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TITLE:

Induction and Characterization of Pulmonary Hypertension in Mice using the Hypoxia/SU5416 Model

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KEYWORDS:

Hypoxia, SU5416, Sugen, pulmonary hypertension, PH, right ventricular pressure, pulmonary vascular remodeling, right ventricular remodeling

SUMMARY:

This protocol describes the induction of pulmonary hypertension (PH) in mouse based on the exposure to hypoxia and injection of a VEGF receptor antagonist. The animals develop PH and right ventricular hypertrophy 3 weeks after the initiation of the protocol. The functional and morphometrical characterization of the model is also presented.

ABSTRACT:

Pulmonary Hypertension (PH) is a pathophysiological condition, defined by a mean pulmonary arterial pressure exceeding 25 mm Hg at rest, as assessed by the right heart catheterization. A broad spectrum of diseases can lead to PH, differing in their etiology, histopathology, clinical presentation, prognosis, and response to the treatment. Despite significant progress in the last years, PH remains an uncured disease. Understanding the underlying mechanisms can pave the way for the development of new therapies. Animal models are important research tools for this goal. Currently, there are several models available for recapitulating PH. This protocol describes a two-hit mouse PH model. The stimuli for PH development are hypoxia and the injection of SU5416, a vascular endothelial growth factor (VEGF) receptor antagonist. Three weeks after initiation of Hypoxia/SU5416, animals develop pulmonary vascular remodeling imitating the histopathological changes observed in human PH (predominantly Group 1). Vascular remodeling in the pulmonary circulation results in the remodeling of the right ventricle. The procedures for measuring right ventricular pressures (using the open chest method), the morphometrical analyses of the right ventricular remodeling (by dissecting and weighing both cardiac ventricles) and the histological assessments of the remodeling (both vascular as well as right ventricular by assessing cardiomyocyte hypertrophy and fibrosis) are described in detail.

The advantages of this protocol are the possibility of the application both in wild type and -if desired- in genetically modified mice, the relatively easy and low-cost implementation, and the quick development of the disease of interest (3 weeks). Limitations of this method are that mice do not develop a severe phenotype and PH is reversible upon return to normoxia. Prevention, as well as therapy studies, can easily be implemented in this model, without the necessity of advanced skills (as opposed to surgical rodent models).

INTRODUCTION:

Pulmonary Hypertension (PH) is a pathophysiological condition, defined by a mean pulmonary arterial (PA) pressure exceeding 25 mm Hg at rest, as assessed by the right heart catheterization[1,2]. There is a variety of diseases that can lead to PH. In an attempt to organize the PH-associated conditions, several classification systems have been developed. The current clinical classification categorizes the multiple PH-associated diseases in 5 different groups[1]. This distinction is of importance due to the fact that the various groups of patients have diseases that differ in their clinical presentation, pathology, prognosis, and response to treatment[2]. **Table 1** summarizes the current classification, complemented with the basic histopathological characteristics of each disease.

[Place **Table 1** here]

Despite significant advances in the treatment of PH-associated diseases, PH still remains without cure, with a 3-year- mortality rate ranging between 20% and 80%[3]. This indicates the imperative need for understanding the underlying mechanisms of PH and, thereafter, the development of novel therapies to prevent, slow down the progression of, and cure the disease. Animal models are of crucial importance to this scope. Currently, various models exist to study PH. The interested reader is referred to the excellent reviews on this topic[2-4]. Bearing in mind the variety of diseases leading to PH, it is obvious that the diverse conditions of human PH cannot be perfectly recapitulated in one animal model. The animal models available can be categorized in i) single-hit and , ii) two-hit models as well as, iii) knockout and iv) overexpression models[3]. In the single-hit models, PH is induced by a single pathological stimulus. Contrary to that, two-hit models combine pathological stimuli with the goal of inducing more severe PH and thus more closely imitate the complex human disease. Besides the etiological differences, the several stimuli result in PH modeling differences that depend also on the species and the genetic background of the animals[4].

One of the most commonly used classic PH rodent models is the Chronic Hypoxia model[2]. Hypoxia is known to induce PH in humans as well as in several animal models. Hypoxia has the advantage of being a physiologic stimulus for PH (**Table 1**). However, while the degree of Hypoxia used for inducing PH in rodents is much more severe than in humans, the single insult (Hypoxia) leads only to a mild form of vascular remodeling. This does not imitate the severity of human disease. The addition of a second-hit, an extra stimulus for inducing PH, showed promising results: injection of the compound SU5416 to rodents combined with the hypoxic stimulus induces a more severe PH phenotype[2,5,6]. SU5416 is an inhibitor of the vascular endothelial growth factor (VEGF) receptor-2. It blocks the VEGF receptors and leads to

endothelial cell apoptosis. Under hypoxic conditions, this stimulates the proliferation of a subset of apoptosis-resistant endothelial cells. Furthermore, SU5416 leads to smooth muscle cell proliferation. The combination of these effects results in pathologic vascular remodeling of the pulmonary circulation and leads to elevated PA pressure and right ventricular remodeling[2,5,7]. The model was first described in rats[6] and later on applied to mice[4,5,7]. The mouse model exhibits less severe vascular remodeling compared to rats. Furthermore, when returned to normoxia, PH continues to progress in rats, while in mice it is partially reversible.

The following protocol describes all the steps for modeling PH in the mouse using the Hypoxia/SU5416 method (planning, timeline, execution). Additionally, the characterization of the model is described in this protocol: functionally (by invasively measuring the right ventricular (RV) pressure using the open chest technique), morphometrically (by dissecting and weighing both the right and left ventricle of the heart), as well as histologically (by evaluating the pulmonary vascular remodeling, right ventricular cardiomyocyte hypertrophy and fibrosis).

All the steps and methods described in this protocol can be easily implemented by investigators at any experience level. While the functional measurements of the RV using the open chest technique (described here) is not the gold standard in the field, the open chest method has the advantage that it can quickly be learned and accurately reproduced even by the less experienced experimenter.

