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S100A1 Gene Therapy in Small and Large Animals

Patrick Most, Philip Raake, Christophe Weber, Hugo A. Katus, and Sven T. Pleger

Abstract

Myocardial in vivo gene delivery is a valuable technique to investigate the relevance of a protein of interest on cardiac contractile function, hypertrophy, and energy state in healthy animals as well as in a variety of models of cardiovascular disease. Rodent models are used to screen effects and to investigate molecular mechanisms, while large animal models, more closely reflecting human anatomy, physiology, and function, are inevitable for translational therapeutic approaches. The gene of interest, whose expression is driven by a non-cardioselective or cardioselective promotor is cloned into a viral vector. This vehicle is then delivered using an appropriate administration route to target the heart and to achieve efficient protein expression in myocardium.

Here we describe myocardial gene therapy in small and large animal models of postischemic heart failure used to reveal the positive inotrope, antihypertrophic, and pro-energetic action of the small calcium sensor protein S100A1.

Key words: S100A1, Calcium, EF-hand, Myocardial gene therapy, Retroinfusion, Antegrade delivery, Adenovirus, Adeno-associated virus, Efficacy, Cardioselective

1. Introduction

There was tremendous progress in the field of in vivo genetic engineering during the last decade contributing significantly to reveal molecular mechanisms and to explore molecular-targeted treatment strategies in cardiovascular disease (1–6). Myocardial gene delivery is an add-on to transgenic animal models offering the possibility to initiate protein (over)expression at a certain time point in healthy animals, and thus circumventing potential side effects such as the possibility of a lethal embryonic phenotype or an evolutionary switch of the mode of action of the protein of interest. Myocardial gene delivery can also be used as a treatment option (gene therapy)

in animal models of cardiovascular disease. The choice of species depends on suitable or existing animal models as well as on the intention to either screen effects or perform translational research and is of utmost importance for the technique of cardiac gene delivery, the administration route, and the viral vector (3, 6–9).

A significant aspect of myocardial gene therapy is analyses of potential detrimental effects of (a) the applied vector, (b) the gene of interest, and (c) the delivery procedure itself. Especially dosing of the vector, the route of vector administration, vector purification, choice of vector, choice of promotor, and mechanical aspects such as injection pressure may be of major importance interpreting observed effects prior clinical translation (10–12).

Here we demonstrate efficient cardioselective S100A1 gene therapy in rat and pig models causing both, increased global cardiac function and cardiac reverse-remodeling in postischemic heart failure (13, 14). Positive inotrope, antihypertrophic and pro-energetic effects of S100A1 gene therapy in heart failure were mediated by calcium dependent direct binding to distinct intracellular target structures: the ryanodine receptor (RyR2), the sarcoplasmic reticulum calcium ATPase (SERCA2a), the sarcomeric titin, and mitochondrial enzymes such as the F1-ATPase (8, 13–26). The described protocols are based on techniques established by Walter Koch et al., Roger Hajjar et al., Kenneth Chien et al., and Peter Boekstegers et al. which were modified to work best in our hands for efficient and reproducible cardiac gene delivery (27–30). To achieve myocardial S100A1 overexpression in different species, distinct AAV serotypes, cardioselective promotors, and administration routes were used (13, 14). Thus, we describe methods to genetically target the whole heart in a rat model, while for anatomy reasons, in pig the target area of gene delivery is the supply zone of the anterior descending coronary artery (LAD) (13, 14).

Adeno-associated virus (AAV) vectors were produced, purified, and quantitated in core facilities of the "Deutsches Krebsforschungszentrum (DKFZ) and the Center for Translational Medicine at Thomas Jefferson University in order to achieve high-titer AAV vector solutions without toxic contamination. The described procedures should enable to reproduce efficient cardioselective gene therapy in small and large animal models and highlight potential pitfalls.

2. Materials

2.1. Myocardial Gene Delivery in Rats

1. Isoflurane condenser (e.g., Dräger or Harvard Apparatus).
2. Surgical cold light source (Harvard Apparatus).
3. Self-made rat laryngoscope (see Note 1).

