with the use of forceps. Throughout the dissection special attention should be paid to the atrium and the walls of the pulmonary artery, as these structures are very fragile and bleeding is invariably fatal. The next step involves placing the ligature behind the pulmonary trunk. This step is aided by the use of a special device. A provisional patent application has been filed by the inventor, Dr. Hu. The device named “a wire and snare” consists of an i.v. catheter with a needle and port. The device was designed to perform minimally invasive transverse aortic banding operations (13). Inquiry regarding the device should be addressed to Dr. Hu or University of Utah Technology Transfer Office. With the help of this device the 7-0 silk suture is moved behind the pulmonary trunk. The suture is then caught with forceps between the pulmonary and aortic trunks and the catheter is removed. A loose double knot is made (7-10 mm diameter loop). For moderate stenosis the suture is tied against a
25 G needle. A more severe stenosis can be created in 23-25 g mice using a 26 gauge needle (0.46 mm OD) (11). The needle held in position using the “needle holder construct” (see the aortic banding section) is introduced into the loop, above and parallel to the pulmonary trunk. For easier access, the operating table with the mouse can be temporarily rotated 90° in a clockwise direction. The loop is then tied around the pulmonary trunk and needle, and secured with a second knot (this should be done very quickly so as not to severely compromise pulmonary blood flow). The needle is immediately removed to provide a lumen with a stenotic pulmonary artery. At this point the heart rate noticeably slows as the right ventricle has difficulty withstanding the build-up in pressure. To improve the heart rate and override this condition, the tail of the mouse is pinched with forceps. This stimulus usually accelerates the heart rate instantly. If the right ventricle cannot compensate for the increase in pressure, the heart rate may not improve, usually resulting in loss of the animal. This is an unavoidable complication of this surgery. Two more knots are made to secure the tie. The chest retractor is removed and the thymus is moved back to its physiological position. The lungs are reinflated, the chest cavity is closed with 6-0 nylon suture, and the muscles and skin are closed layer by layer with 6-0 absorbable and 6-0 nylon sutures, respectively. The entire procedure takes approximately 15-20 minutes. For the sham operation, dissection of the pulmonary trunk is performed using the L-shaped needle but a ligature is not placed or tied. The lungs are then reinflated and the wound is closed as previously described.
Evaluation of ventricular pressure created by aortic and pulmonic banding

a) Left ventricular (LV) Pressure Measurement for the evaluation of ascending aortic banding. Assessment of the pressure in the LV is important for ensuring the aortic banding operation was successful and that the LV pressure is similar in different experimental groups. Due to the position of the band it is not feasible to pass the catheter into the ventricle retrogradely from the right carotid artery. In this case the method of choice is the direct pressure measurement via the left ventricular apex. The mouse is anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) given at 0.1 ml intraperitoneally. The mouse is intubated and fixated in the same manner as described for surgeries. The animal is ventilated with 100% oxygen (as a supplement) at a tidal volume of 200 μl at 133 cycles/min (Harvard Respirator, model 687). The temperature should be maintained at 36-37°C using a temperature controller connected to a rectal probe (Fine Scientific Tools, TR-100). The rectal probe is inserted after the mouse has been intubated and ventilated. The chest is opened via an incision through the left 4th intercostal space and the edges of the wound are spread to the sides by two 5-0 silk sutures. The pericardial sac is carefully pulled apart to visualize the apex of the heart. A purse-string 7-0 silk suture is placed on the apex and a superficial nick is made with an 18-gauge needle inside of the purse ring. A 1.4-Fr high-fidelity pressure catheter (Millar catheter) is placed through the nick into the ventricular chamber. Before tying the purse-string around the catheter it is important to ensure the catheter is correctly positioned. While recording pressure signals at 2 kHz, the catheter should be gently moved back and forth to ensure the catheter is not hitting the ventricular wall or the signal is not being dampened by trabeculae. Pressure signals are then recorded at 2 kHz for 10 min, stored to disk, and analyzed using PowerLab.
software (Chart 4.1.2, ADInstruments). Anesthetics and open-chest surgery are known to depress heart rate (14, 15). Thus, it is important to monitor heart rate during the pressure recordings to ensure pressure measurements were acquired under similar experimental conditions.