PROTOCOL:

Prior to any animal experimentation obtain the local institutional animal care committee authorization. The current experiments were performed after approval by the Institutional Animal Care and Use Committee (IACUC) at the Icahn School of Medicine at Mount Sinai.

1. PH induction

1.1. Preparation

1.1.1. Before beginning the study, carefully plan the experimental design. Ensure that mice are subjected to Hypoxia at the same time point as the first SU5416 injection. An example of the experimental design for inducing PH using the Hypoxia/SU5416 method is shown in **Figure 1A**. Control mice receive only the vehicle. For this model, SU5416 will be injected to the mice once per week for 3 consecutive weeks.

1.1.2. Use eight-ten-week-old C57BL/6 mice for this study. House the animals at 18-20 °C in a 12-h light-dark cycle. Ensure that food and water are accessible *ad libitum*.

1.1.3. Weigh the animals. Assign them randomly to each group: Normoxia and Hypoxia/SU5416.

1.1.4. Prepare the hypoxic chamber as shown in **Figure 1B.** Secure N_2 tanks near the chamber. Set the oxygen controller at a point of 10% Oxygen. Let the system reach a steady state.

1.1.5. Prepare SU5416 for injection (use a dose of 20 mg/kg body weight). SU5416 does not dissolve in aqueous solutions; therefore, dissolve the calculated amount in 100 µL DMSO[8]. e.g., for a 25 g mouse, the amount of SU5416 to be injected is 0.5 mg dissolved in 100 µL solvent (DMSO). The final concentration of SU5416 for this mouse is, therefore, 5 mg/mL.

CAUTION: SU5416 is a hazardous material. Carefully read the Safety Data Sheet accompanied by the product and make sure to take the recommended precautions when handling this substance. Wear protective gloves and (as for any injection) use eye protection. The chemical structure of SU5416 is shown in **Figure 1C.**

NOTE: Calculate an appropriate excess of the solution to compensate the volume lost during injection (e.g. in the syringe, vial etc.). Depending on the syringe used, the dead volume is approximately 200 µL. For a group of 10 mice, calculate an excess of 2 mouse doses.

1.1.6. Prepare the syringes for injection. Use 1 mL syringes with a 25 G x 5/8" needles.

1.2. SU5416 subcutaneous injection

1.2.1. Restrain the animal. Place the mouse on the lid of the cage to assist restraint. Grasp the skin and form a tent parallel to the spine. Make sure to grasp to the back of the head tightly, to avoid the potential bite injury by the mouse.

NOTE: Presence of two investigators makes the procedure faster and more accurate as one can hold the animal while the other performs the injection.

1.2.2. Insert the needle subcutaneously over the flank at the loose fold of the skin. Make sure to insert the needle parallel to the skin. Avoid penetrating the abdominal wall.

1.2.3. Inject the syringe's content (100 µL of dissolved SU5416 or vehicle).

NOTE: In order to avoid leakage after complete delivery, hold the syringe for approximately 10 s and slightly rotate the needle under the skin.

1.2.4. Withdraw the needle and return the animal to its cage. After SU5416 injection, place the cages in the ventilated hypoxia chamber.

1.3. Exposure to Hypoxia

1.3.1. Monitor the ventilation over time. Make sure to maintain 10% of the oxygen supply. Maintain normoxia animals in a semi-sealable chamber in 21% O_2.

1.3.2. Ensure that the chambers are equipped with an oxygen sensor to measure the oxygen level. Avoid extensive opening of the chambers. For cleaning and adding food and water open the chambers for not more than 20 min every 3 days.

1.3.3. Inspect animals daily. Consider stress signals such as piloerection or significant loss of weight.

NOTE: Animals under Hypoxia/SU5416 are expected to lose weight[5]. This is an indication of disease development.

1.3.4. Repeat SU5416 injection weekly for 3 consecutive weeks (see **Figure 1A** for the overview of the experimental design).

NOTE: Varying the site of injection can help reduce skin irritations.

2. Functional characterization by invasive RV pressure measurements

2.1. Preparation

NOTE: Select an anesthetic regime. Injectable or inhalable anesthetics can be used. Since a slight overdose of injectable anesthetics (especially from ketamine/xylazine or pentobarbital) can significantly affect the heart function, the use of gas anesthetics is recommended. It is of great importance to use the same anesthetic for all mice within a study.

2.1.1. Use a vaporizer to assure an accurate anesthetics dose per animal. The dose for isoflurane is as following: induction 3-4%, maintenance 1% mixed with 100% oxygen.

NOTE: Wear personal protective equipment and avoid breathing the vapor.

2.1.2. Prepare a heating pad and/or warming lamps for maintaining body temperature. Prepare a rectal temperature probe for monitoring body temperature.

2.1.3. Ensure proper ventilation. Prepare the ventilator beforehand. Prepare the Y-tube connector and check the function of the ventilator using the manual mode. Ensure the inspiratory pressure is <1 cm H_2O to avoid barotrauma. Set the respiratory rate at 110 breaths/min.

2.1.4. Prepare an endotracheal tube by cutting a 20 G intravascular catheter.

2.1.5. Prepare the instruments needed: small forceps, scissors, elastic hook retractors, vessel cauterizer, and cotton swabs. On a cotton swab adjust a small 25 G x 5/8” needle that will be used to make a small puncture in the right ventricle.

2.1.6. Prepare the Pressure Catheter, the Pressure-volume Control Unit and initiate the data acquisition software. Place the PV Catheter in a 15 mL centrifuge tube filled with PBS at 37 °C for 15 min and calibrate according to the manufacturer’s protocol.

2.1.7. For the perfusion and fixation of the organs prepare PBS and a solution of 50% PBS / 50% OCT. Prepare 2 x 10 mL syringes (with a 25 G needle): one will be used for perfusing the heart and the lung with PBS *in situ* and the other for injecting OCT/ PBS to the lung part selected for histologic examination.