4. Standard rubber band (~10 cm total length).
5. Styrofoam board.
6. Styrofoam shields to protect the hindlimbs of the rat from freezing.
7. Blunt nose tracheal cannula (Havard Apparatus, size depends on rat's weight).
8. Respirator suitable for rodents (Harvard Apparatus).
9. Thermometer.
10. Ice packs (18 × 10 × 1 cm).
11. Heating pad (40 × 20 cm) (Harvard Apparatus).
12. Standard surgical set of sterile drapes and autoclavable scissors, forceps, surgical clamps, and rib stretcher (Hereaus).
13. P-50 tubing (Becton Dickinson).
14. 4-0 and 2-0 silk (Becton Dickinson).
15. Millar pressure transducer system; 2F SPR-320 catheter (Millar instruments).
16. Waterproofed marker.
17. Chapstick (Labello).
18. Adenosine (Glaxo).
19. Substance P (Sigma chemical).
20. Dobutamine Liquid (Fresenius).
21. Syringes (50 ml, 3 ml, 1 ml) and needles (30½ gauge; Becton Dickinson).
22. 2.5×10^{11} total viral particles of an AAV6-transgene.
23. Timer.
24. Defibrillator suitable for kids (Medtronic).
25. Small electrical cauter (Harvard Apparatus).

2.2. Myocardial Gene Delivery in Pigs

1. Respirator suitable for pigs (Dräger).
2. Ketamine, Xylazine, Atropine, Buprenorphine, Heparine, Nitroglycerine, 0.9% saline, and Isoflurane.
3. Tracheal cannula suitable for pigs (Mallinckrodt).
4. ECG monitoring, pulse oximetry monitoring (Dräger).
5. Standard surgical set of sterile drapes and autoclavable scissors, forceps, surgical clamps, and rib stretcher (Hereaus).
6. Percutaneous transluminal coronary artery (PTCA) angioplasty set (Smiths).
7. Contrast dye (Bracco).
8. 3-0 and 4-0 silk (Becton Dickinson).

9. 6F and 9F vascular sheath (Cordis).
10. Peripheral venous line, peripheral venous catheter (20 gauge; Cordis).
11. Coronary sinus catheter (Attain Command 6250 MB or 6250 MPA2; Medtronic).
12. Coronary sinus balloon catheter (Attain 6215-80 cm; Medtronic).
13. 0.0035′ wire (Medex).
14. 6F Judkins Right catheter (Cordis).
15. Angioplasty balloon (3.5 × 12 mm; Maverick).
16. 1.5×10^{13} total viral particles of AAV6- or AAV9-transgene.
17. Cathlab angiopump (Philips).
18. X-ray C-arm (Philips).
19. Timer.
20. Defibrillator (Medtronic).
21. Balance Middleweight PTCA wire (Abbott).

3. Methods

3.1. Myocardial Gene Delivery in Rats

3.1.1. Intubation of the Rat

1. Place the anesthetized rat (250–300 g) on the table (legs upright), on a flat styrofoam board (~2 cm thick), with the rats head towards you. Tape down the fore- and hindlimbs of the rat on the styrofoam board.
2. To continue anesthesia place the isoflurane line (1.2%) into a 50 ml syringe and place the large open end of the syringe over the rat's head.
3. Move a strong cold light source above the rat's upper thorax/lower neck. Turn off the anesthesia to intubate.
4. Open the rat's mouth and lift up the tongue using a self-made laryngoscope and keep the upper incisors down with a rubber band attached to the Styrofoam box. Intubate the rat by advancing a blunt nose cannula into the trachea (see Note 1).
5. Turn on the respirator; 8–9 ml at 45 resparations/min. When the rat is intubated the chest will go up and down in rhythm with the movements of the respirator, otherwise the stomach will swell. Tape-down the respirator hose to fix it.
6. Shave the animal around the larynx and vacuum. The surgical field will be wiped with alcohol and prepared with Chlorhexadine solution

