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Kiyotake Ishikawa *Editor*

Cardiac Gene Therapy

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Preface

Rapid evolutions in vector technologies and identification of key molecular targets have facilitated the use of gene therapy as a vital approach for treating cardiovascular diseases. In the past decade, there has been substantial progress in clinical translation of cardiac gene therapy. Nevertheless, recent early clinical trials using gene therapy as a therapeutic approach to improve heart failure have shown neutral results, and the difficulty of transferring the genes to human hearts has become ever more recognized. Efficient, cardiac-specific, and safe vectors, as well as refined vector delivery methods, are key for successful cardiac gene transfer and eventually for improving patients' outcomes. Newer vectors and more efficient vector delivery methods have the potential to dramatically improve gene transduction efficacy, while novel gene manipulation techniques enforce the therapeutic power and broaden disease targets.

The aim of this book is to provide methodological information on cardiac gene delivery from classic to state-of-the-art technologies and techniques. Detailed and practical protocols described in this volume will be valuable tools for molecular biologists and physiologists in the cardiology field to conduct cardiac gene transfer research, which will ultimately lead to further advancements in the field.

I thank all expert authors for their dedication in describing step-by-step methodologies that will undoubtedly lead to successful cardiac gene therapy. I am very grateful to Dr. Roger J. Hajjar (Icahn School of Medicine at Mount Sinai) for assisting me with the organization of the contents and also for contributing a number of chapters himself. Lastly, I would like to thank John M. Walker, the series editor, who provided me with this opportunity and guiding the volume's preparation process. We hope that the readers find *Cardiac Gene Therapy: Methods and Protocols* to be a useful reference for conducting and improving their projects.

New York, NY, USA *Kiyotake Ishikawa*

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Part I

Overview

Chapter 1

Current Methods in Cardiac Gene Therapy: Overview

Kiyotake Ishikawa and Roger J. Hajjar

Abstract

During the last decade, there has been a significant progress toward clinical translation in the field of cardiac gene therapy based on extensive preclinical data. However, despite encouraging positive results in early phase clinical trials, more recent larger trials reported only neutral results. Nevertheless, the field has gained important knowledge from these trials and is leading to the development of more cardiotropic vectors and improved delivery systems. It has become more evident that humans are more resistant to therapeutic transgene expression compared to experimental animals and thus refinement in gene delivery tools and methods are essential for future success. We provide an overview of the current status of cardiac gene therapy focusing on gene delivery tools and methods. Newer technologies, devices, and approaches will undoubtedly lead to more promising clinical results in the near future.

Key words Cardiac gene therapy, Heart failure, Adeno-associated vectors, Gene delivery, Surgical delivery, Percutaneous delivery, Cardiotropic, Promoters

1 Introduction

From the time I wrote a chapter in this book series in 2003 describing the cardiac gene transfer methods in rodents [1], there has been tremendous progress in the cardiac gene therapy field towards clinical translation. After an early disappointment from the neutral phase II–III angiogenic gene therapy trials using plasmid DNA and adenoviral vectors [2], the field has quickly shifted to more efficient vectors and delivery methods to improve gene transfer efficacy. Application of recombinant adeno-associated virus (rAAV) for cardiac gene delivery is a representative technological advance that enables long-term, efficient, and homogeneous cardiac gene transduction. Numbers of preclinical studies have demonstrated efficient transgene expression and therapeutic efficacy using this vector [3, 4] which led to the initiation of early phase clinical trials [5]. However, after much promise in these early phase trials [5–7], a more recent larger trial reported only neutral results [8]. Similarly, another phase II clinical gene therapy trial utilizing transcatheter

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endocardial injection of plasmid DNA failed to meet the primary efficacy endpoint [9]. These results will delay the application of cardiac gene therapy in daily clinical practice; however, we have gained important knowledge to move forward. Cardiac samples obtained from patients who underwent cardiac transplantation after the rAAV gene therapy has informed us that the vectors indeed transduce the human heart [6]. Notwithstanding, the viral uptake within the myocardium was much lower in humans compared to animal studies which only corresponds to less than 1 % of cardiomyocytes being infected [10]. These results direct us to refine our methods of cardiac gene transfer including a search for better vectors, more robust delivery systems and novel targets. This book has a timely focus on these methodologies to further improve cardiac gene transduction, and covers various novel techniques to produce better vectors that specifically and efficiently target the heart. In this chapter, we provide an overview of currently available cardiac gene delivery vectors and delivery methods.

2 Vectors

One of the most important factors for successful gene therapy is the choice of vectors. Vectors determine the efficiency of transduction, tropism to the targeted tissues, degree of inflammation, and length of transgene expression. Despite the progress in the identification of promising targets for the treatment of a number of cardiovascular diseases, the targeted delivery of therapeutic nucleic acids yet remains a formidable hurdle especially in advanced mammalians. Nonetheless, over the past years, considerable advances have been made in developing and improving several vector platforms. Broadly, these vectors can be classified into two groups: nonviral vectors and recombinant viral vectors. Each of these vector systems has its own set of advantages and disadvantages, and we will briefly discuss the main vectors currently employed in cardiovascular gene therapy.

2.1 Nonviral Gene Delivery

Naked plasmid DNA has been the predominant vector used in the previous cardiac gene transfer trials that have employed nonviral vectors, with only a few trials using lipofection [11]. The major advantages of plasmid DNA include (1) the ease of large scale production, (2) the near absence of a DNA size limit, and (3) the limited innate, cellular and humoral immune response. The lack of a significant humoral immune response against the vector is a great advantage that allows repeat vector administration, which is one of the major limitations of viral vectors. However, repeat vector administration comes with an appreciable risk of serious adverse events due to the administration procedure that often requires invasive procedures. Unfortunately, the Achilles heel of plasmid DNA

as a gene delivery vehicle remains the low transfection efficiencies [12]. Innate immune response to plasmid DNA is considered moderate and can also reduce transfection efficiency [13]. These limitations clearly indicate the necessity of a major breakthrough to improve transfection efficiency to fully realize the potential of plasmid DNA gene transfer.

Recently, promising new nonviral gene transfer methods have emerged. These approaches include modified mRNA [14] (modRNAs) and exosome [15] mediated gene delivery. The use of modified mRNAs has two main advantages: (1) ModRNAs, unlike unmodified nucleic acids, do not bind to Toll-like receptors [16], which could trigger apoptosis of the transfected cells. As a result, modRNAs can transfect the cells very efficiently. (2) Because mRNAs are translated in the cytoplasm, they do not need to be imported into the nucleus for transgene expression, which poses a formidable hurdle for transfection with DNA. ModRNAs trigger high-level transgene expression, and unsurprisingly, transgene expression is relatively short-lived, 2–6 days [14, 17]. Depending on the application, this short, pulse-like expression can be either disadvantageous or beneficial. For example, whereas the short-term expression of proteins deficient in inherited cardiomyopathies would most likely have no long-term therapeutic benefit, the short-term expression of, for instance, growth factors and stem cell recruiting factors [18] might not only be therapeutically optimal but also safer. Recently, Turnbull et al. have shown that modRNA mixed with nanoparticles delivered by direct injection into the myocardium or by intracoronary fashion can induce expression as fast as 20 min following delivery in rodent and in pig hearts [19]. Thus, for short-term and rapid expression, modRNA offers a safe and reliable delivery system to the myocardium.

2.2 Viral Vectors

Recombinant viral vectors are often very efficient in delivering therapeutic genetic material to the targeted cells compared to nonviral vectors. They all have their own characteristics and appropriate vector selection is one of the key components for successful cardiac gene transfer.

To date, the majority of virus-mediated cardiovascular clinical gene therapy trials have used adenoviral vectors. This vector has the advantage of transducing a broad array of cell types, including cardiomyocytes, with a high transgene expression, although transient. However, adenoviral vectors do not have cardiac tropism and the transgene expression cannot be restricted to certain tissues or cell types unless targeted specifically. The most significant limitation of adenoviruses for cardiovascular gene therapy is however, that they trigger a strong immune response [20, 21]. The so-called first-generation adenoviral vectors, which are deficient in only one viral gene (usually E1), trigger a strong cellular immune response [21], presumably as a result of the expression of adenoviral

proteins. However, even after removing most of the viral gene, i.e., gutless adenoviral vectors, a strong innate immune response against the adenoviral capsid was triggered [22], a risk not to be taken lightly in cardiac gene therapy.

Lentiviral vectors have been used experimentally in preclinical cardiac gene therapy studies [23]. In contrast to γ-retroviral vectors, lentiviral vectors can transduce nondividing cells such as cardiomyocytes. Moreover, long-term expression can be achieved in both nondividing and dividing cells, because they integrate their genetic material into the host genome. The immune response is in general moderate [20], but similar to adenoviral vectors, lentiviral vectors have no specific tropism to cells of the cardiovascular system, which likely will require intramyocardial injection as a vector delivery method when targeting cardiac cells. Moreover, lentiviral vectors can cause insertional mutagenesis through the random integration of DNA into the host genome, raising concerns for the aberrant expression of important genes and to tumorigenesis. These limitations have restricted lentivirus use as a vector for in vivo cardiac gene transfer, and it has been mainly used in ex vivo gene transfer to reprogram cells or to induce cardiac progeny in stem cells [23].

AAVs are one of the most promising gene delivery platforms for cardiac gene therapy. AAVs are small, non-enveloped, single-stranded DNA viruses that are nonpathogenic in general. Both dividing and nondividing cells can be transduced by rAAVs and they can trigger long-term transgene expression even in the absence of genome integration in postmitotic tissues, such as the myocardium. One of the main advantages of rAAV vectors for cardiac gene therapy is that multiple AAV serotypes display natural tropism for cardiomyocytes [24, 25]. In small animal models of cardiac diseases, this allows the systemic administration of rAAVs to efficiently transduce the myocardium. Unfortunately, the cardiac tropism of present AAV serotypes and variants is not perfect. As a result, in large animal models—and most importantly in humans—rAAVs carrying therapeutic genes need to be delivered regionally. The cellular immune response against rAAVs is not very strong. In clinical trials using the hepatotropic AAV serotype 8 to deliver factor IX to treat hemophilia B, two patients experienced a transient transaminase increase, putatively as a result of an anti-AAV immune response [26], but the liver transaminase levels rapidly returned to normal after a short regimen of immune suppression. Interestingly, a cellular immune response has not been detected in more than 300 patients in the CUPID 1 and 2 trials [7]. However, despite the limited cellular immune response against rAAVs, the presence of antecedent neutralizing antibodies emerged as a significant obstacle to the broad application of AAV gene therapy. Preexisting neutralizing antibodies against the naturally occurring serotypes, presumably a result of a prior infection with wild-type AAVs, can significantly reduce the transgene efficacy. In fact, more than half the patients (up to 80% in certain regions) who could have been

potentially enrolled into the CUPID trial had to be excluded because of antecedent neutralizing antibodies. Another limitation of this vector is the limited packaging capacity (≤5 kb), which restricts the genes that can be loaded in this vector. Clearly, methods to address these limitations have become increasingly important as cardiac gene therapy moves towards the clinic. Active researches are ongoing to overcome these obstacles [27–29] and rAAVs are arguably the most promising vectors currently available for cardiac gene therapy.

3 Promoters

To achieve strong expression, constitutively active promoters such as CMV (cytomegalovirus) or RSV (respiratory syncytial virus) have been used. More recently, cardiac specific promoters emerged to achieve cardiac specific expression to the heart. These include the myosin heavy chain promoter, the myosin light chain promoter, and troponin T promoter [30]. Unfortunately, even though a higher specificity to the heart was conferred, the resulting expression is weaker compared to constitutively active promoters requiring higher doses of vectors. Novel cardiac-specific cis-acting regulatory modules have been used to further restrict off-target expression. Inducible promoters can be either activated or silenced by a drug or a small molecule [31]. Disease specific promoters such as ANF (atrial natriuretic factor) which are highly expressed in heart failure have also been used experimentally to induce transduction during volume enlargement in a dog model of heart failure [32].

4 Methods of Gene Transfer

Another key to successful cardiac gene therapy is to choose appropriate vector delivery methods. As mentioned previously, even the most cardiac tropic vector, i.e., rAAV is not powerful enough to transduce the heart specifically via intravenous route in large animals. Thus cardiac targeting is necessary to increase the specificity. Cardiac targeting also increases the amount of vectors distributed to the heart and thus important to increase the expression efficacy. Representative cardiac delivery methods are illustrated in Fig. 1. Each method exhibits different gene transfer efficiency, transgene distribution pattern, and invasiveness. Thus, the optimal delivery technique needs to be carefully determined considering the used vector, targeted gene, treating disease, and patients' condition. For example, gene targets that are aimed to be over-expressed locally, such as biological pacemaker may benefit more from focal transgene expression rather than homogeneous gene distribution throughout the myocardium. Surgical delivery may be too invasive for patients with NYHA class IV heart failure, whereas the heart is

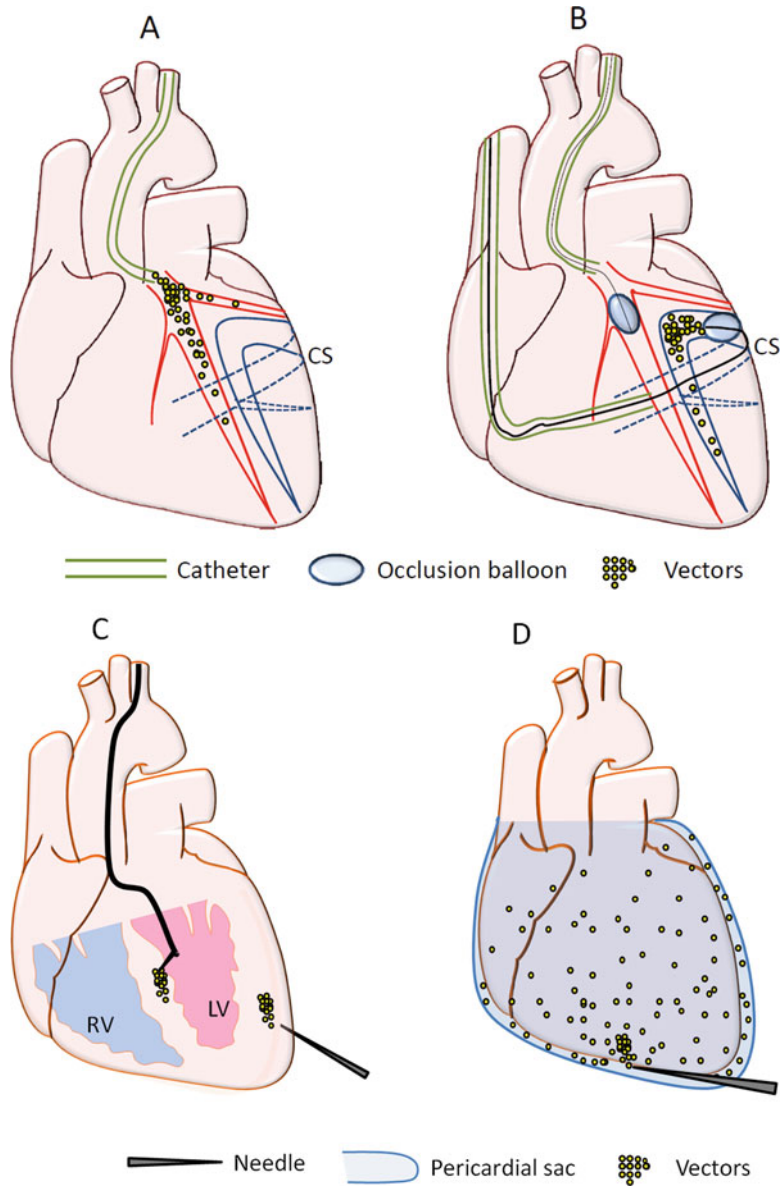

Fig. 1 Methods of myocardial targeted gene delivery. (**a**) Antegrade intracoronary injection. (**b**) Retrograde injection through the coronary sinus with simultaneous blockade of the coronary arterial flow. (**c**) Direct myocardial injections through the left ventricle using catheter based approach (endocardial) and surgical approach (epicardial). (**d**) Intrapericardial injection

readily accessible in patients undergoing elective cardiac surgeries. It is important to note that safety is as important as efficacy in delivering the genes and minimally invasive percutaneous approaches are usually preferred when targeting patients with chronic heart failure. In the following sections, we briefly review representative cardiac targeting delivery methods.