2.2. Intubation

2.2.1. Weigh the mouse and record the health status before anesthesia.

2.2.2. Induce anesthesia with 3-4% isoflurane. Check the anesthesia depth by testing the toe-pinch reflex: pinch the toe of one of the limbs firmly. If the animal withdraws the limb, it is a sign of insufficient anesthesia.

2.2.3. After anesthesia induction, shave the neck and the chest areas.

2.2.4. Place the mouse on the heating pad. Place a rectal temperature probe for monitoring body temperature.

NOTE: Maintenance of body temperature is of importance for the functional measurements. The body temperature should be approximately 36.5-37 °C.

2.2.5. Using curved forceps attach a suture thread to the upper incisors of the mouse, stretch and fix to the heating pad with surgical tape. Secure the limbs of the mouse using surgical tapes.

2.2.6. For intubating the animal make a small incision of approximately 1 cm in the medial cervical skin using small scissors.

NOTE: Oral intubation is an alternative method that requires more experience.

2.2.7. With a cotton-tipped applicator separate bluntly the parotid and submandibular salivary glands at the midlevel. This will expose the muscles overlying the trachea.

2.2.8. Carefully cut these muscles exposing the trachea.

2.2.9. With small scissors make a small incision between the tracheal cartilages and insert the prepared endotracheal tube. Take out the metal guide of the intravascular catheter.

2.2.10. Connect the catheter to the ventilator. Verify the tracheal tube position by manually gently inflating the lungs. Secure the position with tape.

2.2.11. Maintain a 1% isoflurane anesthesia throughout the procedure.

2.2.12. Regularly monitor the depth of anesthesia by testing the toe pinch reflex. Adjust the anesthesia accordingly.

NOTE: The recommended heart rate during the experiments, under 1% isoflurane anesthesia, is approximately 400 beats /min. Maintenance of body temperature and anesthesia are essential for controlling the heart rate. Excess of isoflurane can reduce the heart rate. However, recovery can be achieved by reducing the isoflurane rate.

2.3. RV pressure measurements (open chest approach)

2.3.1. With small scissors perform a skin incision of approximately 1 cm over the xiphoid process and the upper abdominal part. Separate the skin covering the chest and the abdominal wall of the upper abdominal quadrants: start at the middle line, distal to the xiphoid and carefully move laterally on both sides. Use thermocautery to control bleeding.

NOTE: The goal is to have access to the thoracic cavity through the abdominal wall.

2.3.2. Open the abdominal cavity and cut the diaphragm carefully, taking care not to injure the beating heart or the lungs.

NOTE: The goal is to expose the apex and the right ventricle of the heart. Good exposure and view of the heart are of crucial importance for the correct placement of the catheter. It is of great importance to avoid bleeding throughout the procedure. Even small changes in the intravasal volume can change the load of the right heart and affect the recorded parameters.

2.3.3. Gently remove the pericardium using a cotton-tipped applicator.

2.3.4. Just before placing the pressure catheter in the heart, bring the catheter next to the mouse.

2.3.5. Using the prepared cotton-tipped applicator with the needle make a stab wound in the apical distal part of the right ventricle. Carefully remove the needle and insert the pressure catheter in this hole.

NOTE: This should work without applying force. In case this is not possible try making a new hole near the first one, in order to avoid extended injury of the heart. The needle should not be inserted more than approximately 3 mm.

2.3.6. Insert the pressure catheter parallel to the direction of the right ventricle, with the tip facing the pulmonary artery.

2.3.7. Watch the pressure wave tracing to ensure correct positioning of the catheter. Representative tracings are demonstrated in **Figure 2.**

2.3.8. Allow the pressure signal to stabilize. Pause respirations and obtain at least 3 measurements. In between the individual measurements allow the animal to be ventilated.

2.3.9. Once all measurements are recorded remove the catheter and place carefully back to the PBS filled centrifuge tube in the water bath.

NOTE: After the completion of the experiment clean the catheter according to the manufacturer's instructions.

2.4. Euthanasia and lung perfusion

2.4.1. Upon completion of the experiment euthanize the mouse by exsanguination.

2.4.2. Open the chest widely. With scissors cut the entire sternum, paying attention not to injure the heart or the lungs.

2.4.3. With iris scissors make a small incision in the left ventricle to allow blood to leave the chamber.

2.4.4. Place the 25 G needle of a syringe containing 10 mL of PBS in the right ventricle and inject the PBS until lungs are cleared of blood.

2.4.5. Once this step is completed, confirm euthanasia by vital tissue harvest (heart and lungs): cut the cava and aortic attachments and remove the heart and lungs *en block*.

3. Morphometric characterization

3.1 Immediately after removing the heart and lung (Step 2.4.5) isolate the heart and remove both atria. With curved tenotomy scissors dissect carefully the right ventricle (RV) from the left ventricle (LV), leaving the septum (S) with the left ventricle. Weigh RV and LV+S and calculate the Fulton index= RV/LV+ S (**Figure 3**)[5,9].

3.2 Take a part of the right heart and place it in an OCT prefilled embedding mold. Use the other part of the right ventricle for RNA and/or protein analysis. Snap freeze in dry ice and store at -80 °C.

3.3 Use iris scissors to isolate the lungs from the heart and any other remaining tissue.
NOTE: For the preparation of the lungs, the perfusion as described above (Steps 2.4.3-2.4.5) is of great importance.

3.4 Snap freeze part of the lung and store it for RNA, protein extraction or other assays.

3.5 Use the other part of the lung for histological analysis. For this purpose, insert the syringe containing 50% PBS and 50% OCT in a bronchus of the used lobe[10,11]. The experimenter can easily see that the lung gets inflated when the syringe's content is perfused in the tissue.

3.6 Place these pieces of lung in embedding molds prefilled with OCT and snap freeze them in dry ice. Store the samples at -80 °C after they are frozen.