3.1.2. Preparation Prior Gene Delivery in Rat

1. Place a thermometer in the anus of the rat to monitor body temperature; fix it by taping down.
2. Place ice packs ($18 \times 10 \times 1$ cm) on the hindlimbs of the rat. Use two layers of Styrofoam (3 mm thick) to protect the legs from freezing and necrosis (see Note 2).
3. Cool down the rat to below 30°C (between 28 and 30°C) while preparing the carotid artery (see Note 3).
4. Turn on a heating pad and lay aside (will be used after the virus is injected).
5. Using a scalpel blade make an incision from just below the forelimbs anterior to the upper (proximal) third of the sternum along the midline.
6. Grab the tissue with forceps and clamps and expand, do not cut because of blood vessels. There are muscles on either side of the neck that help the head turn, the Sternomastoids, and muscles that lie on top of the trachea, the Sternohyoids, cut the sternohyoids down the center line exposing the ridged trachea.
7. Lift (!) the sternum and cut the upper (proximal) third of the sternum (~ 1 cm) just in the very midline of the sternum to avoid major bleeding by injuring the Aa. Mammaria internae. Cut the sternum with scissors in short snips, significant force is required to cut through, go slow. If some bleeding occurs that's ok, use the electric cauter to stop it. Remember the heart is underneath the sternum.
8. Insert a rib stretcher between the severed bones of the upper third of the sternum. Carefully stretch the upper (proximal) third of the sternum.
9. Open the pericardium and avoid opening of the pleura (!) (see Note 4).
10. Clamp the thymus and deflect to the left side (see Note 5).
11. Identify the right carotid artery and separate it from connecting tissue and the N. Vagus, which runs alongside it.
12. Take a loop of a 4-0 silk suture and slip it underneath the right carotid artery, then split the suture and move one strand towards the heart and the other towards the neck. Make a loop of the suture closest to the heart, but do not tighten.
13. Place a loop of a 2-0 silk suture under the ascending aortic. Loop the aorta ascendens. Don't loop the pulmonary artery or the vena cava superior (see Note 6).

3.1.3. Myocardial Gene Delivery in Rat

Once the body temperature of the rat is below 30°C:

1. Knot the loop on the carotid artery situated towards the head of the rat. Put tension on the silk by taping to the Styrofoam to stretch the carotid artery.

2. Clamp the carotid below the second loop situated towards the heart.
3. Cut a tiny hole (a third of the diameter of the vessel) in the upper carotid artery between the two loops using fine scissors.
4. Insert a 2F Millar pressure transducer (SPR-320) into the right carotid artery and advance it via the ascending aorta and the aortic valve into the left ventricle. Afterwards pull back the pressure transducer just above the aortic valve using the shape of the pressure curve and define the distance between the hole in the carotid artery and the aortic valve. Mark the distance on the pressure transducer and pull out the catheter (see Note 7).
5. Mark the exact distance between the hole of the carotid artery and the aortic valve on the P-50 tubing.
6. Insert the P-50 tubing into the right carotid artery. Advance just above the aortic valve (not in the left ventricular chamber) by using the mark (see Note 8).
7. Reduce the isoflurane to 0.5%.
8. Inject 1.2 mg of adenosine in 400 µl directly into the right ventricle chamber using a 30½ gauge needle. This will induce a temporary atrioventricular blockage and stop the heart beating.
9. Immediately, stop blood flow from the ascending aorta proximal of the tip of the P-50 tubing (which is still between the clamp and the aortic valve) by tightening the 2-0 suture and fix the suture using a small but strong clamp. Once you clamp the aorta start the timer.
10. Rapidly inject half of the prepared virus solution: (2.5×10^{11} total viral particles in a volume of 250 µl, 8 µg of substance P in 80 µl, and 1.6 ml of saline totaling to ~1.9 ml volume in a 3 ml syringe) as a bolus via the P-50 tubing. We were using AAV serotype 6. Expression of our transgene was driven by a cardioselective promotor (elongation factor 1α promotor and the α-cardiac actin enhancer). The heart is now resting and the color of the myocardium turns white/yellow (see Note 9).
11. Wait 1-min.
12. Inject the remaining half of the virus solution and flush the P-50 tubing with saline using an appropriate volume (depends on the length of your tubing) to get the virus solution out of the P-50 tubing.
13. Wait 1-min (the heart may eventually start beating).
14. Inject a bolus of 30 µg of dobutamine via the P-50 tubing to restart the heart.
15. Remove the P-50 tubing and clamp from the ascending aorta.

16. Warm the rat to 37°C using the heating pad and removing the ice packs (see Note 10).
17. Make a knot in the carotid artery close to the incision before closing.
18. Suture the fascia and the muscles
19. Suture the skin.
20. Turn off the oxygen and isoflurane start on room air.
21. When the rat starts breathing spontaneously extubate and place in a cage (care for appropriate pain reliever) (see Note 11).