4.1 Intramyocardial Delivery

The first successful cardiac gene transfer was demonstrated by directly injecting the plasmid DNA into the myocardium [33]. This approach has the advantage of achieving higher vector concentration and presumably higher retention within the injection site. Transgene distribution is focal, usually within 5–10 mm range around the needle track. A surgical approach delivers vectors epicardially, while a catheter-based endovascular approach delivers vectors to the endocardium. Direct visualization of the injection is an advantage of surgical delivery, whereas endovascular approach is likely less invasive. When an endovascular approach is used, site of injection can be determined using electromechanical mapping system, fluoroscopy, or CT. Unfortunately, even direct intramyocardial injection cannot prevent systemic leak of the vectors, because the vectors leave the heart through venous drainage, the lymph system, and from the injection needle holes. Thus, off-target distribution needs to be evaluated similar to other delivery methods. It has been reported that the rate of retention decreases when higher volumes are injected [34]. Thus, lower volume with concentrated vectors seems to be the best option to accomplish efficient gene transduction while minimizing off-target distribution using this approach.

4.2 Intravascular Delivery

Antegrade coronary artery delivery is probably the most clinically familiar approach to deliver the genes with cardiac targeting. Similar devices and techniques used for coronary angiogram or percutaneous coronary interventions can be applied. Homogenous distribution of the transgene expression can be achieved using this method. However, a single bolus injection of vectors through this route results in limited expression. Using ex vivo system, Donahue et al. found that higher coronary flow, longer virus exposure time, and higher virus concentrations are critical factors to improve myocardial vector uptake [35]. The importance of these factors together with perfusion pressure for increasing the transduction efficacy was later confirmed in vivo by us using a cross-clamp technique [36]. Vector administration during brief coronary occlusions can increase the relative vector concentration and exposure time and results in improved transgene efficiency. However, coronary occlusions can cause stunning of the myocardium even with very short ischemia and may predispose patients to adverse events in the context of limited functional reserve in advanced heart failure. Retrograde injection of the vectors into the coronary sinus is another intravascular approach. This approach has been shown to increase the transduction efficiency compared to antegrade delivery, and is currently used in one of the clinical trials using plasmid vectors (ClinicalTrials.gov Identifier: NCT01961726). Transgene distribution using this method is less homogeneous with epicardial dominant expression [37] compared to the antegrade delivery. Because the vectors need to be administered against the antegrade flow, this technique usually requires balloon occlusion

of both the coronary artery and the sinus to prevent rapid washout of the vectors. It is likely that the longer occlusion time would result in higher gene expression, however prolonged occlusion can increase the risk of causing delivery related complications and the benefit of achieving higher efficiency needs to be balanced with its risks. For intracoronary delivery, co-administration of permeability increasing agents (such as nitroglycerin) has been shown to significantly increase the transduction efficacy and several different recipes are reported to be effective [32, 38]. Because the gene delivery vectors are usually very small, they can easily pass through the coronary capillaries unless they attach to the vessel wall. To prevent the escape of vectors from the heart, recirculation of the vectors was proposed using a catheter system whereby the coronary sinus is blocked and blood with the virus is recirculated to the coronary arteries (Osprey system) [39]. The "Molecular Cardiac Surgery with Recirculating Delivery" (MCARD) has been developed as an invasive cardiac surgical technique that uniquely allows for in situ, multiple-pass recirculation of recombinant vector in the isolated coronary circulation in large animals [40]. Both approaches appeared to improve cardiac gene transduction; however, off-target distribution is not completely eliminated by both approaches.

4.3 Intrapericardial Delivery

Percutaneous access to the pericardium space has become popular after successful application of epicardial ablation for epicardial ventricular tachycardia. This space has also been used to deliver vectors, cells, and drugs, and their efficacy has been reported [41]. Although it is a closed space, there is a rapid turnover of the pericardial fluid and the vectors seem not to stay too long in the space after delivery. To increase the retention inside the pericardium, we have injected the vectors with biodegradable gel foam [42]. This approach resulted in epicardial transduction; however, there was no expression in the other layers suggesting very limited transmural penetration using this approach. Thus, additional modification is likely required for targeting the myocardium.

5 Conclusion

Recent clinical trial results in cardiac gene therapy have made us further realize the difficulty of effectively transducing in the myocardium in humans. It is clear that further improvements in gene delivery tools and methods are required (Fig. 2), and newer approaches described in this book will undoubtedly contribute to future success of cardiac gene therapy in patients.

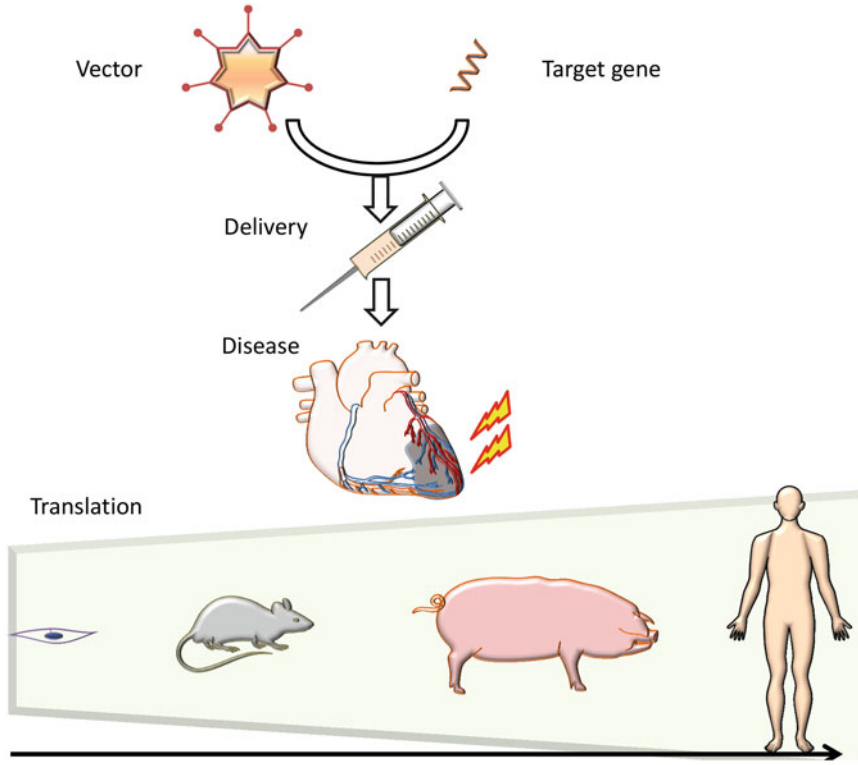

Fig. 2 Important elements for cardiac gene transfer. The appropriate combination of vector, gene target, delivery method, and targeted disease determines the success of cardiac gene therapy. The challenge also underlies the translation into clinics as effective gene transfer appears to be an extremely difficult task in more advanced mammalians

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Part II

Genetic Modification

Chapter 2

Silencing Genes in the Heart

Henry Fechner, Roland Vetter, Jens Kurreck, and Wolfgang Poller

Abstract

Silencing of cardiac genes by RNA interference (RNAi) has developed into a powerful new method to treat cardiac diseases. Small interfering (si)RNAs are the inducers of RNAi, but cultured primary cardiomyocytes and heart are highly resistant to siRNA transfection. This can be overcome by delivery of small hairpin (sh)RNAs or artificial microRNA (amiRNAs) by cardiotropic adeno-associated virus (AAV) vectors. Here we describe as example of the silencing of a cardiac gene, the generation and cloning of shRNA, and amiRNAs directed against the cardiac protein phospholamban. We further describe the generation of AAV shuttle plasmids with self complementary vector genomes, the production of AAV vectors in roller bottles, and their purification via iodixanol gradient centrifugation and concentration with filter systems. Finally we describe the preparation of primary neonatal rat cardiomyocytes (PNRC), the transduction of PNRC with AAV vectors, and the maintenance of the transduced cell culture.

Key words siRNA, shRNA, amiRNA, AAV vectors, Iodixanol gradient centrifugation, Primary neonatal rat cardiomyocytes

1 Introduction

Gene silencing induced by RNA interference (RNAi) is widely used in experimental research and has also shown its suitability for the investigation of cardiac gene function and for the treatment of cardiac diseases [1]. Small interfering (si) RNAs induce RNAi. After transfection into cells, one strand of this roughly 19 bp long double-stranded RNA binds to the mRNA of a target gene in a sequence-specific manner and induces the degradation of its target mRNA, leading to suppression of gene expression [2]. An as yet unsolved problem of the employment of siRNA in the cardiac system is the low transfection efficiency of isolated cardiomyocytes in vitro and of the heart in vivo. Moreover, cardiac specific in vivo delivery of siRNA is challenging with regard to target organ specificity and long-term efficacy. These bottlenecks can be overcome by delivery of small hairpin (shRNA) and artificial microRNAs (amiRNAs), which are processed intracellular to mature siRNAs, by cardiotropic adeno-associated virus (AAV) vectors [3–5].

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Free available computer programs can be used to select siRNA, shRNAs, and amiRNAs, directed against a specific target gene. They can then easily be inserted into expression plasmids and subcloned into AAV shuttle plasmids containing essential AAV genome sequences necessary for packaging of the AAV vector genome into AAV capsids [3, 6]. Over the last decade, huge efforts were made in the development of AAV vectors. Especially pseudotyped AAV vectors, comprising a vector genome derived from AAV2 packaged into the capsid of the AAV serotypes 6 and 9, showed improved cardiotropism [7–9]. The generation of AAV vectors with a self complementary (sc) vector genome represents a further milestone in AAV vector development [10]. Compared to traditional AAV vectors with single-stranded (ss) vector genomes, scAAV vectors express transgenes earlier and reach faster maximal levels in the heart [11]. This is of particular importance for investigation in cultured primary cardiomyocytes, which only survive in culture for a short time [12, 13], as well as for in vivo investigations requiring rapid gene silencing [3]. AAV vectors can be generated in culture plates, flasks, or roller bottles using helper virus-free plasmid-based packaging systems and purified by iodixanol gradient ultracentrifugation and concentrated using specific filter systems. The vectors can then be used directly for in vitro and in vivo applications [14].

Here we describe, as an example of cardiac gene silencing, the development of shRNAs and amiRNAs directed against the cardiac Ca^{2+} regulatory protein phospholamban (PLB), the generation of AAV vector shuttle plasmids with sc vector genomes, the production of pseudotyped AAV2/6 and AAV2/9 vectors in roller bottles, their purification by iodixanol gradient centrifugation and concentration with an Amicon® filter system. We further describe the preparation of primary neonatal rat cardiomyocytes (PNRC), its transduction with AAV vectors and maintenance of the transduced cell culture.

2 Materials

Use double-distilled water (H_2O) for all methods.

2.1 Cloning of shRNAs and amiRNAs

1. siRNA Designer.
2. pSilencer™ neo Kit (Thermo Fisher Scientific).
3. BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (Invitrogen) containing 10× oligo annealing buffer, T4 DNA ligase (1 U/μl), and 5× T4 DNA ligase buffer.
4. *Hind*III (20,000 U/ml), *Bam*HI (20,000 U/ml), and 10× restriction buffer.
5. Agarose.
6. Ethidium bromide.

7. Gel extraction kit.
8. Calf intestine alkaline phosphatase (10,000 U/ml) and 10× CIAP buffer.
9. Oligonucleotides (200 μM).
10. Annealing buffer: 100 mM K-Acetate, 30 mM HEPES, pH 7.4, 2 mM Mg-acetate. Weigh 0.9815 g K-acetate, 0.715 g HEPES and transfer to a glass beaker add 90 ml H_2O and adjust the pH to 7.4 with KOH. Add 0.0285 g Mg-acetate and fill up to 100 ml with H_2O. Stir the solution with a magnet stirrer. Store the solution at 4 °C.
11. T4 Polynucleotide kinase (10,000 U/ml), 10× T4 PNK buffer, ATP (100 mM).
12. T4 DNA ligase (400,000 U/ml) and 10× T4 DNA ligation buffer.
13. Competent *E. coli* cells.
14. Plasmid mini preparation kit.
15. Plasmid maxi preparation kit.

2.2 Generation of AAV Shuttle Plasmids with Self Complementary AAV Vector Genome

1. pAAV-MCS (Stratagene).
2. *Msc*I (5000 U/ml), *Hinc*II (10,000 U/ml), and 10× restriction buffer.
3. Agarose.
4. Ethidium bromide.
5. Gel extraction kit.
6. T4 ligase (400,000 U/ml) and 10× T4 DNA ligation buffer.
7. Competent *E. coli*.
8. Plasmid mini preparation kit.
9. Plasmid maxi preparation kit.

2.3 Production of AAV Vectors in Roller Bottles

2.3.1 Plasmids

1. AAV shuttle plasmids.
2. pDP6rs (PlasmidFactory).
3. pHelper (Agilent Technologies, Inc).
4. p5E18-VD2/9 [3].

2.3.2 Cell Culture Media, Buffers and Solutions

1. HEK293T cells.
2. DMEM-complete medium: DMEM, 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% L-glutamine, 1% sodium pyruvate. Add 50 ml FCS, 5 ml penicillin/streptomycin (10,000 U penicillin/10 mg/ml streptomycin), 5 ml L-glutamine (200 mM), and 5 ml sodium pyruvate (100 mM) to 500 ml DMEM (4.5 g/l glucose, w/o sodium pyruvate, w/o L-glutamine). Store the solution at 4 °C.
3. 1× PBS. Store the solution at 4 °C.

4. Trypsin–EDTA. Store the solution at 4 °C.
5. Collagen Type I from calf skin (Sigma-Aldrich, Cat. No. C8919). Store the solution at 4 °C.
6. 150 mM NaCl: Weigh 0.8766 g NaCl and dissolve it in 100 ml H_2O. Store the solution at room temperature.
7. 2.5 M $CaCl_2$: Weigh 27.75 g $CaCl_2$ and dissolve it in 100 ml H_2O. Store the solution at room temperature.
8. Polyethylenimine (PEI). To have a final concentration of 2.58 µg/µl dissolve 129 mg PEI in 50 ml H_2O. Store PEI at –20 °C.
9. Benzonase nuclease, 25 U/µl. Store the enzyme at –20 °C.
10. 1.86 M NaCl/24 % PEG 8000: Weigh 54.35 g NaCl and transfer it into a glass beaker containing 500 ml H_2O. Add stepwise 120 g PEG and stir the solution strongly with a magnet stirrer (*see* **Note 1**). Store the solution at room temperature.
11. NaCl–Hepes solution: 50 mM Hepes, 150 mM NaCl, 25 mM EDTA. Weigh 1.192 g Hepes and transfer it into a glass beaker. Add 0.3 ml of 5 M NaCl and 0.5 ml of 500 mM EDTA. Fill up to 100 ml with H_2O and mix the solution under continuous stirring using a magnetic stirrer. Store the solution at 4 °C.
12. Iodixanol solution (60 %, with density of 1.32 g/ml (Progen)). Store the solution at room temperature in the dark.
13. PBS-MK: 1× PBS, 1 mM $MgCl_2$, 2.5 mM KCl. Weigh 0.95 g $MgCl_2$ and 0.1864 g KCl and transfer it into a glass beaker containing 1 l 1× PBS. Mix the solution under continuous stirring using a magnetic stirrer and autoclave it. Store the solution at 4 °C.
14. Phenol red solution. Store the solution at 4 °C.

2.3.3 Materials and Equipment

1. Corning® roller bottles (850 cm^2 surface) (Sarstedt).
2. Cannula short (18 G×1½, Braun).
3. Cannula long (21 G×4¾, Braun).
4. Amicon® Ultra 15 ml centrifugal filters (100 K) (Millipore).
5. Polypropylene centrifuge tubes (capacity 29.9 ml, for ultracentrifugation, Optiseal).
6. Conic centrifuge tubes (15 and 50 ml).
7. Microtubes.
8. Bottle turning device 2 levels HC 240 (VWR International GmbH).
9. Heracell™240i CO_2 incubator.