3.7 Prepare 8 μm sections of RV and lung using a cryostat machine. Air dry the sections at room temperature for 30 min.

3.8 Fix the slides at room temperature using 10 % paraformaldehyde (PFA) for 10 min.

NOTE: PFA is a known human carcinogen. Reduce exposure risk by using a chemical fume hood, proper procedures and personal protective equipment. Refer to the Material Safety Data Sheet (MSDS) for further information.

3.9. Vascular remodeling assessment by Hematoxylin/Eosin staining

Note: Perform Hematoxylin/Eosin staining in order to assess the structural changes of the heart and the vascular remodeling in the lung (**Figure 3**).

3.9.1 Stain with Hematoxylin solution for 8 min.

3.9.2 Rinse with running tap water for 5 min followed by a quick rinse in distilled water.

3.9.3 Rinse in 95% EtOH for 1 min and counter-stain in the Eosin solution for 1 min.

3.9.4 Dehydrate (80% Ethanol 10-30 s, 100 Ethanol for 1 min and 100% Toluol for 3 min).

3.9.5 Clean slide with a tissue paper; mount and cover with a coverslip. Dry the slides overnight at room temperature.

NOTE: The solutions used for staining may be hazardous. Reduce exposure risk by using a chemical fume hood, proper procedures and personal protective equipment. Refer to the MSDS for further information.

3.10 Right ventricular fibrosis assessment by Picrosirius Red Staining

NOTE: In the Picrosirius Red Staining, Picrosirius Red, which is acid, binds to collagen[12]. Therefore, this staining can be used for a histological examination of the collagen content.

3.10.1 Incubate the slides in preheated Bouin's Solution at 58 °C for 1 h.

3.10.2 Wash the slides in running tap water to remove yellow color from sections for 10-15 min.

3.10.3 Stain in 0.1% Fast Green for 20 min at room temperature.

3.10.4 Rinse in 1% Acetic Acid for 1 min.

3.10.5 Rinse in tap water for 5 min.

3.10.6 Stain in 0.1% Sirius red for 30 min at room temperature followed by dehydration in Toluol.

CAUTION: The solutions used for staining may be hazardous. Reduce exposure risk by using a chemical fume hood, proper procedures and personal protective equipment. Refer to the MSDS for further information.

3.11. RV cardiomyocyte hypertrophy assessment by WGA Staining

NOTE: Hypertrophy of the right heart at the cellular level can be assessed by performing a Wheat Germ Agglutinin (WGA) staining (**Figure 4**).

3.11.1 Fix the slides in cold Acetone solution for 15 min followed by 3 steps of washing in PBS (5 min each).

3.11.2 Block with 10% goat serum in a Dako solution for 30 min at room temperature.

3.11.3 Incubate the slides with WGA: Add WGA 1:200 and incubate for 1 ½ h at 37 °C in the dark.

3.11.4 Wash the slides three times with PBS.

3.11.5 Incubate the slides with a nucleic acid dye.

3.11.6 Wash the slides three times with PBS.

3.11.7 For mounting, remove the excess liquid and apply mounting media and a coverslip. Dry the slides for 1 hour at room temperature in the dark and store at 4 °C.

NOTE: The solutions used for staining may be hazardous. Reduce exposure risk by using a chemical fume hood, proper procedures and personal protective equipment. Refer to the MSDS for further information.

3.12. Perform Immunochemistry of the lung to further and specifically assess vascular remodeling. For example, smooth muscle cell staining can be used to assess the muscularization of the vessels, while von Willebrand Factor staining can be used to visualize endothelial changes. These methods are described elsewhere[5].

REPRESENTATIVE RESULTS:

In this protocol, we describe in detail the creation of the Hypoxia/SU5416 model for inducing PH in mice. Furthermore, we include all the steps for performing the pulmonary vascular and cardiac evaluation at the end of the observation period.

An overview of the experimental design for this model is shown in **Figure 1A**[13,14]. Mice are subjected to normobaric hypoxia (10% O2) and subcutaneously injected once a week with SU5416 for three consecutive weeks. The stimuli used to induce PH in this protocol are shown in **Figure 1B and 1C**.

The VEGF receptor antagonist SU5416 acts by causing endothelial cell apoptosis and, therefore, allowing the proliferation of apoptosis resistant-endothelial cells. This leads to vascular remodeling in the pulmonary vasculature and increased vascular resistance[5]. The elevated pressure in the pulmonary circulation increases the RV afterload and leads progressively to the right ventricular (RV) dysfunction and failure[9]. In the first step, the success of the Hypoxia/SU5416 protocol can be evaluated by functionally assessing the RV function at the end of the observation period. In this protocol, we describe in detail the invasive assessment of the RV systolic pressure using the open chest RV pressure measurement method. Representative pressure curves and quantitative analysis of the right ventricular pressure are displayed in **Figure 2.**

How can we quantify vascular remodeling, which leads to elevated vascular resistance and consequently PH? Histomorphometry is the gold standard for characterizing the pulmonary vasculature. In this protocol, we describe in detail the Hematoxylin & Eosin Staining (H&E) protocol. After staining and capturing of the images, the pulmonary arteries can be distinguished in small (<50 µm) and larger ones (> 50 µm). Bronchial arteries were excluded from our study. For assessing the medial thickness, the external (ED), as well as the internal diameter (ID) of the arteries, is measured. Representative images of remodeled pulmonary arteries after Hypoxia/SU5416 treatment are shown in **Figure 3A.** The percentage of arteries medial thickness in relation to cross-sectional diameter is shown in **Figure 3B**. The morphometric analysis of distal pulmonary arteries demonstrates a significant increase in medial thickness in Hypoxia/SU5416-treated mice in comparison with Normoxia animals (**Figure 3**).