3.2. Myocardial Gene Delivery in Pig

3.2.1. Preparation Prior Myocardial Gene Delivery in Pig

1. Animals (30–35 kg) will not receive food in the morning of procedure.
2. The animals will be anesthetized with an intramuscular injection of a cocktail containing Ketamine (20 mg/kg), Xylazine (2.0 mg/kg), and Atropine (0.04 mg/kg). Buprenorphine (0.05 mg/kg) will be given before the surgical procedure starts.
3. Upon loss of responsiveness and spontaneous movement, the animal will be intubated and maintained on isoflurane (1–4%) and oxygen. If necessary, pigs may be ventilated using an intermittent positive pressure ventilator. Heart rate, EKG, and oxygenation (pulse oximetry) will be monitored throughout the duration of the procedure.
4. The neck area of each animal will be shaved; the surgical field will be wiped with alcohol and prepared with Chlorhexadine solution. A marginal ear vein will be catheterized for fluid administration and any necessary drug delivery during procedure.

3.2.2. Myocardial Gene Delivery in Pig (Target Area Is the Supply Zone of the LAD)

1. Surgically expose the right carotid artery and the right jugular vein. Carefully prepare the carotid artery and the jugular vein from connecting tissue and the N. vagus.
2. Take a loop of a 3-0 or 4-0 silk suture and slip it underneath the right carotid artery, then split the suture and move one strand towards the heart and the other towards the neck. Make a loop of the suture closest to the heart, but do not tighten. Perform the same procedure to prepare the right jugular vein (see Note 12).
3. Ligate the right carotid artery using the suture towards the head. Carefully stretch the vessel towards the head using a clamp.
4. Ligate the right carotid artery using the suture towards the head. Carefully stretch the vessel towards the head using a clamp.
5. Cut a small hole, both in the upper right carotid artery and the upper right jugular vein between the two loops using fine scissors and insert a vascular sheath (6F or larger for carotid artery and 9F for jugular vein).

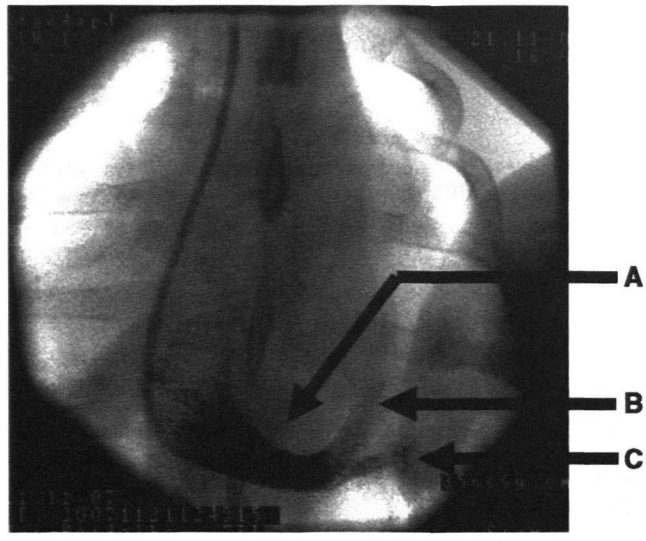

Fig. 1. Visualization of the coronary sinus in German farm pigs. Radioscopic image in anterior posterior projection showing (**A**) the coronary sinus, (**B**) the V. azygos, and (**C**) the vein draining blood from the coronary arteries. Contrast dye is primarily injected into the coronary sinus.

6. Administer heparin (2,500 IU) intravenously using a peripheral line via an ear vein.
7. Advance a coronary sinus catheter (Attain Command 6250 MB2 or 6250 MP A2) into the coronary sinus (Fig. 1; arrow A) and next into the vein draining blood from the coronary arteries (Fig. 1; arrow C) (see Note 13).
8. After the catheter intubates the vein draining blood from the coronary arteries (Fig. 1, arrow C) insert a 0.014′ Balance Middleweight Wire (BMW) or Balance Heavyweight Wire (BHW) into anterior interventricular cardiac vein (AICV) (Figs. 2 and 3) which runs parallel to the left ascending coronary artery (LAD).
9. Advance a coronary sinus balloon catheter (Attain 6215-80 cm or Arrow A1-7F) to the entrance of the AICV (Fig. 2) (see Note 14).
10. Introduce the guiding catheter (6F Judkins right) via the right carotid artery into the ostium of the left coronary artery (LCA) using an LAD 0.035′ standard wire.
11. Via the guiding catheter introduce a 0.014′ BMW wire in the LAD (see Note 15).
12. Introduce a 3.5 mm × 12 mm PTCA balloon into the LAD distal of the first diagonal branch using contrast dye.
13. Occlude LAD distal to the first diagonal branch (see Note 16).