2.4 Preparation of Primary Neonatal Rat Cardiomyocytes

2.4.1 Solutions and Media

1. 10× CIM, 1 l (Ca-free, phosphate-buffered cell isolation medium): 1.2 M NaCl, 45.6 mM KCl, 4.4 mM KH_2PO_4, 8.4 mM $Na_2HPO_4 \cdot 2H_2O$, 250 mM $NaHCO_3$, 55.5 mM glucose, pH 7.5. Weigh 58.44 g NaCl, 74.55 g KCl, 136.09 g KH_2PO_4, 177.95 g $Na_2HPO_4 \cdot 2H_2O$, 84.01 g $NaHCO_3$, and 180.16 g glucose and transfer it into a glass beaker filled with 900 ml H_2O. Mix the solution under continuous stirring using a magnetic stirrer. Adjust pH at room temperature to 7.5. Decant the solution into a 1 l volumetric flask and fill up to exactly 1 l using H_2O. Perform sterile filtration using a vacuum filtration system with a pore size of 0.22 μm. Distribute 50 ml aliquots of 10× CIM into 50 ml sterile tubes under a laminar flow hood. Mark each tube with "10× CIM" and the current date. Store these aliquots at −20 °C not longer than 3 month until use.
2. 20× P/S: Use either lyophilized penicillin/streptomycin (10,000 U/ml/10 mg/ml) or a ready to use solution of penicillin/streptomycin (10,000 U/ml/10 mg/ml). The latter solution can be portioned into 5 ml tubes under sterile conditions and stored at −20 °C until use.
3. 1× CIM with P/S: Mix 10 ml 10× CIM with 5 ml 20× P/S and 85 ml H_2O under sterile conditions to obtain 100 ml 1× CIM with P/S. Store the final solution at 4 °C (*see* **Note 2**).
4. 1× CIM (Ca free, phosphate buffered cell isolation medium): Mix 10 ml 10× CIM with 90 ml H_2O under sterile conditions to obtain 100 ml Ca-free 1× CIM and store this solution at 4 °C (*see* **Note 2**).
5. 10× trypsin (1.25% trypsin stock solution): Dissolve 0.5 g trypsin (*see* **Note 3**) in a 50 ml beaker glass filled with 50 ml H_2O under continuous stirring using a magnetic stirrer. Perform sterile filtration of the resulting solution (*see* **Note 4**) using a vacuum filtration system with a pore size of 0.22 μm.
6. 1000× $CaCl_2$: 1.26 M $CaCl_2$. Dissolve in a 100 ml beaker glass filled with 40 ml H_2O 9.261 g supra pure $CaCl_2 \cdot 2H_2O$ under continuous stirring using a magnetic stirrer. Transfer the solution in a narrow-necked volumetric flask and fill up to exactly 50 ml with H_2O. Perform sterile filtration of the resulting solution using a 50 or 10 ml syringe equipped with a syringe filter with a pore size of 0.22 μm. Distribute 1 ml portions of the solution into sterile 1.5 ml micro tubes and store them at −20 °C until use.
7. 1000× FUDR (5-fluoro-2′-deoxyuridine), 2 mM FUDR: Dissolve 2.924 mg FUDR in 10 ml H_2O, filtrate the solution using a syringe with a syringe filter with a pore size of 0.22 μm. Distribute 1 ml portions of the solution into sterile 1.5 ml micro tubes and store them at −20 °C until use.

8. 500× gentamycin, 10 mg/ml: Dissolve 10 mg lyophilized sterile gentamycin in 1 ml H_2O or use ready to use 10 mg/ml gentamycin solution.
9. FCS: Thaw the 500 ml bottle with frozen FCS at room temperature. Inactivate the thawed FCS (if needed) for 30 min at 56 °C using a water bath. Distribute the volume into portions of either 100 ml of 50 ml under sterile conditions and store at –20 °C.
10. Horse serum (HS): Thaw the 500 ml bottle with frozen HS at room temperature. Inactivate the thawed HS (if needed) for 30 min at 56 °C using a water bath. Distribute the volume into portions of either 100 ml of 50 ml under sterile conditions and store at –20 °C.
11. CMRL1415-ATM cell culture medium (from Biochrom KG, Berlin) (*see* **Note 5**).
12. Complete CMRL1415-ATM cell culture medium: Mix 498 ml CMRL1415-ATM with 0.5 ml 1000× $CaCl_2$, 0.5 ml 1000× FUDR, and 1 ml 500× gentamycin under sterile conditions (*see* **Note 6**).
13. Disinfectant, 1 l each. We recommend using ready-to-use disinfectant Barrycidal (Manfred Sauer GmbH). It consists of a synergistic blend of selected organic nitrogen compounds and provides a broad spectrum of activity against bacteria, yeasts, fungi, and viruses. Alternatively, 70% ethanol can be used as a disinfectant. However, the activity spectrum of the latter is not nearly as broad as Barrycidal.
14. 1× CIM with trypsin. Mix 10 ml 10× CIM, 80 ml H_2O, and 10 ml 10× trypsin (*see* **Note 7**).

2.4.2 Materials and Equipment

1. A stack of autoclaved paper towels.
2. A small beaker glass filled with 70% ethanol for soaking instruments.
3. A large beaker glass filled with 70% ethanol for dipping neonate rats.
4. A spray bottle containing Barricydal or 70% ethanol.
5. An aerosol can filled with disinfectant Barricydal or 70% ethanol.
6. Test tube rack.
7. A bag for the unneeded parts of the animal attached to the hood for easy accessibility.
8. Disposable plastic tissue culture pipettes.
9. Pasteur pipettes.
10. Sterile micropipette tips.

11. Three large weigh boats.
12. Two sterile petri dishes with 20 ml 1× CIM with P/S each.
13. Large forceps for animal transfer.
14. Container with sterile surgery tools (large scissors for animal decapitation, large curved forceps for animal fixation, small scissors for thorax section, small scissors for heart removal, small forceps for transfer of removed hearts).
15. FCS.
16. HS.
17. Various sterile 0.22 µm filtration units.
18. Conic centrifuge tubes (50 ml).
19. 50 mm glass funnel with sterile gauze.
20. Culture flasks (75 cm^2 filter cap), culture plates (round 6-well, 12-well, 24-well on demand); culture flasks (25 cm^2 with filter cap instead of culture plates, if needed).

3 Methods

Use autoclaved double-distilled water (H_2O) for all methods.

3.1 Cloning of shRNAs and amiRNAs

3.1.1 Cloning of shRNAs Against Phospholamban

To enable vector delivery, an siRNA sequence (*see* **Note 8**) can be converted into the corresponding shRNA (Fig. 1). The two strands of the double-stranded RNA molecule are connected by a loop and the shRNA is usually expressed under control of an RNA polymerase III promoter, e.g., the U6 or H1 promoter. The intracellular RNAi machinery will then process the shRNA to give the mature siRNA. Various vectors are commercially available for the expression of shRNAs. An example is the pSilencer 2.1-U6 neo vector from Thermo Fisher Scientific (originally from Ambion). The oligonucleotides encoding the shRNA are cloned between a *BamH*I and a *Hind*III site (*see* **Note 9**). How to clone shRNA is described in the following steps.

1. Mix 3 µg of pSilencer 2.1-U6 neo vector with 0.25 µl *BamH*I, 0.25 µl *Hind*III, 2 µl 10× restriction buffer and H_2O ad 20 µl. Incubate for 2 h at 37 °C.
2. Add 0.5 µl Calf Intestine alkaline phosphatase, 2.5 µl 10× CIAP buffer to the linearized vector and fill up with H_2O to 25 µl.
3. Separate the linearized plasmid by agarose gel electrophoresis using a 1 % agarose gel containing ethidium bromide (0.5 µg/ml). Cut out the gel slice containing vector band (*see* **Note 10**) and isolate the DNA fragments using a gel extraction kit. Estimate the vector concentration on a separate agarose gel with a standardized marker (*see* **Note 11**).

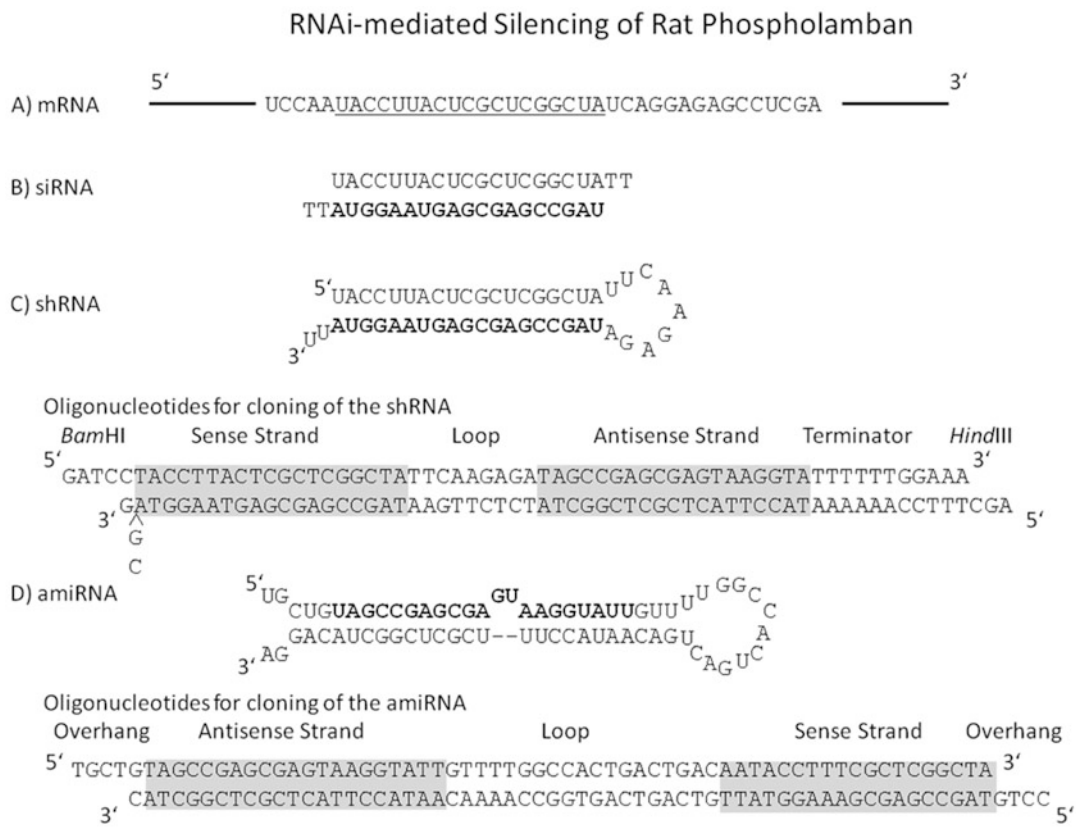

Fig. 1 Schematic of siRNA, shRNA and amiRNA directed against phospholamban [6]. (**a**) The target site in the mRNA is underlined. (**b**) The standard design of an siRNA comprises a 19mer duplex and two deoxythymidine overhangs at both 3′ ends. (**c**) For the shRNA approach, the two strands of the siRNA are connected by a loop. The oligonucleotides encoding the shRNA are shown below the shRNA. Please note that the second oligonucleotide is shown in 3′–5′ direction for clarity in this illustration, but must be in the 5′–3′ direction when ordered. For cloning, a *BamH*I and a *Hind*III site are added to the 5′ and 3′ end, respectively. A GC pair needs to be introduced upstream of the sequence encoding the sense strand in case the siRNA starts with a U or C (as in the case of the shown shRNA targeting phospholamban). A series of six Ts terminates polymerase III transcription. (**d**) For the amiRNA approach, the siRNA sequence is inserted into the sequence environment of a natural microRNA, e.g., the murine miR-155 encoded for example by the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (Invitrogen). The miR-155 flanking sequences were optimized for higher knockdown efficiency and facilitated sequence analysis. The oligonucleotides encoding the amiRNA were designed for cloning into the pcDNA 6.2-GW vector (Invitrogen). Again, the second oligonucleotide is shown in 3′–5′ direction for clarity, but needs to be ordered in the 5′–3′ direction. The active RNA antisense strand is always written in *bold*

4. Mix 100 pmol of each of the oligonucleotides with annealing buffer ad 50 µl. Heat to 95 °C for 4 min; slowly cool to room temperature.
5. Mix 2 µl of oligonucleotides annealing mixture with 1 µl ATP, 1 µl T4 polynucleotide kinase, 1 µl 10× T4 PNK buffer and H_2O ad 10 µl. Incubate for 30 min at 37 °C. Inactivate T4 polynucleotide kinase by heating to 70 °C for 10 min.

6. Mix 50 ng of linearized, dephosphorylated plasmid with 5 µl of mixture with phosphorylated oligonucleotides, 1 µl T4 DNA ligase, 1 µl 10× T4 DNA ligation buffer and H_2O ad 10 µl. Incubate at 16 °C overnight.
7. Transform the ligation mixture into competent *E. coli* cells according to standard procedures.
8. Pick bacteria colonies next day and carry out plasmid preparation using a plasmid miniprep kit. Control the correctness of the plasmid by restriction analysis and sequencing. Carry out plasmid maxi preparation using a plasmid maxiprep kit (*see* **Note 12**).

3.1.2 Cloning of amiRNAs Against Phospholamban

For this procedure the siRNA sequence is inserted into the sequence environment of a naturally occurring microRNA, commonly miR-30 or miR-155 (Fig. 1). This approach has four major advantages: (1) amiRNA systems are highly efficient, as the endogenous miRNA pathway is used. (2) Some RNA polymerase II promoters are—in contrast to RNA polymerase III promoters—tissue-specific. (3) The amiRNA system may be used to co-express a protein-encoding cDNA, e.g., GFP as a reporter. (4) amiRNAs can be inserted into an expression cassette as repetitive copies and together with a transgene. Cloning of an amiRNA directed against PLB with the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (Invitrogen) is described here as an example.

1. The vector pcDNA™6.2-GW/miR is supplied as a linearized plasmid.
2. Mix 5 µl of each of the oligonucleotides (200 µM) with 2 µl of 10× oligo annealing buffer and 8 µl H_2O. Heat the mixture at 95 °C for 4 min. Slowly cool down to room temperature. Dilute the mixture to obtain a final concentration of the double-stranded oligonucleotide of 10 nM.
3. Mix 2 µl of linearized pcDNA™6.2-GW/miR with 4 µl of the double-stranded oligonucleotides, 4 µl of 5× T4 DNA ligase buffer, 1 µl T4 DNA Ligase and 9 µl H_2O. Incubate for 5 min at room temperature.
4. Transform the ligation mixture into competent *E. coli* cells according to standard procedures.
5. Pick bacteria colonies next day and carry out further analysis as described under Subheading 3.1.1, **step 8**.

3.2 Generation of AAV Shuttle Plasmids with Self Complementary (sc) AAV Vector Genome

To express a transgene, AAV vectors with single-stranded (ss) vector genomes need to intracellularly convert their single-stranded DNA genome into double-stranded DNA genomes [15]. This takes days to weeks and is the main reason for delayed onset of transgene expression after transduction. In contrast, transgene expression rapidly increases when scAAV vector are used [11].

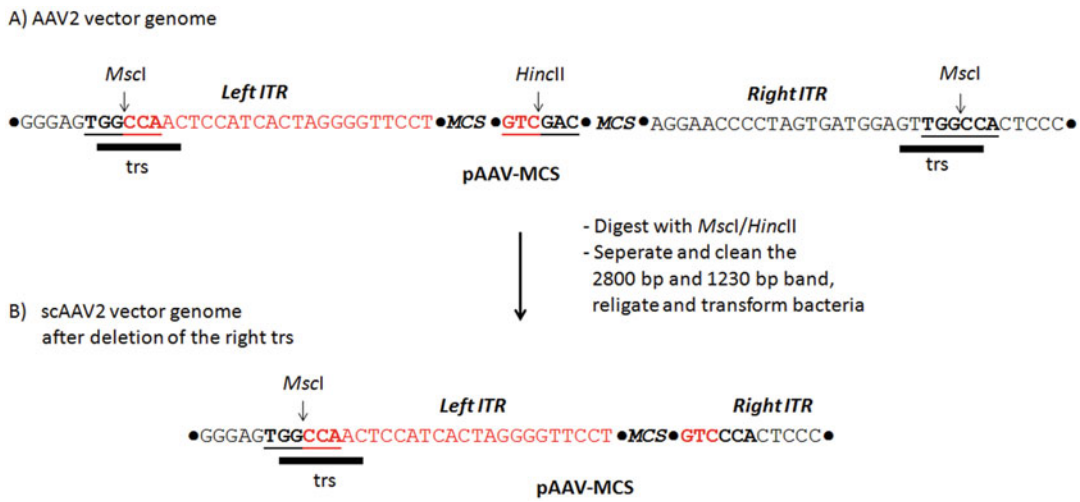

Fig. 2 Generation of AAV shuttle plasmid by deletion of terminal resolution site in pAAV-MCS (**a**) AAV vector genome sequence of left and right AAV2 ITR with trs and *Msc*I and *Hinc*II restriction sites. (**b**) Partial sequence of left and right AAV2 ITRs after insertion of a 1230 bp long *Msc*I/*Hinc*II fragment (*red*) into the 2800 bp long plasmid backbone of *Msc*I/*Hinc*II digested pAAV-MCS. *MCS* multi cloning site, *trs* terminal resolution site; *Dots*, stretches of unwritten nucleotides

In these vectors both DNA strands are packaged as a single molecule, forming a dimeric vector genome, which can directly be used for transcription. scAAV vectors can be generated by deleting the terminal resolution site (trs) sequence in one AAV ITR. How to delete the trs is described in the following steps (*see* also Fig. 2).