The increased afterload leads to RV hypertrophy and as the disease progresses to RV fibrosis[9,15]. RV hypertrophy can be assessed morphometrically by measuring the Fulton Index (RV/LV+Septum) as well as by measuring cardiomyocyte (CM) hypertrophy. The weight ratio of the right ventricle (RV) to the left ventricle (LV) plus septum [RV/(LV+S)] is calculated as an index of right ventricular hypertrophy. Representative results from the Fulton Index in Hypoxia/SU5416 and Normoxia mice are shown in **Figure 4B**. The method described here for assessing CM hypertrophy is the staining of right ventricular sections with Wheat Germ Agglutinin (WGA). WGA binds to glycoproteins of the cell membrane and can be used for determining the cross-sectional area of myocytes[16,17]. Representative images of right

ventricular sections stained with WGA are shown in **Figure 4A**. Quantifications of CM area in both diseased and control mice are shown In **Figure 4A.** Hypoxia/SU5416 exposure results in a marked increase in cardiomyocyte size and right ventricular hypertrophy (**Figure 4**). Others and we have previously shown that, when compared to the single hit (only Hypoxia), Hypoxia/SU5416 aggravates the RV phenotype[5,18].

FIGURE AND TABLE LEGENDS:

Table 1: Overview of the clinical classification of PH, along with the main histopathological features within the groups. Suitability of the Hypoxia/SU5416 protocol for modeling PH. This table has been modified from [19]).

Figure 1: Overview of the Hypoxia/SU5416 method. (A) Experimental design for the Hypoxia/SU5416 mouse model. SU5416 is injected subcutaneously once a week for 3 consecutive weeks. **(B)** Schematic representation of the hypoxia system. The controller senses and regulates oxygen inside the chamber by infusing Nitrogen through the gas infusion tube. (**C**) Chemical structure of SU5416.

Figure 2: Right ventricular pressure in mice exposed to chronic hypoxia combined with SU5416 injection. (**A**) Representative tracings of invasive pressure measurements of the right ventricle (RV). (**B**) RV systolic pressure in Hypoxia/SU5416 mice and control animals exposed to Normoxia. n = 6-8 mice per group. *** $p < 0.001$. All quantitative data are reported as means $\pm$ SEM.

Figure 3: Hypoxia/SU5416 induces pulmonary vascular remodeling. (A) Representative Hematoxylin/Eosin-stained sections of lungs from the indicated groups demonstrate increased media wall thickness in pulmonary arteries of Hypoxia/SU5416 mice. Scale bar: 50 μm. **(B)** Percentage of arteries medial thickness in relation to cross-sectional diameter. n = 5 mice per group. *** $p < 0.001$. All quantitative data are reported as means $\pm$ SEM.

Figure 4: Right ventricular hypertrophy in mice exposed to chronic hypoxia combined with SU5416 injection. (**A**) (Left) Representative WGA (Wheat Germ Agglutinin) staining of right ventricle tissue after the indicated treatment. Scale bar: 50 μm. (Right) Quantitative analysis of the data. n = 5 mice per group. (**B**) RV hypertrophy reflected by the RV weight over LV plus interventricular septum (S) weight ratio (Fulton index= RV/LV+ S) in each group. n = 8 mice per group. *** $p < 0.001$. All quantitative data are reported as means $\pm$ SEM.

DISCUSSION:

This protocol describes how to model PH in mice by combining two pathological stimuli: chronic hypoxia and SU5416 injection (Hypoxia/SU5416)[18]. In an attempt to correlate this mouse model with the human PH condition, one inevitably must look at the current PH classification, shown in **Table 1**. PH in almost all forms is characterized by pulmonary vasoconstriction and aberrant proliferation of endothelial and smooth muscle cells. This leads to elevated pressure in the pulmonary arteries and consequently to increased afterload of the right ventricle.

Every attempt to characterize an animal model for PH should include the evidence for the histopathological remodeling of a) the pulmonary vasculature and b) the right ventricle. The single-hit hypoxia mouse model leads to a mild form of vasculature remodeling[2,3]. These pathological findings include the muscularization of previously non-muscularized vessels, accompanied by an endothelial, smooth muscle cell and fibroblast proliferation. These findings are aggravated by the addition of the second hit (SU5416 injection). The effects are reversible in the single-hit (Hypoxia) model and only partly reversible in the Hypoxia/SU5416 model.

The main cause of death for PH patients is the right ventricular failure (RVF)[4,20]. Pulmonary vascular remodeling in animal models is not always accompanied by RVF. In order to characterize an animal model in terms of RVF morphological, functional and molecular data should be analyzed. The latter is beyond the scope of this protocol. RV morphological remodeling includes both macro- and microscopical aspects. At the macroscopical level, the main index for RV hypertrophy is the Fulton index, defined as the weight of RV divided by the left ventricular (LV) and Septum (S) weight (RV/LV+S). At the microscopical level, fibrosis, inflammation, and hypertrophy can be assessed by Sirius red, Hematoxylin/Eosin and WGA staining, respectively.

The mouse Hypoxia/SU5146 model (which is described here) shows an RV dysfunction, as measured by elevated systolic pressures and morphological criteria. Regarding the pulmonary vascular remodeling, medial hypertrophy is observed three weeks after initiation of the protocol. Compared to the Hypoxia/SU5416 model in rats, the mouse model does not cause RV Failure (only moderate dysfunction), does not lead to severe obliterative angiopathy, as observed in severely diseased humans, and the pulmonary pathology ameliorates after return to normoxia. Overall, the mouse Hypoxia/SU5416 model is suitable for imitating vascular injury as encountered in PH, predominantly Group I (partially Group III, see **Table 1**)[1,19]. The advantage of this model is the application in wild type (genetically unmodified) mice, the relatively easy and low-cost implementation, the relatively low mortality of the diseased animals, and the quick development of the disease of interest (3 weeks). PH prevention, as well as therapy (proof of concept), studies can easily be implemented in this model, without the necessity of advanced skills as opposed to surgical rodent models.