**b) RV Pressure Measurement for the evaluation of pulmonary artery banding.** This technique is similar to the one described for the left ventricle, but the surgical access is different. An incision is made in the left third intercostal space down to the level of the sternum (cautery is used at this point to stop bleeding). Three 5-0 silk sutures are placed on the ribs (two on the second and one on the third rib) to spread the edges of the wound apart. The whole heart is now visualized covered from the top with thymus. Thymus and the pericardium are removed to facilitate the view. The front wall of the right ventricle should be visualized in the wound. A superficial incision is made at the mid portion of the RV with an 18-gauge needle prior to insertion of a 1.4-Fr high-fidelity pressure catheter. Since the wall of the right ventricle is much thinner than that of the left, a common complication can be that the right ventricle is punctured during this process (i.e. before inserting the catheter while making the superficial nick) resulting in bleeding. Bleeding can be stopped with cotton applicators. The catheter is secured in place with a purse-string 7-0 silk suture. Pressure signals are recorded at 2 kHz for 10 min, stored to disk, and analyzed using PowerLab software (Chart 4.1.2, ADInstruments).
Operation III: Myocardial Infarction (MI) Model & Ischemia Reperfusion.

In the United States approximately 1 million patients annually suffer from acute MI, and in nearly all cases this results from coronary atherosclerosis (3). In a variety of animal models this condition has been mimicked by ligation of the left coronary artery. Reperfusion of the ischemic heart has become an important therapeutic intervention for myocardial infarction. However, the process of reperfusion, although beneficial in terms of myocardial salvage, also results in cell death and scar formation due to a process known as reperfusion injury (3). The underlying mechanisms involved in ischemia-reperfusion are not well understood. The murine model of myocardial infarction and ischemia reperfusion has been widely described in the literature (16-21) and the procedure was described in reasonable detail by Michael et al. (21). However, the technique of correctly visualizing the left anterior descending (LAD) coronary artery, and temporarily occluding the LAD during reperfusion can be very difficult. In the current publication we have placed more emphasis on technical approaches and devices utilized in this surgery which significantly aid in the success and reproducibility of this surgical technique.

a) Myocardial Infarction

The landmark for the incision is the left armpit. 0.1 ml of 0.1% lidocaine is injected subcutaneously. An oblique 8 mm incision is made 2 mm away from the left sternal border towards the left armpit (1-2 mm below it) (Fig. 2B). The muscles are separated as previously described (see Operation I: Ascending Aortic Banding) with caution taken to avoid damaging blood vessels. The rib cage and moving left lung are then visualized. The 4th intercostal space represents the area between those ribs where the lowest part of the lung
is observed. The chest cavity is then opened taking caution not to damage the lung. The chest retractor is inserted and opened gently to spread the wound 8-10 mm in width. The heart partially covered by the lung is then visualized. The pericardium is gently picked up with curved and straight forceps, pulled apart and placed behind the arms of the retractor. This maneuver pushes the lung up slightly, mobilizing it and providing better exposure of the heart. The LAD coronary artery should then be visualized as a pulsating bright red spike, running in the midst of the heart wall from underneath of the left atrium towards the apex. Certain experience is needed to properly visualize the artery, since it can easily be confused with the vein (correct lighting is critical for this step, it should be focused but not too bright). If the LAD artery cannot be visualized, the left atrium can be lifted so that the origin of the LAD artery from the aorta is located.