1. Mix 5 µg pAAV-MCS (*see* **Note 13**) with 1 µl (5 U) *Msc*I, 0.5 µl *Hinc*II, 5 µl 10× restriction buffer and H_2O ad 50 µl and incubate for 2 h at 37 °C.
2. Separate the digested plasmid fragments by agarose gel electrophoresis using a 1% agarose gel containing ethidium bromide (0.5 µg/ml). Cut out gel slices containing a 2800 and 1230 bp band (*see* **Note 10**) and isolate the DNA fragments using a gel extraction kit. Estimate the concentration of each DNA fragment on a separate agarose gel by comparison with a standardized marker.
3. Mix 100 ng of the 2800 bp fragment with 250 ng of the 1230 bp fragment (total volume ≤ 17 µl), add 1 µl T4 DNA ligase, 2 µl 10× T4 DNA ligation buffer and H_2O ad 20 µl and incubate for 1 h at room temperature.
4. Transform competent *E. coli* with the mixture (*see* **Notes 14** and **15**) and transfer the bacteria solution to agar plates according to standard procedures.
5. Plasmid isolation: Pick positive colonies next day and carry out plasmid minipreparation using commercial plasmid miniprep

kits and control correctness of the plasmid by restriction analysis and sequencing. Use plasmid maxiprep kits for maxi-preparations of large amounts of the scAAV shuttle plasmid (*see* **Note 16**).

6. To generate shRNA and amiRNA expressing scAAV shuttle plasmids, the respective expression cassettes can be cut out from respective plasmids (*see* Subheadings 3.1.1 and 3.1.2) and subcloned into the scAAV shuttle plasmid (*see* **Note 17**) using standard cloning procedures.

3.3 Production of AAV Vectors in Roller Bottles (see Note 18)

Recombinant AAV vectors can be produced by co-transfection of the 293T host cell line with an AAV shuttle vector plasmid, a helper plasmid containing genetic information of adenoviral helper gene products (E2A, E4, VA RNA) and another plasmid expressing AAV replicase and capsid gene products (e.g., for generation of scAAV2/9 vectors). Alternatively the adenoviral helper function and the AAV replicase and capsid genes may be expressed from a single plasmid. Here only two plasmids, the helper and the AAV shuttle plasmid, are necessary to generate AAV vectors (e.g., for generation of scAAV2/6 vectors). The following protocol can be used for the production of ssAAV vectors and scAAV vectors. Generated vectors can be used directly for in vitro and in vivo transduction.

1. Coat roller bottle with 35 ml collagen solution (*see* **Note 19**) and incubate it in a Heracell 240i CO_2 incubator on the bottle turning device for 30 min at 37 °C. Roller bottles should spin with 0.2 rpm during this and all further incubation steps (*see* **Note 20**).
2. Prior to cell seeding, wash the coated surface of the roller bottles with 25 ml 1× PBS. For wash steps the bottle can also be spun by hand. To seed the cells, carefully add 6×10^7 HEK293T cells to the bottom of the bottle (*see* **Note 21**). Add 200 ml DMEM complete medium to the cells and put the bottle onto the bottle turning device in the Heracell 240i CO_2 incubator. Let cells grow at 37 °C, 5% CO_2 for 48 h. Check the confluence of the cells. If cell density reaches about 80%, the cells can be transfected.
3. According to the desired vector, prepare the transfection solutions Mix A and Mix B as described in Table 1 using PEI (*see* **Note 22**). Consolidate Mix B and Mix A, vortex and incubate the transfection mix at room temperature for 15 min.
4. Remove 60 ml media from the roller bottle and store it as conditioned media.
5. Add 14 ml of the transfection mix to the roller bottle and incubate at 37 °C, 5% CO_2 for 72 h.
6. Decant the supernatants (about 154 ml) into 200 ml Erlenmeyer flask (*see* **Note 24**) and wash the cells with 25 ml 1× PBS.

Table 1
Preparation of transfection solution for 1 roller bottle (*see* Note 23)

Vector	Mix A	Mix B
scAAV2/6	7 ml 150 mM NaCl 30 µg pscAAV-shRNA or 30 µg pscAAV-amiRNA 90 µg pDP6rs	7 ml 150 mM NaCl 120 µl PEI
scAAV2/9	7 ml 150 mM NaCl 50 µg pscAAV-shRNA or 50 µg pscAAV-amiRNA 90 µg pHelper 90 µg p5E18VD2/9	7 ml 150 mM NaCl 230 µl PEI

7. Add 10 ml trypsin–EDTA into the roller bottle and incubate for 10 min at 37 °C and 5 % CO_2 on the bottle turning device.
8. Stop cell detachment with 25 ml conditioned media from **step 4** (*see* **Note 25**).
9. Transfer the suspension to 50 ml centrifuge tubes or larger centrifugation vessels if applicable.
10. Centrifuge for 20 min, 1200 × *g* at room temperature (*see* **Note 26**).
11. Resuspend the cell pellet in 14 ml 1× PBS and transfer it into a 50 ml centrifuge tube.
12. Centrifuge at 1200 × *g* for 20 min at room temperature and discard the supernatant.
13. Resuspend the pellets in 3.5 ml 1× PBS (*see* **Note 27**).
14. Disrupt the cells by 4 cycles of repeated freezing (−80 °C) and thawing (37 °C in a water bath).
15. Centrifuge for 30 min at 3900 × *g* and transfer the supernatant to a 15 ml centrifuge tube.
16. Centrifuge for 30 min at 3900 × *g* and transfer the supernatant to a new 15 ml centrifuge tube.
17. Add 35 µl benzonase nuclease (final concentration 250 U/ml) and incubate for 1 h at 37 °C. Mix the solution from time to time.
18. Centrifuge for 20 min at 3900 × *g* and transfer the supernatant to a new 15 ml centrifuge tube. Mix the supernatant 1:1 with 1× PBS. The final volume is about 7 ml (*see* **Note 28**).

3.4 Precipitation of AAV Vectors from Supernatant (see Note 29)

1. Add 2 ml 2.5 M $CaCl_2$ per 100 ml supernatant, mix and cool on ice for 1 h.
2. Distribute the solution into 50 ml centrifuge tubes or larger centrifugation vessels.

3. Centrifuge for 30 min at 3900 × *g* and transfer the supernatant into a 400 ml Erlenmeyer flask.
4. Add 60 ml 1.86 M NaCl/24% PEG per 100 ml supernatant, mix and incubate for 72 h at 4 °C.
5. Centrifuge for 30 min at 3000 × *g* and discard the supernatant.
6. Resuspend the pellet in 5 ml NaCl–Hepes solution.
7. Centrifuge 15 min at 10,000 × *g* and transfer the supernatant into a 15 ml centrifuge tube.
8. Add 50 µl Benzonase Nuclease to the supernatant (final concentration 250 U/ml) and incubate for 1 h at 37 °C. Mix from time to time.
9. Centrifuge for 20 min at 3900 × *g* and transfer the supernatant to a new 15 ml centrifuge tube. Mix the supernatant 1:1 with 1× PBS. The final volume is about 10 ml (*see* **Note 30**).

3.5 Filtration of AAV Vectors with Iodixanol Gradient System

1. Prepare iodixanol dilutions as shown in Table 2.
2. Add 3.5 ml 15% iodixanol to a 22 ml polypropylene centrifuge tube.
3. Underlay 15% iodixanol with 3.5 ml 25% iodixanol, then with 4.5 ml 40% iodixanol and finally with 6 ml 54% iodixanol using a long cannula.
4. Overlay the gradient with the AAV solution produced under Subheadings 3.3 and 3.4 (*see* **Note 31**).
5. Fill-up the tube with PBS-MK to the mark on the tube (*see* **Note 32**).
6. Centrifuge at 300,000 × *g* at 18 °C under vacuum. Use a slow deceleration protocol to avoid swirling of the layers.
7. Take the tube out of the centrifuge, dispose its cap and remove the AAV-containing layer by pricking the tube with a short cannula laterally into the tube immediately above the 54% iodixanol layer and extract the layer between 25 and 54% iodixanol layers (approximately 3 ml) (*see* **Note 33**, Fig. 3).

Table 2
Dilution of iodixanol

	15% iodixanol	25% iodixanol	40% iodixanol	54% iodixanol
PBS-MK	37.5 ml	28 ml	16.5 ml	5 ml
Iodixanol	12.5 ml	20 ml	33 ml	45 ml
Phenol red	–	100 µl	–	100 µl

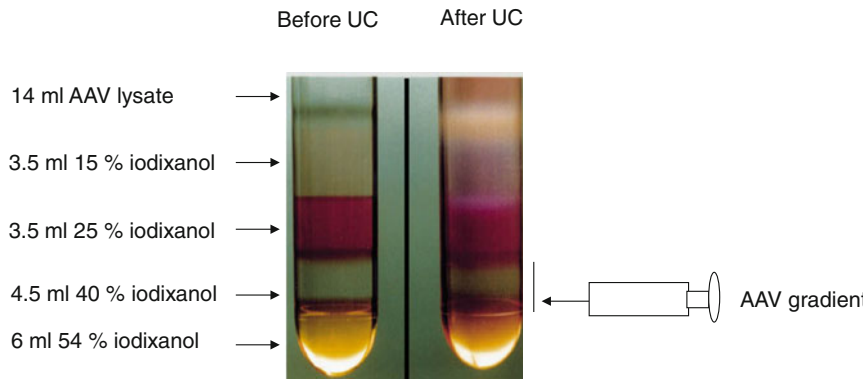

Fig. 3 Iodixanol gradient before and after centrifugation. Positioning of the cannula for harvesting the AAV vector is shown on the right site. *UC* ultra centrifugation

3.6 Concentration of AAV Vectors

1. Dilute scAAV vector preparation 1:10 with PBS-MK.
2. Fill 15 ml of the scAAV vector preparation into an Amicon® Ultra 15 ml centrifugal filter.
3. Centrifuge for 15 min at 4000×*g*. Discard flow-through and repeat until the entire preparation has been centrifuged (*see* **Notes 34** and **35**).
4. Rinse the filter areas of the Amicon® Ultra 15 ml centrifugal filters with the remaining buffer (about 150 µl) several times to resuspend the vector particles, if necessary dilute with PBS-MK (*see* **Note 36**).

3.7 Isolation of Primary Neonatal rat Cardiomyocytes (PNRC), Its Transduction with AAV Vectors and Maintenance of the Transduced Cell Culture

To characterize the posttranscriptional inhibition of selected cardiac targets (e.g., PLB) using small interfering RNAs such as shRNA or amiRNAs, cultured primary neonatal rat cardiomyocytes (PNRC) have been proven to be a valuable experimental model [4, 6, 13]. Therefore, the preparation of primary neonatal heart cell cultures from ventricular tissue of 1–3-day-old rat pups, described by Vetter et al. [12] earlier, will be specified here as a detailed protocol.

1. Wipe down a hood with either Barrycidal or 70% ethanol.
2. Pour 20 ml ice-cold 1× CIM with P/S into each of two or three sterile 100 mm petri dishes.
3. Wash five neonatal rats (*see* **Note 37**) at a time in two sequential weigh boats with 70% ethanol.
4. Holding a pup with large forceps, dip briefly into 70% ethanol, decapitate with large scissors, place the decapitated neonate on its back on the stack of paper towels. Repeat this procedure for the remaining four pups. Before continuing, wash another set of five pups with 70% ethanol and transfer into the proper weigh boats. Hold the decapitated animals down with forceps,

dab the ventral thorax area with a sterile swab using another pair of forceps and make a midline incision through the sternum using a small pair of scissors (*see* **Note 38**). Press downward with the large forceps to pop the heart out through this incision. Clip the heart out with the small pair of scissors and carefully transfer to the petri dish filled with 20 ml 1× CIM with P/S with the small forceps. Repeat this procedure with the other pups. Up to 25 hearts per dish can be pooled.

5. After all the hearts have been collected, trim away the atria and any other tissue such as connective tissue or fat from each heart. Transfer the remaining heart (consisting now of the right and left ventricle only) to a second petri dish with 20 ml ice-cold 1× CIM with P/C, where the remaining heart should be cut into 3–4 tissue pieces using small forceps and scissors. Alternatively, cutting into pieces can also be performed using two scalpels. Finally, pool all tissue pieces of the dissected hearts in the latter dish (*see* **Note 39**).
6. Carefully withdraw 1× CIM with P/C from the petri dish using a Pasteur pipette. Add 20 ml ice-cold 1× CIM to wash the tissue pieces and withdraw the solution from the petri dish again. Repeat the latter procedure once more. Thereafter, add 15 ml 1× CIM into the dish (*see* **Note 40**).
7. Transfer the complete content of the petri dish including all tissue pieces into a sterile spinner flask. Remove the buffer with a sterile Pasteur pipette and add depending on the number of hearts used 8–15 ml antibiotic-free 1× CIM with trypsin as indicated in Table 3.
8. Incubate the content in the spinner flask jacketed with 36–37 °C thermostatic H_2O under continuous stirring with 150 rpm. Remove and discard the digestion buffer after 3–5 min (*see* **Note 41**).
9. Add 10 ml 1× CIM with trypsin and incubate at 36–37 °C. Remove the digestion buffer carefully after 15 min

Table 3
Used 1× CIM with trypsin per number of hearts

Number of hearts	1× CIM with trypsin, ml
0–14	8
15–19	9
20–25	10
25–29	11
30–34	13
35–50	15

using a sterile culture pipette and transfer the content into a prepared 50 ml conic centrifuge tube filled with 5 ml ice-cold HS (In case you have added 15 ml 1× CIM with trypsin, the removed digestion buffer should be transferred into a prepared 50 ml conic centrifuge tube filled with 6.5 ml ice-cold HS). Mix the content of the centrifuge tube carefully. Add another 10 or 15 ml 1× CIM with trypsin to the Spinner flask and incubate as indicated before. While this next digestion is running, centrifuge the centrifuge tube at 300–400 × *g* for 8 min using centrifuge equipped with a swing out rotor. Set the centrifugation conditions to slow acceleration, minimal brake, and room temperature. Use this schedule to perform another five digestion steps, with subsequent cell collection, using centrifugation in each step (*see* **Note 42**).

10. Carefully remove and discard the supernatant from the centrifuge tube after centrifugation. Add 1 ml FCS plus 1 ml CMRL1515-ATM to this tube and resuspend the cell pellet carefully. Perform this procedure for all cell pellets in the six centrifuge tubes obtained after centrifugation and pool the contents of the six tubes into a single tube.
11. Filter the pooled crude cell suspension through two layers of gauze into one sterile 50 ml conic centrifuge tube using a sterile 40 mm glass funnel. Centrifuge the tube at 300–400 × *g* for 8 min as indicated under **step 9**. Remove and discard the supernatant carefully after centrifugation (*see* **Note 43**).
12. Resuspend the obtained cell pellet in complete gentamycin-containing CMRL1415-ATM cell culture medium supplemented with 10% of FCS and 10% HS (FCS/HS-CMRL1415-ATM) each and transfer the crude cell suspension into a 175 ml culture flask.
13. Incubate the cell suspension in an incubator for 90 min at 37 °C for selective attachment of non-muscular cells. In general, use 1 ml of this medium per one heart used for digestion, i.e., for 10 hearts use 10 ml medium, for 20 hearts use 20 ml, etc. At the end of the incubation period, carefully remove the cardiomyocyte-enriched medium from the culture flask and transfer it into a 50 ml conic centrifuge tube using a disposable plastic tissue culture pipette.
14. Pipette 50 µl of the obtained cell suspension into a micro tube together with 50 µl 1× PBS and 50 µl Trypan blue and mix the contents carefully. Immediately use a sample of this mixture to count the number of PNRC using a Neubauer improved hemocytometer and a microscope. Count the number of PNRC in each of four quadrants and calculate the mean value. For final calculation of the cell density, take into consideration the 1:3 dilution of the original cardiomyocyte-enriched medium obtained under **step 13**.