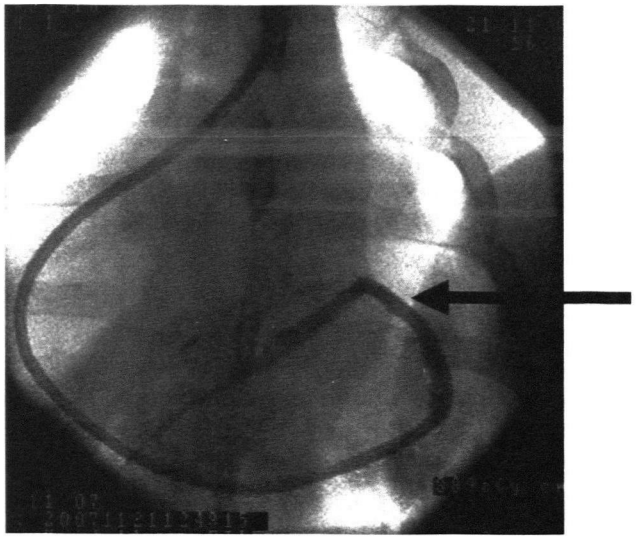

Fig. 2. Visualization of the anterior interventricular cardiac vein (AICV). Radioscopic image in anterior posterior projection. The *arrow* marks the entrance of the AICV draining blood from the left anterior descending coronary artery (LAD). At the entrance of the AICV the coronary sinus balloon catheter should be situated and virus solution should be retrogradely injected in the AICV. The AICV runs towards the *lower left corner* of the image.

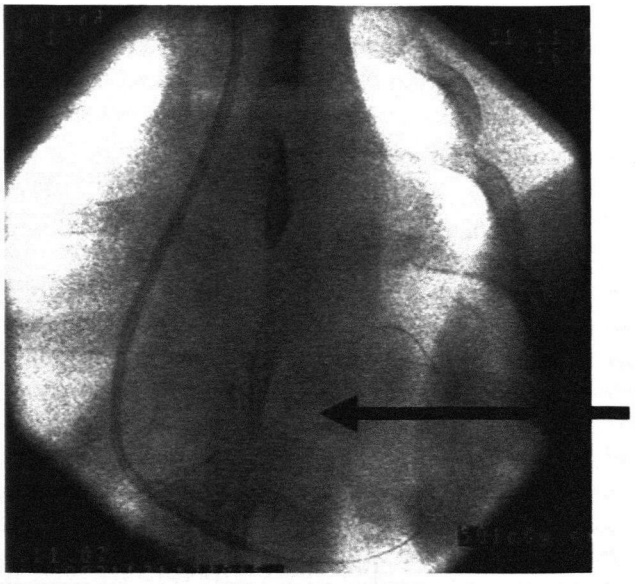

Fig. 3. Visualization of the anterior interventricular cardiac vein (AICV). Radioscopic image in anterior posterior projection. The *arrow* marks a wire situated in the AICV running parallel to the LAD.

14. Immediately, occlude the AICV using the coronary sinus balloon at the entrance of the AICV (Fig. 2).
15. Immediately, inject 0.2 mg of nitroglycerine (NTG) into the AICV (see Note 17).
16. Immediately, inject a third of the virus solution containing 1.5 x 10*13 tvp of AAV6 or AAV9-transgene using a cardioselective promotor (cytomegalovirus/myosin light chain 0.26 (CMV/MLC0.26)), 0.4 mg of NTG and 0.9% saline totaling to a volume of 50 ml using a 50 ml syringe (see Note 18). Start timer.
17. Wait 45 s from the start of virus delivery. Then deflate the balloons in the LAD and in the AICV (see Note 19).
18. Wait 3 min.
19. Repeat steps 14–17 twice to inject the whole virus solution.
20. Remove catheters and balloons from the LAD and the AICV. Perform a follow-up coronary angiography of the LCA to ensure integrity of the vessel.
21. Close the incision using three layers of suture (fascia, muscle, and skin). Care for appropriate pain reliever.

4. Notes

1. This is a difficult procedure that must be done quickly. Use a strong cold light source placed over the neck, not the top of the throat, but the neck near the top of the thorax. The trachea and the vocal cords are quite apparent with the light. Don't push too hard. There may be some resistance, but it should not feel like your pushing through the body.

   It is important to use bright focused surgical light. The light used during this procedure must be a cold light source since the brain will not tolerate the procedure at a temperature above 30°C.