The position of the ligation depends on the volume of infarction desired. For most studies the LAD artery is ligated 1-2 mm below the tip of the left auricle in its normal position, which induces roughly 40-50% ischemia of the left ventricle (Fig. 5). Once the site of ligation has been determined the curved forceps are used to gently press on the artery a little below the subsequent ligation (this will enhance the view of the artery and stabilize the heart). Next, with a tapered needle a 7-0 silk ligature is passed underneath the LAD coronary artery (Fig. 5A). For easier and smoother passage the needle should be bent in advance to make the curvature rounder. When placing the ligature under the artery it is important not to enter the cavity of the ventricle with the needle, but at the same time not to be too superficial as the ligature will cut through the wall of the ventricle. The ligature is then tied with three knots. Occlusion is confirmed by the change of color (becoming pale) of the anterior wall of the left ventricle. The retractor is removed and the lungs are re-
inflated by shutting off the ventilator outflow as previously described (see *Operation I: Ascending Aortic Banding*). The chest cavity is closed by bringing together the 4th and the 5th ribs with one or two 6-0 nylon sutures (with pressure applied to the chest wall to reduce the volume of free air). The muscles and skin are closed layer by layer with 6-0 absorbable and nylon sutures, respectively. The duration of the whole procedure takes approximately 12-15 minutes. The sham operated mice undergo the same procedure without tying the suture but moving it behind the LAD artery. The chest is closed as described above.

b) *Ischemia-Reperfusion.*

The goal of this operation is to create transient ischemia of the left ventricle by the temporary occlusion of the LAD coronary artery. We routinely occlude the LAD coronary artery for 60 minutes. An immediate complication of this surgery is cardiac arrhythmia, which may occur after the restoration of blood flow (reperfusion). To prevent arrhythmia, two intraperitoneal doses of lidocaine may be given at specific time points during the procedure (see below). For the ischemia-reperfusion model it is essential to monitor body temperature for 2 reasons. Firstly, the procedure is considerably longer than that described for the other operations, thus the body temperature of the mouse will fall dramatically during this period. Secondly, infarct size is dependent on the time of the occlusion as well as body temperature. Thus, it is critical that both are kept constant during this procedure. Body temperature should be maintained at 36-37 °C with a heating pad monitored by a rectal probe, connected to a temperature controlling device (Fine Science Tools). The rectal probe is inserted after the mouse has been intubated and ventilated.
In the ischemia-reperfusion operation the occlusion is made around fine PE-10 tubing (0.28 mm ID, 0.61 mm OD; VWR Cat #63018-623) to minimize the damage of the artery and facilitate the removal of the ligating suture. All the steps are the same as described for the MI model up to the point at which a 7-0 silk ligature is passed underneath the LAD coronary artery. Then a loose double knot is made to leave a 5-6 mm diameter loop and the PE-10 tubing is placed into the loop. The correct positioning of the tube on the beating heart can be difficult. To aid in this procedure we designed a construct. A 5-6 cm piece of PE-10 tubing is attached to a 30-Gauge needle, which has been bent to a 60° angle. The needle (with the tubing) is placed on a 1ml syringe and is attached to a stand by two rods and a 2-ball joint connector (Fig. 3B). The tubing is then introduced into the loop from the distal side directly above and parallel to the LAD artery until it touches the left auricle (1-2 mm above the level of the suture). The loop is tied around the artery and tubing and secured with two additional knots (the operator should be careful not to cut through the ventricle wall). Next, the tubing is cut with durable curved scissors (Roboz Cat #RS-5983) 1-2 mm distal from the suture to leave a piece 3-4 mm in length. Occlusion is confirmed by the dramatic change in color of the anterior wall of the left ventricle (becomes very pale). The first dose of lidocaine (6 mg/kg) is then given intraperitoneally. The chest retractor is removed, the lungs are re-inflated and the wound is temporarily closed with two interrupted 6-0 nylon sutures on the skin. An additional dose of sodium pentobarbital (30 mg/kg) should then be given intraperitoneally. The mouse remains on the ventilator for the duration of the 60 min LAD artery occlusion.