15. Adjust the cell density cardiomyocyte-enriched medium (obtained under **step 13**) to 4×10^5 cells/ml using FCS/HS-CMRL1415-ATM with gentamycin. Distribute this cardiomyocyte containing medium either to cell culture plates or cell culture flasks depending on the demands of the planned experiments. For later transduction of the cultured cardiomyocytes with viral vectors a seeding cell density of 1.25×10^5 cells/cm^2 is recommended. To reach this density, volumes of 3.0 ml, 1.5 ml, and 1 ml/well of the cardiomyocyte-enriched medium with 4×10^5 cells/ml are needed if distributed to round 6-well, 12-well, or 24-well culture plates, respectively (*see* **Note 44**).
16. Incubate the cell culture plates for 24 h at 37 °C in an incubator (*see* **Note 45**).
17. After the latter incubation period remove and discard the medium and add to each well of the culture plates the indicated volumes (as described under Subheading 3.7, **step 15**) of complete CMRL1415-ATM cell culture medium with 10% FCS containing also 2 μM FUDR in addition to 20 μg/ml gentamycin (*see* **Note 46**).
18. After another 48 h of incubation replace the medium by fresh CMRL1415-ATM cell culture medium with 10% FCS, 2 μM FUDR, and 20 μg/ml gentamycin and transduce the cells with scAAV vectors by directly adding the vector into the cell culture medium and incubate at 37 °C as before (*see* **Note 47**).
19. Twenty four hours later the cell culture medium containing the viral vector can be removed and discarded. The wells of the plates are washed once with 1 ml, 1.5 ml, or 3 ml 1× PBS, depending on the type of plates used, respectively, to get rid of the non-absorbed viral vector. Thereafter, add fresh CMRL1415-ATM cell culture medium with 10% FCS, 2 μM FUDR, and 20 μg/ml gentamycin to the wells and incubate at 37 °C as indicated above. Using medium changes every 24–48 h, the spontaneously contracting PNRC can be cultured until they are harvested after 4–14 days in culture (*see* **Note 48**).

4 Notes

1. Using this procedure the volume of the solution will be increased by 150 ml.
2. Do not store this glucose containing solution longer than 3 days at 4 °C.
3. Most commercially available trypsin preparations for cell cultures purposes will not work properly for neonatal cardiomyocyte preparation. Best results can be obtained with crude trypsin preparations, although not all crude enzyme prepara-

tions will work properly and vary from lot to lot. Therefore, it is necessary to prescreen several lots of crude trypsin before selecting a lot to purchase. We have successfully used crude porcine trypsin 1:250 from (Belger Biochemie, Kleinmachnow, Germany, Lot 0110795).

4. First use a 20 or 50 ml syringe with a disposable filter unit (0.45 μM pore size) to filter the entire volume. Thereafter, use a 10 ml syringe with another disposable filter unit (0.22 μM pore size) to filter 10 ml portions to sterility of the prefiltered 10× trypsin. This procedure is recommended to prevent plugging of the 0.22 μM filter.
5. CMRL stands for Connaught Medical Research Laboratories and ATM stands for atmosphere. The bicarbonate-free medium has a high buffering capacity and a 5 % CO_2 atm and thus a CO_2 incubator is not needed for cell cultures growing in this medium. Moreover, cell cultures can be handled outside the incubator without substantial changes in the pH of the medium.
6. Usually 500 ml CMRL1415-ATM are delivered by the supplier in 500 ml bottles. It is recommended to remove 2 ml from such a bottle. Thereafter, add the indicated volumes of ingredients of 1000× $CaCl_2$ and 1000× FUDR to the CMRL1415-ATM containing bottle. This will guarantee the correct final concentrations of $CaCl_2$ (1.26 mM), FUDR (2 μM), and gentamycin (10 μg/ml).
7. We routinely calculate approx. 25 ml of this solution for isolation of cardiomyocytes from five neonatal rat hearts. Accordingly, a higher volume is needed if higher numbers of hearts are used, e.g., prepare 250 ml of this solution for 50 hearts.
8. Various criteria for the design of efficient siRNAs have been suggested, including a GC content between 30 and 52 %, A or U in positions 15–19, lack of internal repeats, A at positions 3 and 19, U at position 10, G or C at position 19 and G at position 13 [16]. In addition, structural restrictions of the target site have to be taken into consideration [17]. Various programs are provided at no cost to support the design of siRNAs, e.g.: http://dharmacon.gelifesciences.com/design-center/ or http://rnaidesigner.thermofisher.com/rnaiexpress/design.do. Alternatively, use one of the known active siRNAs published for most human and rodent genes in recent years. Furthermore, various companies offer predesigned and validated siRNAs with a silencing guarantee. It is still advisable to test three to four siRNAs for a given target to find the most efficient candidate. The standard design for siRNAs comprises a 19mer duplex with two nucleotide overhangs at both 3′ ends. Figure 1 shows an example of an active siRNA targeting phospholamban.

9. As the prices for DNA synthesis have dropped dramatically in recent years, one may chose the option not to clone oligonucleotides downstream of the promoter, but rather to have parts or the entire expression cassette synthesized. This strategy is usually much faster, less labor-intensive and only slightly more expensive.
10. Put the agarose gel on a UV transilluminator to visualize the bands and cut out the gel slices with a scalpel. Be careful to protect your eyes with UV protective glasses and wear protective gloves to prevent contact with the gel, as ethidium bromide, used to make the DNA bands visible, is toxic. The vector backbone (2800 bp DNA fragment) can be dephosphorylated to prevent the *Msc*I sites from religating.
11. Dephosphorylation enhances the efficiency of cloning by preventing religation of vector DNA which was only cleaved by one of the restriction enzymes.
12. Plasmid maxipreparation yield large amounts of plasmid DNA, making it easier to subclone an expression cassette into AAV shuttle plasmids. In principle, plasmids isolated by plasmid mini preparation can also be used for subcloning.
13. pAAV-MCS is a plasmid containing an AAV vector genome which consists of the 5′ and 3′ ITRs from AAV2.
14. We suggest using recombination deficient (*recA*) bacteria, e.g., XL10-Gold Ultracompetent cells (Stratagene) for cloning to prevent recombination of AAV ITRs.
15. After restriction with *Msc*I/*Hinc*II, the 1230 bp DNA fragment has blunt ends. Therefore it is possible that after ligation the fragment is inserted in two different orientations in the plasmid, leading to destruction of either the left or right AAV ITR. Either plasmid, containing a deletion of the right or left ITR, can be used as AAV shuttle plasmid to generate scAAV vectors.
16. Plasmid mini- and maxiprep kits are supplied by several companies. The plasmid maxiprep kits are typically used to produce high amounts of plasmid DNA which are necessary for AAV preparation. To prevent possible toxic side effects during AAV production, use endotoxin free plasmid maxiprep kits.
17. The vector DNA present in the AAV shuttle plasmid (representing the nucleotide number from the 5′ end of the left to the 3′ end of the right ITR in the AAV shuttle plasmid) should not exceed 2.4 kb for reliable packaging. Despite reports that larger AAV vector genomes can be packaged, we were unable to consistently package scAAV genomes exceeding this critical threshold. ShRNAs and amiRNA expression cassettes are typically smaller than this size, and therefore packaging of the respective expression cassettes in AAV genomes should be unproblematic. The lower limit of scAAV vector genome size

is currently unknown. We found that scAAV vector genomes with total length of 975 bp can be successfully packaged.

18. The method is described for production of AAV vectors in one roller bottle. One roller bottle is sufficient for production of a total of 1×10^{10}–1×10^{11} scAAV vectors (determined as vector genomes (vg) by quantitative PCR). For large-scale production we generally use a minimum of 6–10 roller bottles. You can also use standard disposable cell culture vessels (flasks or plates) for production. One roller bottle replaces approximately 10 cell culture plates (14.5 cm diameter).
19. The collagen is available from Sigma-Aldrich (Cat. No. C8919). For details of preparation of the collagen solution go to: http://www.sigmaaldrich.com/technical-documents/articles/biofiles/collagen-product-protocols.html#sthash.Y5GaCDUp.dpuf
20. Removed collagen solution can be reused. We have reused it for up to 10 times. Coated roller bottles can be stored at 4 °C for several days.
21. In each step when a solution is added/removed from the roller bottle set down the bottle gently to avoid shaking off cells. Also avoid spraying added solutions onto the walls of the bottle where cells are growing, as this may lead to unintentional detachment of the cells.
22. Before use, PEI must be warmed up at 65 °C for 10 min to ensure the PEI is completely dissolved.
23. For packaging of pseudotyped scAAV2/6 vectors (containing ITRs of AAV2 and capsids of AAV6) the pDP6 plasmid is used. It contains essential adenoviral helper functions and the replicase gene of AAV2 and the capsid gene of AAV6. For packaging of pseudotyped scAAV2/9 vectors (containing ITRs of AAV2 and capsids of AAV9) the adenoviral helper functions (E2a, E4, and VA RNA) and the replicase gene of AAV2 and the capsid gene of AAV9 are on separate plasmids, pHelper and p5E18VD2/9, respectively.
24. The supernatant also contains AAV vectors, but the yield is usually low compared to the cells collected from the roller bottle. Therefore isolation of AAV vectors from the supernatant (*see* Subheading 3.4) is often not recommended.
25. Rinse the lateral surfaces of the roller bottle with the cell solution while gently spinning the bottle by hand to detach all cells from the walls.
26. Prolonged centrifugation (30 min) leads to more compact pellets. Be careful with decantation to avoid discarding the cell pellet.
27. The suspension can directly be used in the next step or be stored at −80 °C until use.

28. The solution can be stored at −80 °C or directly filtered with the iodixanol gradient system.
29. In most cases this step will not lead to a drastic increase of total vector yield. Therefore this step is optional.
30. Store the solution at −80 °C or go to filtration with iodixanol gradient system.
31. Let the AAV solution run down on the wall of the tube very slowly to avoid swirls within the iodixanol layers. The maximum capacity of the tube for AAV solution is about 14 ml. Do not overload the gradients: A ratio of 2 roller bottles of AAV isolates per gradient should not be exceeded.
32. The centrifuge tube has an obvious mark on its neck. Be sure that the tube is filled exactly to the mark, otherwise the tube will collapse during ultracentrifugation. Tare the centrifuge tubes as exactly as possible (e.g., to the fourth digit on a milligram scale).
33. If the cannula is pricked into the tube, the open angle of the cannula must point upwards. When you remove the cannula from the tube, the contents of the tube will leak out the punctured site. Therefore, we hold the tube over a 50 ml centrifuge tube and drop it into the tube immediately after pulling out the cannula. AAV vector solution can be stored at −80 °C.
34. If solution remains in the tube and does not pass through (which is a result of remaining iodixanol in the preparation), resuspend and rinse the membrane of the Amicon® Ultra 15 ml centrifugal filters with the remaining volume or extend centrifugation time.
35. For large volumes of AAV, several Amicon® Ultra 15 ml centrifugal filters can be used in parallel. After centrifugation the concentrated AAV solutions can be combined and concentrated further using a fresh Amicon® Ultra 15 ml centrifugal filter. Be careful if an Amicon® Ultra 15 ml centrifugal filter is centrifuged several times. The more often a column is centrifuged, the higher the probability of leaks and therefore loss of AAVs.
36. Use a suitable micropipette for resuspension to avoid formation of foam. Concentrated scAAV vectors can be stored at −80 °C.
37. Newborn Sprague Dawley or Wistar rats can be used. However, use 1–3 day old pups only. Do not use neonates older than 3 days, because the hearts of these animals have a more extensive extracellular matrix, which hampers its trypsin-catalyzed digestion and needs more digestion time. Extending digestion times may lead to enhanced trypsin-related damage of PNRC. As a consequence the number of viable PNRC will decline and an excessive number of non-surviving cells will be found in culture.

38. The length of the incision should be less than half of the length of the body. It should not extend across the thorax–abdomen border.
39. Complete removal of atria is very important. Contaminating atrial pace maker cells in the culture of spontaneously beating PNRC will result in a markedly increased contraction rate.
40. These washing steps are necessary to get rid of penicillin and streptomycin which could be harmful to the PNRC during the following enzymatic digestion.
41. This first digestion allows the removal of broken cells as well as blood cells. The temperature during this digestion step as well as the following ones should be continuously checked and protocoled. It should not exceed 37 °C. Otherwise trypsin-catalyzed digestion will result in excessive cell damage leading to an increased number of dead cells in the sought culture.
42. Too rapid acceleration of the centrifuge rotor may damage the enzymatically isolated PNRC, while too rapid deceleration will result in loss of PNRC due to turbulence causing some pelleted cells to be lost to the supernatant.
43. This filtration step through sterile gauze is used to remove feasible cell debris of larger size from the cell suspension.
44. For firm attachment and growth of the isolated PNRC we strongly recommend using flasks and cell culture multidishes with Nunclon™ Delta surface coating. If you intend to use other types of plastic culture material for attachment and growth of the PNRC, they must first be tested. In some cases premature surface coating with collagen or another suitable surface coating substance may be needed.
45. Using CMRL1415-ATM medium during incubation no CO_2 supply is needed. Therefore, if a CO_2-incubator is being used, adjust the CO_2 supply to zero. This also holds true for all following incubation steps.
46. The mitosis inhibitor FUDR is added to reduce division and growth of contaminating fibroblasts, which, in contrast to PNRC, will otherwise proliferate substantially in the serum-containing medium.
47. At this time point spontaneously contracting PNRC usually will have reached a confluence of about 70–80 %.
48. We have been successful keeping contracting PNRC in culture for up to 21 days. However, keep in mind that the number of fibroblasts in the culture will increase continuously, even in the presence of 2 μM FUDR. Higher concentrations of the inhibitor to achieve complete suppression of fibroblast mitosis should not be used, due to its toxicity to PNRC.

Acknowledgments

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Chapter 3

Generation of Efficient miRNA Inhibitors Using Tough Decoy Constructs

Jimeen Yoo, Roger J. Hajjar, and Dongtak Jeong

Abstract

Over the last decade a previously unappreciated mechanism of gene regulation has been uncovered that is mediated by a large class of small noncoding RNAs known as microRNAs (miRNAs), and this mechanism is utilized by organisms ranging from plants to humans. MiRNAs are important downregulators of gene expression and are seen to be dysregulated in disease development. Thus inhibition of aberrantly upregulated miRNAs as a therapeutic approach has become a promising field.

Many models of miRNA inhibitors currently exist, with decoy models being the most successful in current research. A promising inhibition model is the tough decoy (TuD) RNAs inhibitor, which uses antisense sequences to bind to target miRNAs, preventing them from binding to their endogenous targets. Since the TuD inhibitors have the ability to be successfully used in vitro and in vivo studies, this is a covetable inhibition method. In this chapter, we introduce how to design and generate miRNA tough decoy inhibitors with an adeno-associated viral construct. TuD inhibitors will have two miRNA binding sites. The TuD will include stem sequences, a miRNA binding site, and linkers. In vitro validation experiments to confirm the effectiveness of the TuD to inhibit miRNA are described. We also propose some practical approaches for making a TuD for miRNA of interest. We hope this chapter facilitates readers to create a simpler method to generate TuD that can be used for miRNA loss of function studies.