   An iron or aluminum sheet sized 7 cm (length) and 6 mm (wide) can be used to construct a rat laryngoscope (create a flattened S-shape of the sheet).
2. Without Styrofoam to protect the legs you will find necrosis at the hindlimbs of the rat eventually causing loss of toes.
3. A body temperature below 30°C is required to perform the procedure without brain and myocardial damage due to the cardiac arrest for ~2 min. Further reduction of the body temperature (below 28°C) is possible but will cause a significant longer period of time for the rat to wake up and recover.

4. Open the pericardium only at the basis of heart. Do not open the pleura since otherwise the lungs collapse.
5. The thymus may be a large organ especially in young rats. Cutting the thymus will cause significant bleeding. Avoid cutting, mobilize the thymus by using electrical cauterization on the right side just enough to get access to the ascending aorta and deflect the thymus to the left side.
6. A 90°C needle holder with a thin but blunt proximal tip is useful for this procedure.
7. This procedure is necessary since the virus solution need to be injected between the aortic valve and the silk-loop around the ascending aorta to allow perfusion of the coronary arteries. Injection of the virus solution into the left ventricular chamber (having a resting heart) will not cause myocardial gene delivery.
8. This is not easy, take your time, carotid is flexible. Slide the tubing into the carotid without rupturing the carotid artery and advance the catheter past the clamp. Determine the position of the catheter in the ascending aorta using forceps. Advance just above the aortic valve (not in the left ventricle) by using the mark. Use chapstick on the outer side of the P-50 tubing to reduce friction.
9. Myocardial gene delivery will only be efficient in a resting heart, some beats may occur.
10. Because substance P causes the rat to secrete fluid you eventually need to dry the mouth and nose using cotton tips during the following 10 min.
11. During the first hour after the procedure some rats will develop ventricular fibrillation. The animal might suddenly cramp and faint. Use the defibrillator to convert into sinus rhythm.
12. Do not rotate the jugular vein. Otherwise you may occlude the vessel.
13. Carefully turn the catheter to the left side in the proximal third of the right atrium to intubate the coronary sinus (Fig. 1; arrow A). Pigs have a prominent V. azygos (Fig. 1; arrow B) draining into the coronary sinus (Fig. 1; arrow A). To intubate the vein draining blood from the coronary arteries (Fig. 1, arrow C) slightly rotate and advance the catheter clockwise. Sometimes there is a valve in front of the vein draining blood from the coronary arteries (Fig. 1, arrow C). Try to visualize by using contrast dye. Overall aim is to intubate the anterior intraventricular coronary vein (AICV) (Fig. 2; arrow) draining venous blood from the supply zone of the anterior descending coronary artery (LAD). Retrograde delivery of the virus solution via

the AICV will cause myocardial gene delivery to the anterior/septal myocardium (supply zone of the LAD) which is the target zone for cardiac gene delivery described in this protocol.

14. Alternatively (to steps 7–9) use a multipurpose catheter (7F) to intubate the vein draining blood from the coronary arteries (Fig. 1; arrow C); advance a 260 cm 0.014′ or 0.018′ wire into the AICV. Using the wire in place remove the multipurpose catheter and introduce the coronary sinus balloon catheter (Attain 6215-80 cm or Arrow A1 -7F) into the entrance of the AICV (Fig. 2).
15. The wires in the LAD and the AICV now run in parallel. Of note, there is considerable variation in the anatomy of the AICV depending on the breed of the pigs. In German farm pigs, we always observed an anatomy as described here and also shown in Figs. 1, 2, and 3. However, Yucatan pigs and cross-breeding pigs between Yorkshire pigs and Yucatan pigs sometimes show draining of the AICV directly into the V. cava superior making retrograde myocardial gene delivery as described in this protocol impossible.
16. This will block antegrade perfusion of the target area and facilitate myocardial gene delivery. Further proximal temporary occlusion of the LAD will cause a significant higher rate of ventricular fibrillation and eventually myocardial damage.
17. To reduce the endothelial barrier.
18. Use an angiopump which is standard in every cathlab to control injection velocity and maximal pressure within the vessel. Use a ramp to avoid a pressure peak and thus damage of the AICV. Possible injection velocity may vary between 16 ml within 15–45 s and allowing a maximum injection pressure of 100 mmHg.
19. Injection of the virus solution is divided into three portions to reduce the time of myocardial ischemia but to allow a prolonged time of incubation of the virus within the vessel.

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