The second dose of lidocaine (6 mg/kg) is injected intraperitoneally immediately before the reperfusion. The temporary sutures are removed and the chest cavity is opened
with the chest retractor. The pericardium is moved apart to expose the heart. Holding the tubing with curved forceps, the ligating suture is gently cut with a #15 sterile surgical blade and the suture and PE-10 tubing are removed. Reperfusion is confirmed by return of the pink-red color of the anterior wall of the left ventricle (it may take 15-20 seconds). Next, the lungs are re-inflated, and the chest cavity, muscles and skin are closed layer by layer with 6-0 absorbable and nylon sutures, respectively. The duration of the whole procedure amounts to approximately 85-90 minutes.

When performing ischemia/reperfusion and making comparison of infarct size, it is important to verify that the area at risk was similar between groups. The area at risk is determined by the site of placement of the ligature around the coronary artery. During surgery, the ligature suture can be a slip knot, left in place during reperfusion after removal of the PE-10 tubing. Upon sacrifice and assessment of the area at risk/infarct size, the slip knot and suture that was left in place can be retied and the heart perfused with ink to delineate the area at risk.

Post-Operative Care.

Post-operative care for all the surgeries described is critical to ensure full recovery after surgery. The measures taken are directed to alleviate pain, provide supplementary heat to prevent hypothermia, and control respiratory depression (22). Immediately after the operation 0.5 ml of 37°C saline can be given i.p., and a dose of analgesic (Buprenorphine 0.1 mg/kg) is given subcutaneously and then every 8 hours for the next 48 hours. The mouse is moved to another ventilator in a designated recovery area and 100% oxygen is connected to its inflow. Heat is provided by a regular 60-watt lamp from a distance of 25-
30 cm (10-12 inches). After approximately 45-60 minutes the mouse should start making attempts to breath spontaneously. At this point it can be taken off the ventilator with the intubation tube still kept in the trachea and supplementary oxygen is provided for another 10-15 minutes (the mouse is placed next to the source of oxygen). After the mouse resumes a normal breathing pattern it can be extubated (the tube should be removed slowly to avoid aspiration). To make sure the mouse is not in any respiratory distress it can breath oxygen for another 5-10 minutes before returning to a cage.

The mouse should be placed in a clean cage by itself to prevent possible contamination of the surgical site or injury caused by other animals. To prevent accidental aspiration it is wise not to put the mouse directly on the bedding of the cage, but place a piece of paper towel underneath the mouse for the time of recovery (22). The cage is placed on a regular heating pad on a low setting. The temperature of the heating pad should not exceed 40 °C to prevent possible burns to the animal. In 1.5-2 hours after surgery the mouse should be completely awake and move around in the cage. The cage can then be taken off the heating pad. Nylon sutures should be removed from the skin 10 days after surgery.

**RESULTS**

*Peri-operative survival*

In the current study peri-operative survival rates for aortic constriction, pulmonary artery constriction (severe stenosis: 26G), myocardial infarction, and ischemia-reperfusion were 93.0% (n ≈ 250), 81.2% (n ≈ 100), 96.5% (n ≈ 250) and 96.2% (n ≈ 100), respectively. The lower survival rate for pulmonary artery banding reflects the inability of the right
ventricle to compensate for the increase in ventricular pressure immediately after placement of the band in some mice.

**Aortic banding and pulmonary artery banding**

Ascending aortic banding and pulmonary artery banding are important tools for examining the responses of transgenic and knock-out mice to a pathological stress. The pressures developed in the ventricles using aortic banding and pulmonary artery banding are presented in Table 2. The peak systolic pressures and heart rates were relatively low in the present study. This can be explained by the effects of anesthesia and the invasive technique used to measure LV pressures in open-chest mice. It is noteworthy that our values for LV pressures are similar to those reported in a previous study using similar open-chest techniques (15). Mice subjected to pulmonary artery banding display a significant increase in right ventricular weight after 4 days, 1 week and 2 weeks compared to sham operated mice (Table 3), and dilation of the right ventricle is obvious on histological examination (Fig. 6.).