When implementing the protocol there are some critical steps, which one should keep in mind.
When planning the study, one should keep in mind that in the Hypoxia/SU5416 Group the mortality of the animals varies between 5-10 % (unpublished observations). Therefore, in order to reach statistical power and avoid underpowered studies, at least 10 mice per group is recommended. The solubility of SU5416 is low. Therefore, DMSO or another solvent (e.g. CMC, Ciuclan) have to be used. DMSO in high doses can be toxic. The LD_{50} for subcutaneous (s.c.) use in mice has been reported to be 13.9 – 25.6 g/kg[21,22]. LD_{50} is defined as the dose required to kill 50% of the members of a tested population after a specified test duration[21,22]. For a mouse that weighs 25 g, 4.4 g/Kg of DMSO is used (calculations based on DMSO density of 1.1 g/mL and 0.1 mL applied s.c./mouse). Therefore, the subcutaneously given dose is much lower than the LD_{50} value. In our hands, application of SU5416 dissolved in DMSO, as described here, can cause skin irritation in some cases, but no other toxic effects are observed. However, several reports

recommend the use of CMC (Carboxymethyl cellulose) as an alternative vehicle to SU5416[14]. When performing the RV functional measurements, close attention has to be paid at the body temperature, bleeding, as well as depth of anesthesia, as assessed by testing the mouse reflexes. The open chest technique for assessing the RV pressure as described here has the advantage of easily being implemented even by an inexperienced user. The closed chest method (described elsewhere[23-25]) has the advantage of being less invasive and can, therefore, be implemented also in non-terminal experiments. It requires though a high level of expertise.

After the first description of the Hypoxia/SU5416 model in rats, the mouse model has been successfully used in several studies[5,9,13]. However, there is evidence that the results depend on the genetic background and sex of the mice, the manufacturer of SU5416 and the frequency of SU5416 injection[26]. While injecting SU5416 over three consecutive weeks leads to PH in mice, a single dose would not induce PH[4]. Furthermore, other forms of PH, such as those associated with left heart disease or due to chronic thromboembolic disease, require etiology-related models. New therapies should be tested in at least 2 different animal models, before being able to pave the way to translational studies.

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DISCLOSURES:

The authors have nothing to declare.

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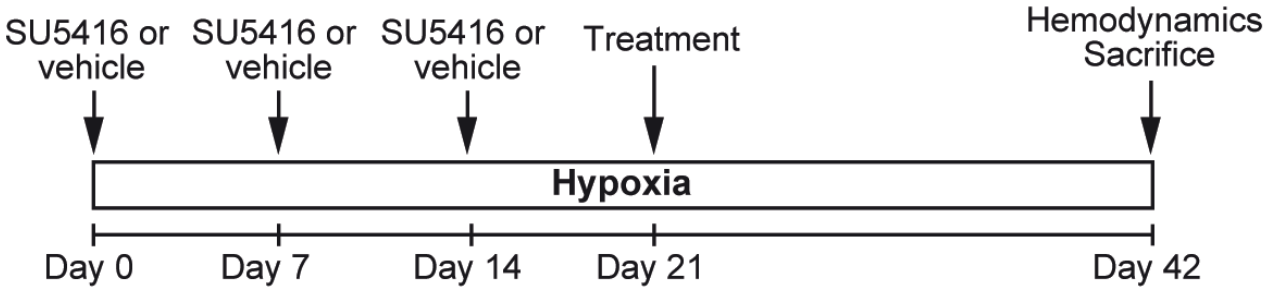
Figure 1

A



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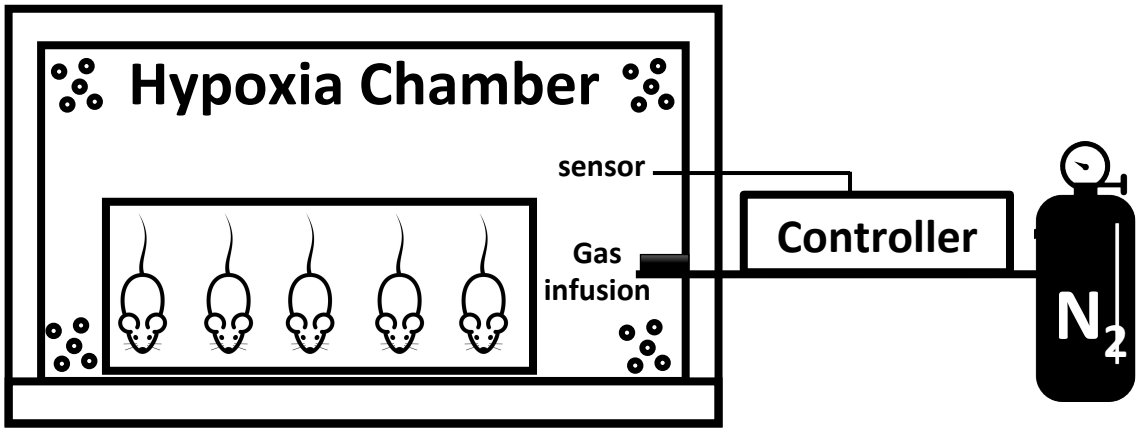

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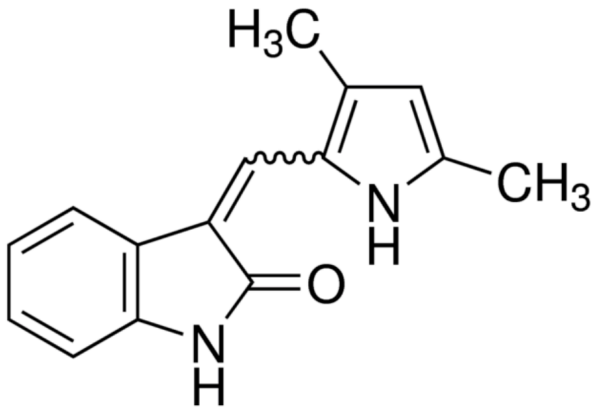