Key words miRNA, Tough decoy, Gene regulation, miRNA inhibition

1 Introduction

1.1 miRNA Function and Biogenesis

Noncoding intron regions of mRNA have been previously ignored as important regions encoding genetic information but the discovery of microRNA (miRNA) has shed light into these once disregarded regions. MiRNAs are small noncoding RNA molecules that are about 21–26 nucleotides in length and mainly downregulates gene expression by binding to mRNA targets and preventing translation [1]. Depending on miRNA levels, phenotypes can be profoundly altered in the cell. Thus this short sequence plays an important regulatory role in RNA silencing and posttranscriptional modification ranging from development to disease pathophysiology. The functional ability of miRNA has rendered it as a potential

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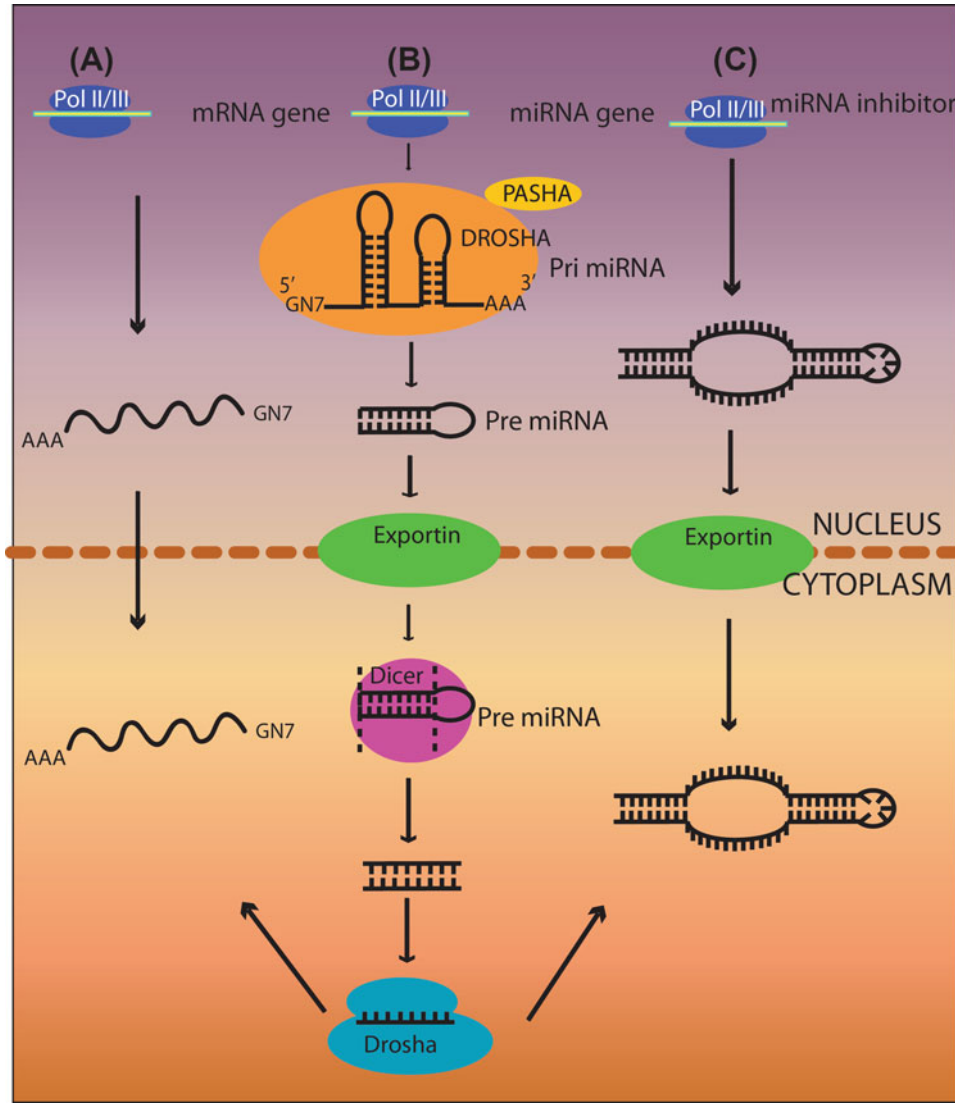


Fig. 1 Biogenesis of mRNA, miRNA, and miRNA inhibitor. (**a**) The target mRNA molecule is processed by RNA Pol II or III and transported to the cytoplasm where it can interact with the miRNA RISC complex. (**b**) The miRNA is transcribed by RNA Pol II or III forming a pri-miRNA complex. (**c**) The miRNA inhibitor of choice, in this case a tough decoy-type inhibitor, is transcribed from the vector delivered inhibitor cassette

therapeutic target. The biogenesis of miRNA is comparable to that of mRNA since both share regulatory elements, and often also shares a primary transcript (Fig. 1). MiRNA can be derived from either its own gene or from the intron of pre-mRNA. The gene or intron is transcribed by an RNA polymerase. Most miRNA were found to be transcribed by RNA Polymerase II (RNA Pol II) with exceptions of *Alu*-repetitive sequences which are transcribed by RNA Pol III [2]. The initial transcription sequence, the pri-miRNA, folds on itself to create one or more of a stem-loop precursor. This sequence can

contain more than one functional miRNA. The pri-mRNA, which contains a 5′ guanosine-triphosphate cap and a polyadenosine tail, is then ready for splicing. Then the pri-mRNA is processed in the nucleus by the heterotetramer microprocessor complex which includes two RNase III Drosha and two Digeorge Syndrome Critical Region 8 (DGCR8/pasha). The microprocessor works in conjunction with cofactors such as the DEAD box RNA helicases p68 and p72 and heterogeneous ribonucleoproteins. The RNase III Drosha is an enzyme and the DGCR8 acts as the pri-miRNA recognition and binding site. The DGCR8/Drosha complex cleaves the RNA to form a stem-loop pre-microRNA complex that is about 70 nucleotides long. Exportin-5 then recognizes the pre-miRNA by a two nucleotide overhang at the 3′ end and exports it to the cytoplasm through a GTP-GDP gradient [3]. In some noncanonical pathways, miRNA biogenesis occurs without the microprocessor step [4].

Once the pre-miRNA enters the cytoplasm, a dicer complex containing two RNase domains is able to excise the miRNA to generate products with 2-nucleotide overhangs on the 3′ end. This miRNA duplex unwinds to yield two mature miRNAs which are 22–26 nucleotides in length. One gets incorporated into the RISC complex while the other is often degraded. In some cases, the complementary strand is also used as a functional miRNA that binds to other mRNA strands. The miRNA then binds to the RNA-induced silencing complex (RISC) through the argonaute family of proteins. The RISC complex allows for interaction between the mature miRNA and the target mRNA.

Since miRNA are short sequences, they lack specificity and can have many target mRNA. MiRNAs binds to its target mRNA by imperfect or near perfect to perfect base pairing that mostly occurs in the 3′-untranslated region (3′ UTR) with some found in the 5′-untraslated region or the coding region. When miRNA binds to the 3′ UTR it can effect stability, localization, and translation of the target mRNA. This occurs by the inhibitor binding to the seed region of the miRNA, which are the 2′–7′ nucleotides on the 5′ end. Although miRNA composition and mechanism of action still remains uncertain, miRNA-mediated inhibition is known to heavily rely on base pairing between the miRNA and mRNA. In imperfect complementarity, degradation or suppression of mRNA occurs, while perfect to near perfect complementarity promotes mRNA cleavage [5]. Thus the ability of miRNA to modulate mRNA activity has given rise to interest in miRNA regulating oligonucleotides.

1.2 miRNA Inhibitors Can Manipulate miRNA Function

Oligonucleotide manipulation methods have been widely studied and used as an effective approach to produce miRNA inhibitors. Current widely used available techniques to inhibit miRNA function include but are not limited to: chemically modified short single stranded oligonucleotides, antagomiRs, locked nucleic acids, microRNA sponge, and miRNA decoy models (Fig. 2).

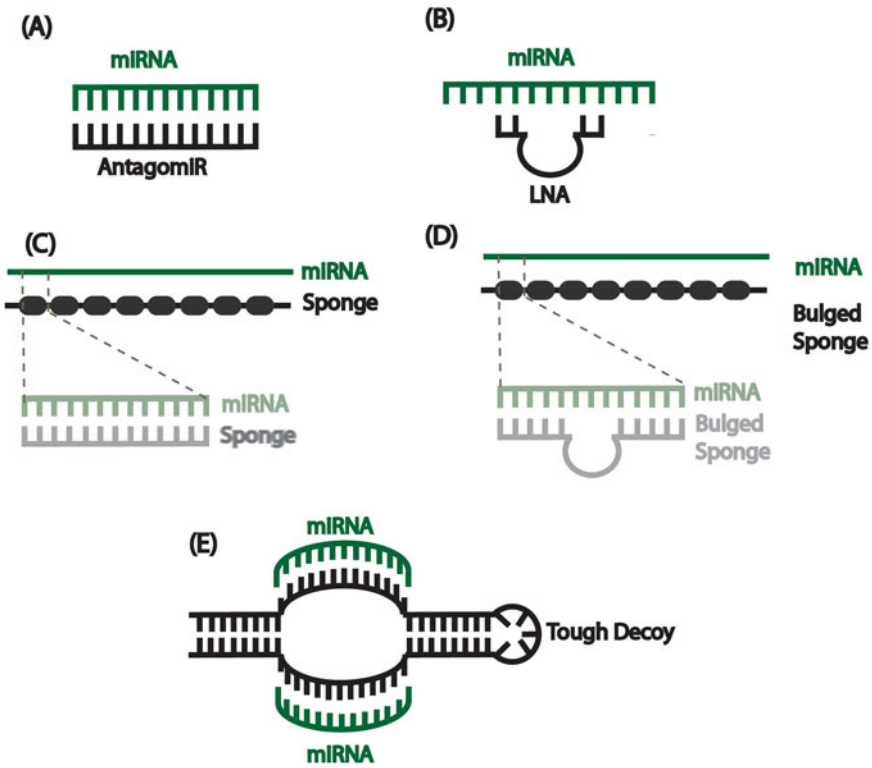


Fig. 2 Method of action for miRNA inhibitors: (**a**) antagomiR, (**b**) LNA, (**c**) sponge, (**d**) bulged sponge, and (**e**) tough decoy. (**a**) AntagomiR with characteristic single perfect miRNA target site. (**b**) LNA has shorter complementation sequences which allow for binding to families of miRNA. (**c**) Sponge inhibitor with eight consecutive perfect miRNA binding sites. (**d**) Bulged sponge inhibitor with eight consecutive perfect miRNA binding sites. (**e**) Tough Decoy inhibitor with characteristic hairpin structure containing two bulged miRNA target sites

These techniques bind to miRNA, inhibiting miRNAs from binding to mRNAs. However, these targeting strategies vary in mechanism of inhibition.

Chemically modified oligonucleotides, also known as anti-miRs, have sequences complementarity beyond the seed region and have successfully been used to block miRNA function in pre-clinical animal models [6, 7]. AntagomiRs often contain 2′-OMe modifications, asymmetric phosphorothiate modification on the 3′ and 5′ ends, and a cholesterol tail which improves in vivo pharmacology and performance [7]. Still, antagomiRs are transient and need to be continuously administered to maintain efficacy. Initial studies of antisense DNA oligonucleotides used as inhibitors of miRNA function showed that they were unstable and prone to degradation [8].

LNA modifications are potent miRNA-inhibitors, having activity even at low doses [9–11]. Thus the exceptional binding of LNA oligonucleotides make it possible to use shorter sequences that only bind to the seed regions of target miRNAs [12]. This method

can be used to target a family of miRNA without having to account for the 3′ sequence.

MicroRNA sponges are antisense inhibitors that competitively bind to their target miRNA [13]. They have been observed to target whole miRNA families through complementarity to seed sequences. These had eight RNA binding sites with perfect complementarity to their target miRNA which allows for specificity [14]. The bulged sponge inhibitor introduces a bulge in the central region of the target site in addition to the eight miRNA recognition sites which decreases endonucleolytic target cleavage, thus improving binding ability to its target mRNA [5].

Still limitations exist for current sponge inhibition models. Increased number of binding sites has been shown to make bulged sponge inhibitors more prone to deletion and mutation, making them less stable [15, 16]. Additionally, low levels of miRNA inhibitors as stable transfectants make this inhibitor short-lived despite initial effectivity.

1.3 Tough Decoy Inhibitors

The tough decoy inhibitor is a 60 base pair long hairpin shaped inhibitor with a large internal bulge containing two miRNA recognition sites [17]. Like the sponge inhibitor, tough decoy inhibitors inhibit whole miRNA families through seed sequence base pairing [5]. The bulge prevents it from endonucleolytic target [5]. Additionally, the double stranded structure is thought to cause RISC mediated destabilization of the inhibitor to be impaired. The initial design by Haraguchi et al. used a vector model designed to be expressed with RNA Pol III. This method allowed for the miRNA inhibitor constructed to be exported with exportin-5 easily because of the short 3′ overhang like that of miRNA. However, tough decoy inhibitors can also be expressed with RNA Pol II. Inhibitors expressed by Pol II maintained their inhibition capacity and had an added benefit of higher tissue specificity [5].

Furthermore, in comparison studies of inhibitors of miR-16 and miR-203, tough decoy inhibitors were shown to be the most potent among antagomiR, stabilized antagomiR, tough decoy, RISC-loaded shRNA, sponge, bulged sponge, and mask inhibition methods [5]. This shows that despite only two recognition sites existing, the use of tough decoy inhibitor is a highly effective method. Furthermore, tough decoy variations can also improve efficiency of tough decoy models. Clustered tough decoy, miRNA inhibition increased efficiency of tough decoy models. Clustering tough decoy inhibitors increases the number of recognition sites and parallel suppression of miRNAs can be documented with single molecules [18]. These suggest that families of miRNA can be suppressed through inhibition by a single clustered tough decoy model.

This inhibition method has been shown to be less effective when adverse sterics caused by the secondary structure lead to compromised functionality [5]. Furthermore, there has been an

issue in which the two target sites of the tough decoy try to mutually anneal to the targeted miRNAs, leading to a partial collapse of the structure, and thus decreasing efficacy of the tough decoy [17]. However, more experimental evidence is needed to understand the mechanistic differences of miRNA inhibitors to determine designs that can be improved.

1.4 Tough Decoy and Bulged Sponge Inhibitor

Currently, two decoy type inhibition methods, bulged sponge and tough decoy type inhibitors show the most potential as miRNA inhibition methods. Depending on the miRNA, the two decoy type inhibition methods are comparable in its efficiency. Studies determining efficiency of these decoy inhibitors in five different miRNAs shows that one was inhibited best by tough decoy, another by the bulged sponge, and three found both equally effective [5]. This shows that inhibition mechanisms need to be sensitive to features of target recognition in which a single method is not a surefire mechanism of inhibition for all miRNA.

Tough decoy gene transfer using viral vectors is a widely used method for creating miRNA inhibitors. Previously, sponge models have been well described and constructed through methodologies given by Kluiver et al. and Ebert et al. [14, 19]. These constructs use tissue specific promoters in in vivo models. However, the lack of a canonical protocol has made it difficult for tough decoy gene transfer production. This methodology chapter aims to introduce a simpler method for tough decoy inhibitor production and gene transfer in various models that can be employed in future experiments.

2 Materials

2.1 Generation of miRNA Tough Decoy and Cloning

1. Oligonucleotide pairs (sense and antisense) containing miRNA binding sites (MBS) and linkers with overhangs compatible with the restriction enzymes.
2. As a negative control, an appropriately scrambled tough decoy sequence.
3. pds-adeno-associated virus (AAV)2 empty vector with U6 promoter (*see* **Note 1**).
4. DNA extraction kit.
5. Restriction enzymes and T4 DNA ligase.
6. Competent cells.
7. SOC medium.
8. Water bath.
9. Antibiotic agar plate.
10. Incubator.

2.2 AAV Production

1. HEK293T cells (ATCC).
2. Growth medium: DMEM supplemented with 10% fetal bovine serum (FBS), 1× penicillin–streptomycin.
3. DMEM without antibiotics for the transfection reaction.
4. Transfection reagent: polyethylenimine (PEI) linear MW 25,000, PBS.
5. DNase digestion buffer: 10 mM Tris–HCl pH 7.5, 10 mM MgCl 2, 50 U/ml DNase I.
6. OptiPrep™ (Iodixanol 60%, 1.320 ± 0.001 g/ml, Axis-Shield PoC AS, Oslo, Norway).
7. Beckman Optiseal polyallomer (26 mm × 77 mm) centrifuge tubes and Plug.
8. Ultracentrifuge and fixed angle rotor.
9. Q-PCR reagents including buffers, enzymes, primers, and detection methods (i.e., SYBR green or probe-based detection) (*see* **Note 2**).
10. Q-PCR machine.