Using the surgical procedures described in this publication, ascending aortic banding results in an increase in the heart weight/ body weight ratio of 40-45 % in non-transgenic mice as compared to sham-operated mice [normalized heart weight/ body weight = 1.00 ± 0.02, n=10; aortic band: normalized heart weight/ body weight = 1.43 ± 0.05, n=13 (23, 24)]. Furthermore, aortic banding was associated with interstitial fibrosis and re-expression of fetal genes, eg atrial natriuretic peptide and brain natriuretic peptide, none of which was detected in sham-operated mice (23, 24). Utilizing this aortic banding model we were able to demonstrate that mice expressing a dominant negative mutant of phosphoinositol-3
kinase (dnPI3K) are more susceptible to a pathological stress than non-transgenic littermates (24). At baseline, cardiac function (assessed by echocardiography) of sham operated dnPI3K transgenics and non-transgenic mice was normal (dnPI3K- fractional shortening: 51 ± 3 %, n=5; non-transgenics- fractional shortening: 53 ± 2 %, n=10), however after aortic banding the cardiac function of dnPI3K transgenics was depressed compared to non-transgenic mice (dnPI3K: 33 ± 3 %, n=8; non-transgenic: 49 ± 4 %, n=13; P<0.05)(24).

**Myocardial Infarction**

Myocardial infarction is associated with extensive remodeling and thinning of the left ventricle after 48 h and extensive collagen deposition is apparent in the infarcted area after 1 week (Fig.7A). The collagen fibers stain blue on the Masson’s trichrome stain. Assessment of determining infarct size has been well described in the literature (25-30), and will not be described here.

To gain valuable information from genomic studies it is critical that variation between mice subjected to the same procedure is kept to a minimum. Within group comparisons from three independent biological replicates should ideally have higher correlation coefficients compared to samples from between groups eg sham operation versus MI. A large variation in phenotype will result in low correlation coefficients. Using hearts from mice subjected to MI or the sham operation, Pearson correlation coefficients from microarray data were calculated.

Within group comparison of microarray data derived from three independent biological replicates of mice subjected to myocardial infarction or the sham operation
resulted in mean correlation coefficients of 0.973±0.021 and 0.989±0.005, respectively. These correlation coefficients are similar to those observed for within group comparisons of microarray experiments from transgenic mice (mean correlation coefficient = 0.987±0.009) and non-transgenic mice (mean correlation coefficient = 0.985±0.003). In contrast, the mean correlation coefficient for the comparison between sham and MI at the one week time point, which is clearly associated with marked differences in phenotype, was relatively low (correlation coefficient = 0.698±0.088). Within group and between group variation of the MI data set are also presented visually on a color correlation matrix (Fig. 7B). Based on the color of the grids illustrating normalized expression, there is good reproducibility for within group comparison and significant differences between sham and MI.

Minimizing within group variation allows for the identification of a greater number of genes which may be critical for the phenotype observed i.e sham versus MI. At 48 h post surgery, 1395 genes were differentially expressed between sham operated mice and those subjected to MI (infarcted area) at a P value of 0.01. The raw data generated from GeneChip arrays were analyzed using Affymetrix Microarray Analysis Suite as described (www.cardiogenomics.org).

**Further Applications**

Further applications of the surgical methods can be found at www.cardiogenomics.org (Animal Models of Cardiomyopathy: Current Animal Models), including details about experimental procedures, histology images, and microarray data.
DISCUSSION

Microarray analysis is becoming a standard tool for detecting genes that are differentially expressed in various disease states. The most recent generation of microarrays can evaluate the expression of up to 34,000 genes. Due to the vast amount of data that is being generated in such studies, statistical quantification is a standard requirement for analysis and interpretation, rather than the qualitative description of observations. Microarray experiments are generally costly and time consuming (31), therefore, many investigators choose to perform only a limited number of independent biological replicates. Microarray studies are confounded by a number of sources of variation, ranging from measurement error, technical variation induced during the RNA extraction, probe labeling, and hybridization, to biological variation. It has been shown that the cumulative contribution of these sources of variation in a cDNA microarray experiment can cause the correlations between samples obtained from individual inbred mice to be as low as 30% (32).