Figure 2

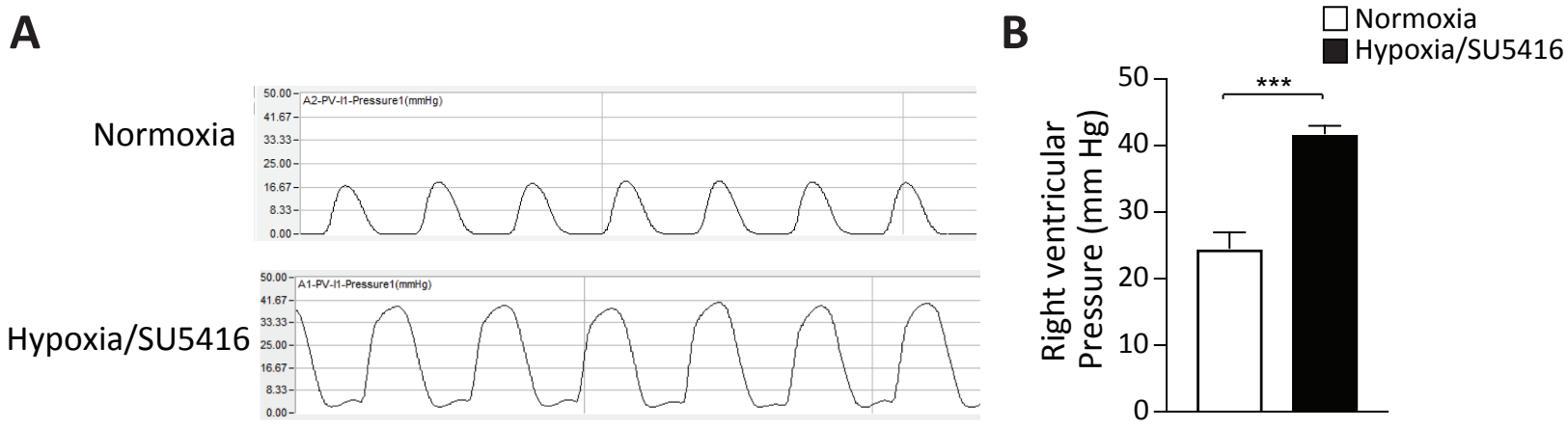


Figure 3

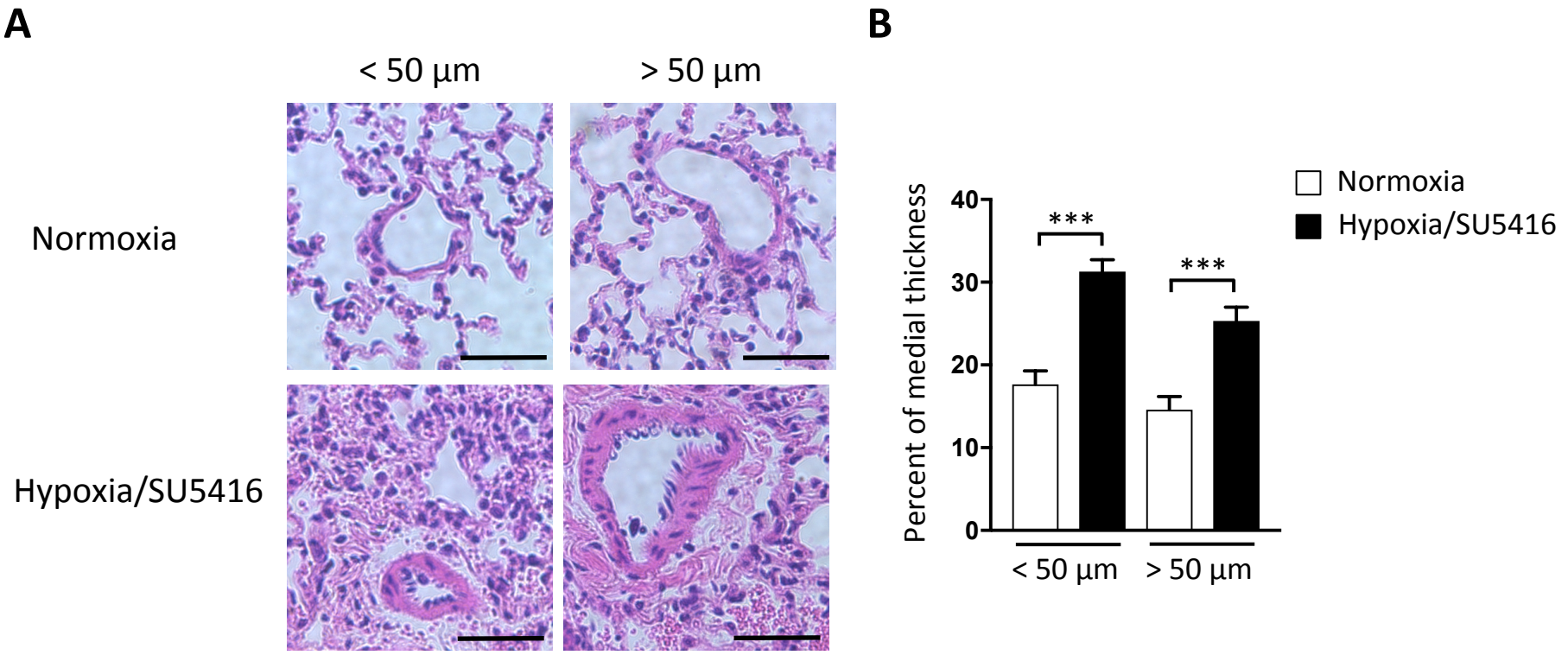
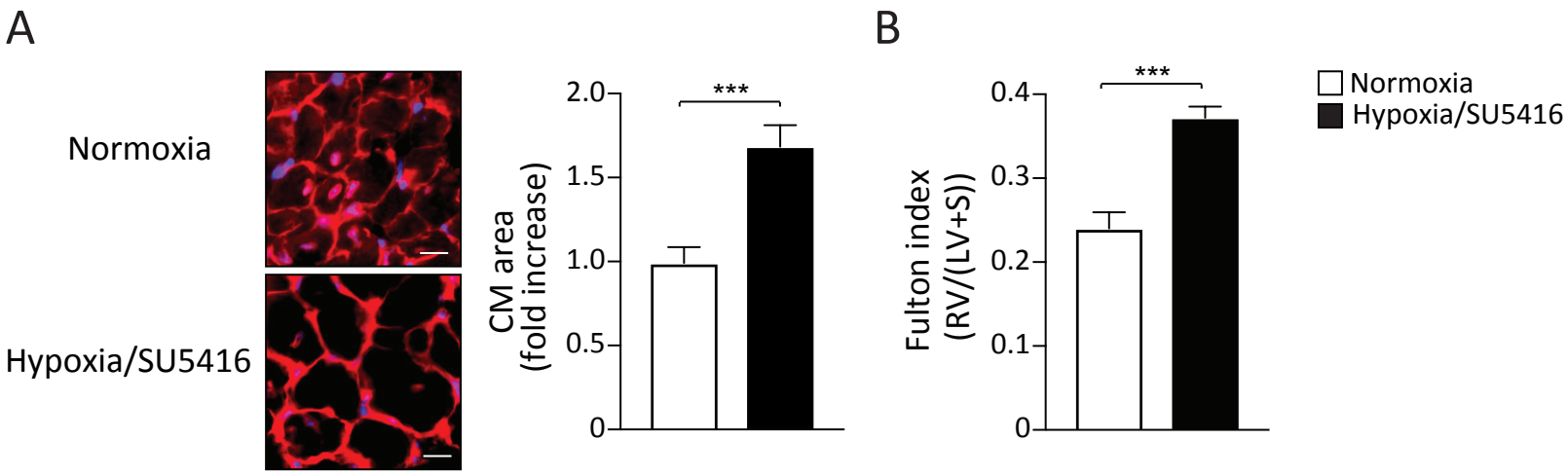