2.3 Isolation of Mouse Adult Myocyte [20]

1. Mouse model of your interest (8–10 weeks of age, 25–30 g).
2. Heparin (50 U).
3. Ketamine (100 mg/ml).
4. Calcium-free Tyrode buffer: 137 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES [pH 7.4], 10 mM 2, 3-butanedione monoxime, 5 mM taurine.
5. O_2 gas.
6. Collagenase type B (300 U/ml) and hyaluronidase (0.1 mg/ml).
7. Cell strainer (100 μm pore size).

2.4 In Vitro Validation of Tough Decoy Efficiency Luciferase Assay

1. pMirtarget vector containing gene of interest 3′-UTR (pMirtarget vector, OriGene, USA).
2. HEK 293 and H9C2 cells for transfection.
3. Luciferin (substrate for luciferase assay).
4. Luminometer.

2.5 In Vivo Validation of Tough Decoy Efficiency

1. AAV9 target miRNA decoy.
2. Transverse aortic constriction (TAC)-induced HF mouse model.
3. Echocardiography equipments.
4. 1.2Fr pressure-volume (PV) conductance catheter.

3 Methods

3.1 MiRNA Tough Decoy Design (See Note 2)

There are many designs and prototypes of tough decoy inhibitors but currently the best construction for a single target miRNA is a two stem structure with a single MBS structure linked by linkers (Fig. 3). The other designs and prototypes have been shown to be effective in other scenarios and are further explored by Haraguchi et al. [17]. The website below is useful to predict RNA folding and target site accessibility: http://genie.weizmann.ac.il/pubs/mir07/ [21].

The MBS in the antisense sequence of the miRNA to be studied will contain a central mismatch in the center of the MBS. The oligonucleotide can contain two of the same or different MBS sequences. The bulge is created by designing non-complementation between the central nucleotides. We then inserted three nucleotide linkers between the stem sequence and MBS.

3.2 MiRNA Tough Decoy Cloning into Self-Complementary AAV Viral System (See Fig. 4)

1. Prepare pds-AAV2 vector and a plasmid containing your target miRNA decoy sequences using either PCR method of proper restriction enzymes, which are available on both plasmids if there is no conflict on the orientation for the cloning.

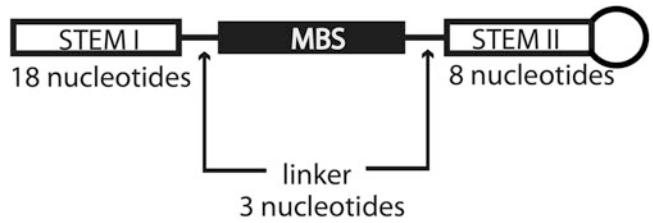


Fig. 3 Representative structure of Tough Decoy RNA with recommended efficient nucleotide lengths

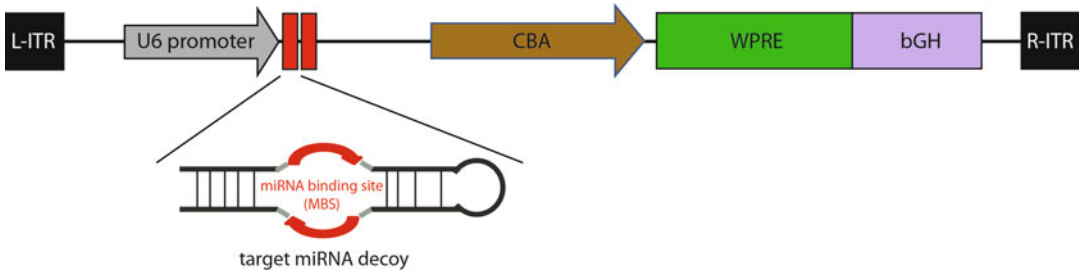
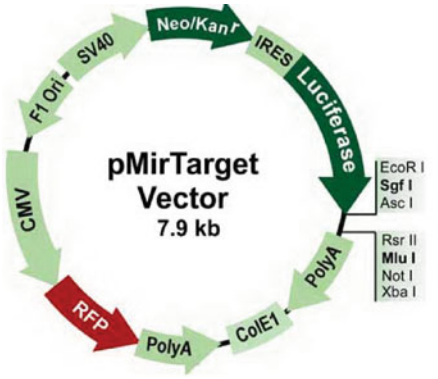


Fig. 4 Vector map for pds-AAV2 target miRNA decoy tough decoy construct (**a**) U6 promoter is subcloned for decoy expression. (**b**) Two tandem repeats of decoy sequence are used to generate AAV virus (*CBA* chicken beta actin promoter, *WPRE* woodchuck hepatitis virus posttranscriptional regulatory element, *bGH* bovine growth hormone gene poly A tail, *ITR* internal terminal repeat)

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>SC210032 3'UTR clone of NM_001681
The sequence shown below is from the reference sequence of NM_001681. The
complete sequence of this clone may contain minor differences, such as SNPs.
Red=Cloning site Blue=Stop Codon

CAATTGGCAGAGCTCAGAATTCAAGCGATCGC

CAACTACCTGGAACCTGCAATACTGGAGTAACCGCTTCCTAAACCATTTTGCAGAAATGTAAGGGTGTTC
GGTTGCGTGCATGTGCGTTTTTAGCAACACATCTACCAACCCTGTGCATGACTGATGTTGGGGAAAAAGA
AAAGTAAAAAACTTCCCAACTCACTTTGTGTTATGTGGAGGAAATGTGTATTACCAATGGGGTTGTTAGC
TTTTAAATCAAAATACTGATTACAGATGTACAATTTAGCTTAATCAGAAAGCCTCTCCAGAGAAGTTTGG
TTTCTTTGCTGCAAGAGGAATGAGGCTCTGTAACCTTATCTAAGAACTTGGAAGCCGTCAGCCAAGTCGC
CACATTTCTCTGCAAAATGTCATAGCTTATATAAATGTACAGTATTCAATTGTAATGCATGCCTTCGGTT
GTAAGTAGCCAGATCCCTCTCCAGTGACATTGGAACATGCTACTTTTTAATTGGCCCTGTACAGTTTGCT
TATTTATAAATTCATTAAAAACACTACAGGTGTTGAATGGTTAAAATGTAGGCCTCCAGTTCATTTTCAG
TTATTTTCTGAGTGTGCAGACAGCTATTTCGCACTGTATTAAATGTAACTTATTTAATGAAATCAGAAGC
AGTAGACAGATGTTGGTGCAATACAAATATTGTGATGCATTTATCTTAATAAAATGCTAAATGTCAATTT
ATCACTGCGCATGTTTGACTTTAGACTGTAAATAGAGATCAGTTTGTTTCTTTCTGTGCTGGTAACAATG
AGCGTCGCACAGACAT