Biological variation is one of the largest contributing factors to variation in microarray experiments. Therefore, it is critical that experimental variation is kept to a minimum so that small sample sizes can provide statistically significant results. We show here that the application of the described methods leads to (a) a post-surgical survival rate of 93% and higher for aortic banding and LAD ligation procedures, and of 81% for pulmonary ligation; (b) consistent hypertrophy development after pulmonary or aortic banding with a low degree of variability; (c) development of a myocardial infarct after LAD ligation that comprises 30-40% of the free left ventricular area with extensive remodeling at 48 hours and beyond (a detailed time series can be found in the web tutorial of myocardial infarction at www.cardiogenomics.org); and (d) a mean correlation coefficient of 0.973 for a
comparison of microarray data derived from three independent biological replicates of mice with myocardial infarction. This correlation coefficient is similar to the correlation observed in microarray experiments on transgenic mice (0.987) and non-transgenic mice (0.985).

Comparison of gene expression between mice with myocardial infarction and sham-operated mice at 48 hours post surgery revealed 1395 genes that are differentially expressed between these two groups with a p-value of 0.01. Similar studies using mouse models of experimental MI have previously identified between 37 and 230 differentially genes (33-36). However, these studies vary wildly with regard to the number of replicates, experimental design, technology platform, and analysis strategy that has been used, which makes it impossible to perform a direct comparison.

The motivation for writing this publication was to provide a detailed description of surgical techniques used in cardiac surgery so that other investigators could generate these models and attain reproducible results with low experimental variation. A number of investigators have performed some of these surgical procedures but due to journal space constraints it has largely been impractical for authors to provide descriptions in sufficient detail for these techniques to be successfully duplicated. Consequently, the large majority of papers utilizing mouse cardiac surgery have come from a relatively small number of laboratories, and other laboratories have looked for simpler alternatives (13).

Here we have described three major surgical procedures used in cardiovascular surgery i.e. aortic constriction, pulmonary artery banding, and myocardial infarction and ischemia-reperfusion. We have also described the construction of some devices which we developed or improved and found critical for obtaining reproducible results. We are not
suggesting that our methods are more successful than some of those previously described by pioneers in this field (10, 11, 21). However, such publications lack information regarding the complications and pitfalls which can be associated with each of the techniques described. Yet, knowledge of these complications is critical for the final outcome of the surgery, as well as for obtaining reproducible results. The recommendations described here are based on optimizing the survival rate and obtaining consistent results from each of the models. Ultimately, this is cost efficient and reduces the number or animals required for such studies.

The mouse models described in this publication have been critical for evaluating the differential expression of genes in cardiac pathological states, and are likely to be important for the development of more effective therapeutics for the treatment of patients with heart disease.

ACKNOWLEDGMENTS

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REFERENCES


FIGURE LEGENDS

Fig. 1. A: Positioning the mouse for intubation (the arrows show the direction of the flaps of tape). B: Intubation tube, made from a 20-gauge catheter, with the connector (PE-190 tubing) and the extender (PE-240 tubing). C: Position of the operator’s hands for the mouse intubation (the head of the mouse is at eye level of the operator). D: Y-shaped connector (the lower edge has been cut short with PE-240 adapter inserted).

Fig. 2. Fixation of the mouse for surgery and location of the incision for A: pressure-overload model (2nd intercostal space), and B: myocardial infarction model (4th intercostal space).

Fig. 3 A: Construct to hold needle for the pressure-overload model. B: Construct to hold tubing for the ischemia-reperfusion model.

Fig. 4. Ascending aortic banding. A: 7-0 silk suture is tied around the aorta and 25 gauge needle. B: Position of the suture on the aorta.

Figure 5. Myocardial infarction. A: The needle with 7-0 silk suture is passed underneath the LAD coronary artery for subsequent ligation. The visibility of the artery has been digitally enhanced. B: The position of the suture on the LAD coronary artery (illustrated by the red cross). C: 24 hours after myocardial infarction operation: position of the suture, and the size of the area at risk (pale zone) after injection of 2% Evans Blue into the left renal vein.
Figure 6. Histological analysis of hearts subjected to constriction of the pulmonary artery reveals dilation of the right ventricle and size reduction of the left ventricle starting as early as 4 days after surgery. Hematoxylin-eosin staining of tissue sections.