Figure 4



PH group	Histopathological Features	Animal model
Group 1 PAH	Early phase: Medial hypertrophy intima proliferation Muscularization of normally nonmuscular arteries	Hypoxia/Sugen mouse
	Late phase: Intima fibrosis Loss of luminal vascular volume Plexiform lesions Recanalization of arteries Fibrinoid necrosis	Hypoxia/Sugen rat
Group 1' Pulmonary venooclusive disease	Congestion of pulmonary parenchyma, Hemosiderosis, Fibrosis of small veins/venules	
Group 2 PH with left heart disease	Arterialization of large or middle-sized pulmonary veins Interstitial edema and fibrosis Hemosiderosis Medial hypertrophy/adventitial thickening of pulmonary arteries	
Group 3 PH associated with lung disease and/or hypoxemia	3.1 and 3.3–3.5. Hypoxic pulmonary vasculopathy Muscularization of arterioles Medial hypertrophy of muscular pulmonary arteries 3.2. Pulmonary vasculopathy associated with interstitial lung disease Features of hypoxic pulmonary vasculopathy Intimal fibrosis of arteries	Hypoxia/Sugen rat
Group 4 PH due to chronic thrombotic/embolic disease	Thromboembolic obstruction of distal pulmonary arteries Eccentric intimal fibrosis	
Group 5 Miscellaneous	Heterogeneous group of disorders: some showing features of congestive vasculopathy some postthrombotic vasculopathy	

Name of Material/ Equipment	Company	Catalog Number
Acetic acid glacial	Roth	3738.1
Acetone, Histology Grade	The Lab Depot	VT110D
ADVantage Pressure-Volume System	Transonic	ADV500
Bouin's solution	Sigma	Ht10132
Cautery System	Fine Science Tools	18000-00
Connection tubing and valves		
Cotton-Tipped Applicators	Covidien	8884541300
Coverslips, 24 x50 mm	Roth	1871
Data Acquisition and Analysis	Emka	iox2
Direct Red 80	Sigma	365548-5G
DMSO (Dimethyl Sulfoxide)	Sigma Aldrich	276855
Dry ice		
Dumont # 5 forceps	Fine Science Tools	11251-10
Dumont # 7 Fine Forceps	Fine Science Tools	11274-20
Embedding molds	Sigma Aldrich	E-6032
Eosin Solution Aqueous	Sigma	HT110216
Ethanol, laboratory Grade	Carolina Biological Supply Company	861285
Fast Green FCF	Sigma	F7252-5G
Fine scissors	Fine Science Tools	14090-09
Goat Serum	invitrogen	16210-064
Heating pad	Gaymar	T/Pump
Hematoxylin 2	Thermo Scientific	7231
Hypoxic chamber	Biospherix	A30274P
Induction chamber	DRE Veterinary	12570
Intubation catheter (i.v. catheter SurFlash (20 G x 1"))	Terumo	SR*FF2025
Iris scissors	Fine Science Tools	14084-08
Isoflurane	Baxter	NDC-10019-360-40
Isoflurane vaporizer	DRE Veterinary	12432
Mice (C57BL/6)	Charles River	

Needles 25 G x 5/8"	BD	305122
OCT	Tissue Tek	4583
PBS (Phosphate Buffered Saline)	Corning	21-031-CV
Piric Acid- Saturated Solution 1.3 %	Sigma	P6744-1GA
Pressure volume catheter	Transonic	FTH-1212B-4018
Retractor	Kent Scientific	SURGI-5001
Static oxygen Controller ProOx 360	Biospherix	P360
SU 5416	Sigma Aldrich	S8442
Surgical Suture, black braided silk, 5.0	Surgical Specialties Corp.	SP116
Surgical tape	3M	1527-1
Syringe 10 ml	BD	303134
Syringes with needle 1 ml	BD	309626
Sytox Green Nuclein Acid Stain	Thermo Scientific	S7020
Tenotomy scissors	Pricon	60-521
Toluol	Roth	9558.3
Ventilator	CWE	SAR-830/P
WGA Alexa Fluor	Thermo Scientific	W11261
Xylene	Roth	

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1. The editor has formatted the manuscript to match the journal's style. Please retain the same.
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