Figure 7. A. Histological analysis of hearts subjected to MI for 48 h and 1 week. Collagen fibers stain blue on the Masson’s trichrome stain. B. Color correlation matrix of microarray data from mice subjected to myocardial infarction or the sham operation. Each box represents an individual sample. Increasingly positive correlations are represented with reds of increasing intensity, and increasingly negative correlations are represented with blues of increasing intensity.
Table 1: *Tidal volumes and ventilation rates for mice (20-60 g).*

<table>
<thead>
<tr>
<th>Mass (g)</th>
<th>Vt (cc) *</th>
<th>Ventilation rate (breaths/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.12</td>
<td>148</td>
</tr>
<tr>
<td>30</td>
<td>0.18</td>
<td>133</td>
</tr>
<tr>
<td>40</td>
<td>0.24</td>
<td>124</td>
</tr>
<tr>
<td>50</td>
<td>0.30</td>
<td>117</td>
</tr>
<tr>
<td>60</td>
<td>0.36</td>
<td>111</td>
</tr>
</tbody>
</table>

* Does not account for system dead space.
Table 2: Ventricular pressure measurements in mice subjected to ascending aortic constriction and pulmonary aortic constriction (severe stenosis: 26G).

<table>
<thead>
<tr>
<th></th>
<th>LV Overload Ascending Aortic Banding</th>
<th>RV Overload Pulmonary Artery Banding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Band</td>
</tr>
<tr>
<td>Number</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>229±8</td>
<td>276±29</td>
</tr>
<tr>
<td>Ventricular systolic pressure, mmHg</td>
<td>69.8±2.7</td>
<td>136.9±11.4 *</td>
</tr>
<tr>
<td>Ventricular diastolic pressure, mmHg</td>
<td>0.9±0.6</td>
<td>5.1±1.3 *</td>
</tr>
</tbody>
</table>

All pressure measurements were made using a 1.4-Fr high-fidelity pressure catheter (Millar catheter). Statistical analysis was performed using an unpaired t-test. * P<0.05 compared to sham.
Table 3: *Postmortem analysis of mice subjected to pulmonary artery banding (severe stenosis: 26G).*

<table>
<thead>
<tr>
<th>Time point</th>
<th>Sham</th>
<th>Pulmonary Artery Banding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 days</td>
<td>1 week</td>
</tr>
<tr>
<td>Number</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>26.6 ± 0.3</td>
<td>18.4 ± 1.2 *</td>
</tr>
<tr>
<td>Tibial length (mm)</td>
<td>15.5 ± 0.1</td>
<td>15.5 ± 0.2</td>
</tr>
<tr>
<td>Right ventricle (mg)</td>
<td>24.2 ± 1.6</td>
<td>36.0 ± 2.0 *</td>
</tr>
<tr>
<td>RV/ BW (mg/g)</td>
<td>0.91 ± 0.06</td>
<td>1.99 ± 0.18 *</td>
</tr>
<tr>
<td>RV/TL (mg/mm)</td>
<td>1.56 ± 0.11</td>
<td>2.33 ± 0.15 *</td>
</tr>
</tbody>
</table>

Right ventricle (RV), body weight (BW), tibial length (TL). Statistical analysis was performed using one-way ANOVA followed by Fishers’s PLSD. *P<0.05 compared to sham.
Fig. 1.
Fig. 2.

A

B
Fig. 3.
Fig. 4.
Fig. 6.

<table>
<thead>
<tr>
<th>Sham operated</th>
<th>4 days</th>
<th>1 week</th>
<th>2 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pulmonary artery banding</td>
</tr>
</tbody>
</table>


Fig. 7.

A

48 h 1 week

B

Low

High

Normalized Expression