ACGCGTAAGCGGCCGCGGCATCTAGATTCGAAGAAAATGACCG
```

Fig. 5 Plasmid for validation of Tough decoy. (**a**) pMirTarget Vector from OriGene. (**b**) Example sequence: SERCA2 3′-UTR sequence. SgfI and MluI restriction enzymes were selected for the cloning

2. Perform the ligation using enzyme digested pds-AAV2 empty vector and your target miRNA decoy insert and transform to XL-10 gold competent cells (Life Tech).
3. Select the bacterial colonies and extract DNA with correct size.
4. Sequence and confirm the final target plasmid using a sequencing primer (usually a primer for U6-forward promoter).

3.3 Validation of Tough Decoy Specificity and Binding Efficiency

1. Subclone 3′ UTR of target gene into pMirtarget vector from Origene and perform a co-transfection experiment to validate the target decoy sequence of target miRNA (Fig. 5).
2. Prepare the HEK293 cells 1 day before transfection (2E5 cells per 60 mm dish).
3. Freshly extract pds-AAV2-(target miR) decoy plasmid, pds-AAV2-scrambled plasmid (control), and pMirTarget with target gene 3′ UTR vector with the endotoxin free midi DNA Purification kit (Qiagen).
4. On the day of transfection, prepare a total of 3.5 μg of plasmids for each transfection which includes 0.5 μg of pMirTarget plasmid with target gene 3′ UTR. For the dose dependent inhibition assay, prepare samples with control and decoy plasmids as follows: (0 μg, 3 μg), (1.5 μg, 1.5 μg), and (3 μg, 0 μg).
5. Transfect cells using PEI reagent.
6. Twenty-four hours later, harvest the cells and obtain protein lysates by RIPA buffer containing protein/phosphatase inhibitor cocktail.
7. Measure the luciferase activity by luminescence using 10 μg of protein for each group.

3.4 In Vitro Validation of Tough Decoy Efficiency

Two cell types are tested for in vitro validation.

1. H9C2 cardiomyoblasts (*see* **Note 3**).
   (a) Prepare H9C2 cells in 6 well culture plate 1 day before transfection.
   (b) Freshly extract pds-AAV2-target miR decoy plasmid and pds-AAV2-scrambled plasmid (control) with the endotoxin free midi DNA Purification kit (Qiagen).
   (c) Prepare at least three different concentrations to confirm dose-dependent activity (e.g., 1, 2, and 4 μg).
   (d) Transfect the cells with PEI reagent.
   (e) Forty-eight hours later, harvest the cells and obtain protein lysates by RIPA buffer containing protein/phosphatase inhibitor cocktail.
2. H9C2 is notorious for its transfection efficiency. For this reason, PEI solution is a superior alternative as a transfection reagent.

Protocol for the production of PEI transfection reagent is described below. However, PEI is also toxic for the cell if overused (recommended dose: 30 μl for 100 mm culture dish) (1E7 cells).

3.4.1 Protocol for PEI Stock Solution

1. Pour ~200 ml of Milli-Q H_2O into a 500 ml glass beaker.
2. Add 250 mg of PEI to the beaker with stirring.
3. Add concentrated HCl dropwise to the solution to pH = 1.9.
4. Stir at least 24 h until the PEI is completely dissolved. Maintain the pH < 2.0 throughout (dissolution appears to occur in 30–40 min but is not, maintain stirring for 24 h). Approximately 400 μl of 12 M HCl will be required for full PEI dissolution. There may still be some small fiber particles that will not dissolve.
5. Neutralize by adding concentrated NaOH dropwise to the solution pH 7.0.
6. Pour the solution into a 500 ml glass cylinder. Adjust the final volume to 250 ml with Milli-Q H_2O.
7. Filter-sterilize the solution through one 0.22-μm membrane.
8. Store aliquots of the desired volume at −80 °C.

3.4.2 Isolated Mouse Adult Cardiomyocytes

Freshly isolate the mouse adult cardiomyocytes from a mouse on the day of infection (*see* **Note 4**).

1. Inject heparin (50 U).
2. Anesthetize the mouse with intraperitoneal ketamine (100 mg, g21).
3. Quickly remove the heart from the chest and retrogradely perfuse the aorta at 37 °C for 3 min with calcium-free Tyrode buffer gassed with 100% O_2.

4. Initiate the enzymatic digestion by adding collagenase type B (300 U/ml; Worthington) and hyaluronidase (0.1 mg/ml; Worthington) to the perfusion solution.
5. Quickly remove the left ventricle when the heart becomes swollen after 10 min of digestion, cut into several chunks, and further digest in a shaker (60–70 rpm) for 10 min at 37 °C in the same enzyme solution.
6. Filter the cell suspension through a cell strainer and gently centrifuge at 500 rpm for 1 min.
7. Plate the pellet containing myocyte fraction right after centrifugation to further remove non-myocyte fraction (mainly fibroblasts).
8. Replate the cells on laminin coated culture dishes.
9. Infect isolated cells with purified AAV9-target miR decoy with at least three different concentrations (i.e., 1E4 viral genome (vg), 5E4 vg, and 1E5 vg per cell).
10. After 4 days, harvest cells and obtain RNAs using mirVana™ miRNA Isolation Kit (ThermoFisher Scientific) and extract protein lysates by RIPA buffer containing protein/phosphatase inhibitor cocktail.

3.5 In Vivo Validation of Tough Decoy Efficiency

Inject AAV9 target miR decoy intraveneously through the tail vein. After a 4 week incubation period of the virus, evaluate decoy function using Q-PCR and western blot analysis.

1. HF mouse is immobilized with a single mouse restrainer.
2. Inject AAV9 target miR decoy intravenously through the tail vein.
3. House the mice for at least 4 weeks to allow sufficient transgene expression.
4. Euthanize the mice humanely and harvest different organs to identify tissue specificity (e.g., heart, liver, kidney, lung, brain).
5. Extract DNAs to confirm genomic incorporation and obtain RNAs and proteins from each tissue to perform Q-PCR and western blot analysis.

4 Notes

1. *Cloning for AAV vector*

   Usually U6 promoter is used to generate miRNA and miRNA inhibitor, however, CMV promoter is also acceptable and works similarly efficient as U6 promoter.

   However, selection of the most optimal promoters depends on the cell type of interest and the application.

2. *Design Errors causing ineffective Tough Decoys*

   Design of a miRNA tough decoy inhibitor is the most critical step in tough decoy gene transfer. It depends on what miRNA will be used and how the protein of interest should be targeted. Additionally, an appropriate scrambled tough decoy sequence also needs to be constructed to act as a negative control (Refer to couple of previously published paper, [17] and [19]).

3. *Anesthetic agent usage for ECHO and PV loop*

   HF animals are very sensitive against all anesthetic reagents, so start with a half dose of ordinary usage.

4. *Myocyte isolation*

   Some genetically modified mice are very sensitive to collagenase concentration. Determine the working concentration before use.

   Also, following procedures are extremely critical for cell viability.

   (a) Suspend the heart on the Langendorff heart perfusion system as soon as possible, ideally in less than 2 min.

   (b) Oxidize all buffers thoroughly for 30 min prior to this experiment.

   (c) Keep the aorta intact when removing the heart in order to thoroughly circulate the buffer through the coronary artery to maximize the enzyme effect.

   (d) Pipet minimally after enzyme digestion to prevent damage to the cell (do not cut the tissue using a razor blade or scissor).

   (e) Plate the cell after cell filtration procedure using 2% bovine serum albumin (BSA) containing Tyrode buffer and exchange it with normal growth medium 2 h later.

Acknowledgements

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Chapter 4

Efficient Genome Editing in Induced Pluripotent Stem Cells with Engineered Nucleases In Vitro

Vittavat Termglinchan, Timon Seeger, Caressa Chen, Joseph C. Wu, and Ioannis Karakikes

Abstract

Precision genome engineering is rapidly advancing the application of the induced pluripotent stem cells (iPSCs) technology for in vitro disease modeling of cardiovascular diseases. Targeted genome editing using engineered nucleases is a powerful tool that allows for reverse genetics, genome engineering, and targeted transgene integration experiments to be performed in a precise and predictable manner. However, nuclease-mediated homologous recombination is an inefficient process. Herein, we describe the development of an optimized method combining site-specific nucleases and the *piggyBac* transposon system for "seamless" genome editing in pluripotent stem cells with high efficiency and fidelity in vitro.

Key words Genome engineering, Homology-directed repair, TALEN, CRISPR/Cas9, *PiggyBac* transposon system

1 Introduction

The advent of nuclease-mediated genome editing is a technological breakthrough for targeted genetic modifications [1]. Programmable nucleases generate double-strand breaks (DSBs) at precise loci, which stimulate the endogenous repair machinery for either nonhomologous end joining (NHEJ) or homology-directed repair (HDR) pathways. The error-prone NHEJ pathway generates random insertions or deletions at the site of DSBs, while HDR employs homologous donor DNA sequences to introduce genetic modifications. Both NHEJ and HDR are utilized for different aspects of genome engineering: NHEJ leads to targeted mutagenesis, whereas HDR allows for precise gene insertions, corrections, deletions, or base substitutions.

To date, three major classes of programmable nucleases—zinc finger nucleases (ZFNs) [2, 3], transcription activator-like effector nucleases (TALENs) [4], and the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9

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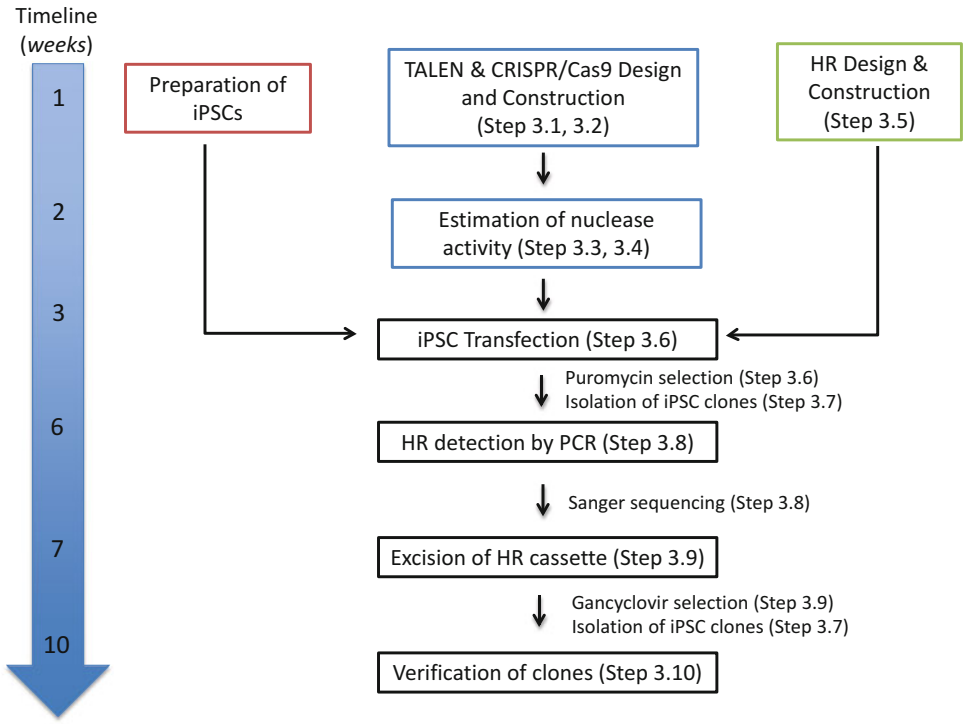

Fig. 1 Flowchart and timeline of nuclease-mediated genome editing in pluripotent stem cells

(Cas9) systems [5, 6]—have been developed to enable site-specific genome engineering. Although desired sequences have been inserted, mutations have been introduced and/or corrected in human iPSC-based models of dilated cardiomyopathy (DCM) [7], Barth syndrome (BTHS) [8], and long QT (LQT) syndrome [9], existing protocols are inefficient—rendering a technically challenging and laborious process. Herein, we describe an optimized methodology utilizing the *piggyBac* transposon system that allows rapid, precise, and "seamless" directed gene editing in pluripotent stem cells (Fig. 1).

2 Materials

2.1 iPSC Culture

1. Essential 8™ Medium (E8).
2. ROCK inhibitor (Y-27632).
3. ACCUTASE™.
4. Gentle Cell Dissociation Reagent.
5. Corning® Matrigel® hESC-Qualified Matrix.

2.2 HEK293 Cell Culture

1. DMEM, high glucose, GlutaMAX™ Supplement (DMEM).
2. Fetal bovine serum (FBS).

2.3 TALEN and CRISPR/Cas9 Components

1. TALEN assembly kit: Musunuru/Cowan Lab TALEN Kit (Addgene, 1000000034).
2. Conventional CRISPR/Cas9 Plasmid (Cut): pSpCas9(BB)-2A-GFP (Addgene, Plasmid 48138).
3. Nickase CRISPR/Cas9 Plasmid: Single-strand break (Nick): pSpCas9n(BB)-2A-GFP (Addgene, Plasmid 48140).

2.4 piggyBac Transposon System

1. Homologous recombination vector: Footprint-Free™ Gene Editing Vector containing Puromycin (Puro) and Thymidine Kinase (TK) selection cassette driven by a phosphoglycerate kinase (PGK) promoter (Transposagen, MV-PGK-Puro-TK) or Footprint-Free™ Gene Editing Vector containing Neomycin and Thymidine Kinase selection cassette driven by a PGK promoter (Transposagen, MV-PGK-Neo-TK).
2. *piggyBac* transposase: Excision only *piggyBac*™ transposase mRNA (Transposagen, PBx-m).

2.5 Cell Transfection Components

1. Opti-MEM® Media.
2. TurboFect™ Transfection Reagent.
3. Lipofectamine® 3000 Reagent.
4. Lipofectamine® MessengerMAX™.

2.6 Surveyor Assay

1. Surveyor® Mutation Detection Kit.

2.7 Plasmid Preparation

1. Plasmid Miniprep Kit.
2. Plasmid Maxiprep Kit.

2.8 Genomic DNA Extraction

1. DNeasy Blood & Tissue Kit.

2.9 Restriction Enzymes

1. NotI.
2. BsiWI.
3. NsiI.
4. AscI.

2.10 PCR Reagents

1. PrimeSTAR® GXL DNA Polymerase.

2.11 Molecular Cloning Reagents

1. Ampicillin.
2. Agar.
3. LB Growth Medium.
4. Chemically Competent *E. coli*.
5. Blunt PCR Cloning Kit.
6. QuikChange II Site-Directed Mutagenesis Kit.

2.12 Selection Drugs

1. Puromycin.
2. Geneticin (G418).
3. Ganciclovir or Fialuridine (FIAU).

3 Methods

3.1 TALEN Design and Construction

1. TALEN design: We recommend using the TALEN Targeter tool to design TALENs (https://tale-nt.cac.cornell.edu/tutorials/talentargeterupdated). The TALEN cutting site should be within 50 bp of the targeted mutation. Each TALEN binding site is 15 bp long with 13–18bp (16bp is optimal) between the forward and reverse TALEN binding sites. The position 0 nucleotide (the nucleotide just 5′ to the first position of the TALEN binding site) should be a T for both TALENs.
2. TALEN construction: We recommend using the protocol provided with the Musunuru/Cowan Lab TALEN Kit (http://www.stembook.org/node/1438.html) to construct the TALEN constructs.
3. TALEN vector sequencing primers:

   TALEN_Fw: 5′-GGCCAGTTGCTGAAGATCG-3′

   TALEN_Rv: 5′-CGCTACAAGATGATCATTAGTG-3′

3.2 CRISPR/Cas9 Design and Construction

1. We recommend using the Feng Zhang's lab CRISPR Design tool to design the guide RNAs (gRNA) for either conventional CRISPR/Cas9 or nickase CRISPR/Cas9 (http://crispr.mit.edu). The CRISPR/Cas9 cutting site should be within 50 bp of the target sequence.
2. We recommend using the protocol from the Fen Zhang's lab (http://www.genome-engineering.org/).
3. CRISPR/Cas9 sequencing primers:

   U6_F: 5′-CAAGGCTGTTAGAGAGATAATTGGA-3′

3.3 Estimation of Cleavage Efficiency

1. *Day 0*: Dissociate HEK293 cells with Accutase. Plate 80,000 cells per well in a 24-well plate in 0.5 mL growth media (90% DMEM with 10% FBS) (*see* **Note 1**).
2. *Day 1*: Perform transfection. The passaged cells should be 25–30% confluent after 24 h.

   Prepare the transfection mix for 1 well of the 24-well plate. In one tube add 1.5 μL of Turbofect Mix in 50 μL Opti-MEM. In a separate tube resuspend the plasmid DNA in 50 μL Opti-MEM as follows:

(a) For TALENs or nickase CRISPR/Cas9 reaction: We recommend using 0.3 μg of each TALEN or nickase CRISPR/Cas9 (total of 0.6 μg/pair).

(b) For conventional CRISPR/Cas9 reaction: We recommend using 0.6 μg of the CRISPR/Cas9.

3. Mix the contents of the two tubes (100 μL total per transfection reaction) and incubate for 10 min at room temperature. Next, gently add the transfection mix dropwise in one well.
4. *Day 2*: Replace media with fresh growth media. Check the transfected cells under a fluorescent microscope for double-positive (GFP+/RFP+) cells for the TALEN transfection, or single-positive (GFP+) cells for the CRISPR/Cas9 transfection (*see* **Note 2**).
5. *Day 3*: Collect the transfected cells and extract the genomic DNA.
6. Proceed to Subheading 3.4.

3.4 Surveyor Assay

1. Follow the manufacturer's instructions to detect the TALEN or CRISPR/Cas9 cleavage efficiency (Fig. 2; *see* **Note 3**).

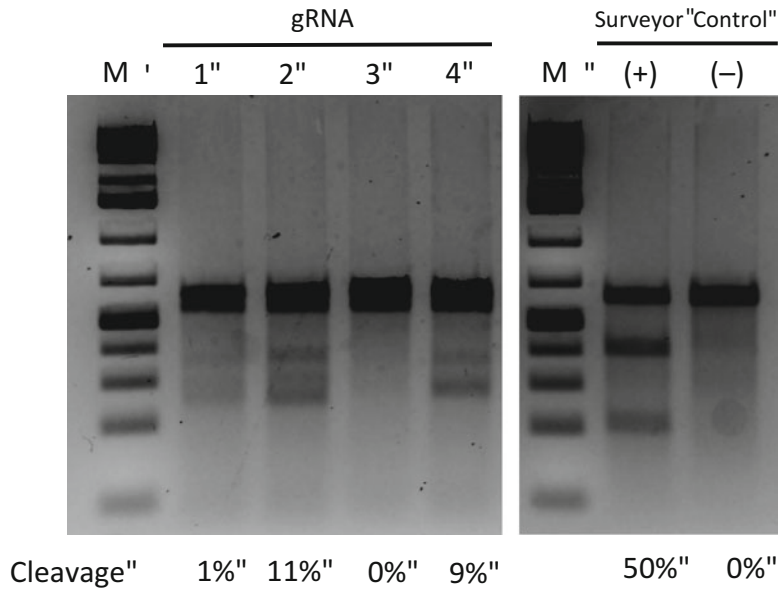

Fig. 2 Surveyor assay for indel frequency analysis. Left panel: the cleavage efficiency of four CRISPR gRNAs was tested by transfecting HEK293 cells with the expression plasmids for Cas9 and the indicated CRISPR gRNA. The genomic DNA was extracted 48 h later and was subjected to the Surveyor assay and the cleavage products were resolved by gel electrophoresis. The best gRNA for the targeted locus was defined as the gRNA that resulted in the highest percentage of cleavage products. The cleavage efficiency of different gRNAs ranged from 0 to 11 %. Right panel: Control G/C heteroduplex/homoduplex DNA (+) and Control G homoduplex DNA (–) were digested in PCR buffer with 1 μL of Surveyor® Nuclease S. Surveyor Nuclease cleavage at the mismatches produces products of 217 and 416 bp. Digestion products were analyzed by gel electrophoresis on a 2.5 % agarose gel in 1× TBE buffer. Lane M: Marker DNA

3.5 HR Vector Construct

1. Design: Download the genomic sequence starting from at least 1 kb upstream and ending with 1 kb downstream of the site to be modified from a public database. We recommend using the RefSeq annotation from NCBI (http://www.ncbi.nlm.nih.gov/gene/).
2. Search for a TTAA sequence near the genomic region to be modified. TTAA should be within 50–75 bp of the TALEN or CRISPR/Cas9 cutting site. If a natural occurring TTAA sequence is not available near the modification site, an artificial TTAA sequence can be created by introducing a silent mutation (*see* **Note 4**).
3. Design the homology arms on either side of the TTAA site to be around 500 bp in length. The 500 bp upstream of TTAA is the 5′-end arm of the HR vector, and the 500 bp downstream of TTAA is the 3′-end arm of the HR vector (Fig. 3).
4. Modify the DNA sequence in the homology arms to correct, or introduce the desired genetic modification.
5. Optional: Additional silent mutations at the TALEN or CRISPR/Cas9 binding site may be introduced in the HR vector to prevent TALEN or CRISPR/Cas9 from cutting the recombinant DNA vector (*see* **Note 5**).
6. Incorporate the appropriate sequences for the transposon-specific inverted terminal repeats (ITR) and the compatible restriction sites to the end of the 5′-end and 3′-end homology arms (Fig. 3; *see* **Note 6**).
7. We recommend using GeneArt™ Gene Synthesis (ThermoFisher) to synthesize both homology arms (*see* **Note 7**).
8. Cloning: Set up restriction digests for the 5′-end insert, and the *piggyBac* plasmid (NotI and BsiWI enzymes).
9. Purify the digested DNA insert with a column-based purification kit.

Fig. 3 Design the homology arms. The *piggyBac* Inverted Terminal Repeats (ITRs) each contain unique restriction sites (BsiWI and NsiI) near their ends, which can be used for cloning the homology arms. The homology arms on either side of the TTAA site are approximately 500 bp in length. The 500 bp upstream of TTAA is the 5′-end arm of the HR vector, and the 500 bp downstream of TTAA is the 3′-end arm of the HR vector

10. Run the *piggyBac* HR vector on a 1% agarose gel, cut the vector band (6 kb), and purify using column-based purification kit.
11. Perform DNA ligation. We recommend a plasmid to insert ratio of 1:5 with 50 ng of the linearized plasmid.
12. Proceed with the transformation of the cloning reaction using chemically competent *E. coli* cells. Follow the manufacturer's instructions.
13. Plate *E. coli* cells on ampicillin-containing agar plates. Incubate overnight at 37 °C.
14. Pick 6–10 individual bacterial colonies and screen for successful ligations. Grow single clones in 2 mL growth medium overnight at 37 °C. On the following day, isolate plasmid DNA (Miniprep). To screen for the ligated insert, perform a restriction digestion (NotI and BsiWI enzymes). Run the digested DNA on an agarose gel (1%). A successfully ligated insert should generate two bands, one corresponding to the vector (6.0 kb) and one the size of the insert (~500 bp).
15. Verify the 5′-end arm of the plasmid by Sanger sequencing (PB_F: 5′-TGTAAAACGACGGCCAGT-3′).
16. Repeat steps **8–14** to clone in the 3′-end insert to the *piggyBac* HR vector containing the 5′-end arm derived in **step 15**, using the 3′-end arm-specific restriction enzymes (NsiI and AscI) (*see* **Note 8**).
17. Verify the 3′-end arm of the plasmid by Sanger sequencing (PB_R: 5′-CAGGAAACAGCTATGAC-3′).
18. Prepare a maxiprep of the completed *piggyBac* HR vector.
19. Repeat Sanger sequencing to confirm that both HR vector arms are correct.

3.6 iPSCs Transfection

1. *Day 0*: Dissociate iPSCs with Accutase™ at 37 °C for 5 min, and plate 3.30×10^5 cells per well in a 6-well plate with 2 mL of E8 media containing 1 μM of ROCK inhibitor (Y-27632). Incubate at 37 °C overnight.
2. *Day 1*: Replace media with fresh E8 media (1.75 mL/well). At this stage, the cells should be 25–30% confluent. Prepare the transfection mix for 2 wells of a 6-well plate. First, mix 10 μL of Lipofectamine 3000 in 250 μL of Opti-MEM in one tube. In a separate tube, first mix 14 μL of P3000 reagent in 250 μL of Opti-MEM, and then add the TALEN or CRISPR/Cas9 and HR vector as follows:
   (a) For TALENs or nickase CRISPR/Cas9: 0.8 μg of each TALEN or nickase CRISPR/Cas9 (total of 1.6 μg/pair) and 5.4 μg of HR vector.
   (b) For conventional CRISPR/Cas9: use 1.34 μg of CRISPR/Cas9 with 5.66 μg of HR vector.

3. Combine the contents of both tubes (500 μL total volume) and incubate for 10 min at room temperature.
4. Add 250 μL of the transfection mix dropwise to the iPSCs in each well of the 6-well plate.
5. *Day 2*: Aspirate the media and add fresh E8 media. Check the transfected cells under a fluorescent microscope for double-positive (GFP+/RFP+) cells for the TALEN transfection, or single-positive (GFP+) cells for the CRISPR/Cas9 transfection (*see* **Note 9**).
6. *Day 3*: Passage the transfected iPSCs from each well using Accutase™. Resuspend the iPSCs in 12 mL of E8 media containing 1 μM Y-27632, and plate in 6 wells of a new 6-well coated with Matrigel (1:6 split).
7. *Day 4*: Begin changing E8 media every day.
8. *Day 5*: Start clone selection with puromycin at 0.10–0.25 μg/mL for the MV-PGK-Puro-TK HR vector or G418 at 50–200 μg/mL for the MV-PGK-Neo-TK vector. Change media every day (*see* **Note 10**).
9. *Day 12*: Individual colonies should appear after 6–8 days of selection. Proceed to Subheading 3.7.

3.7 Isolation of iPSC Clones

1. Pick individual iPSC clones with the assistance of a microscope, and transfer each clone into a separate well of a 48-well plate (Matrigel-coated) containing 250 μL of E8 media supplemented with 1 μM Y-27632. We recommend picking 20–30 clones.
2. Collect the remaining cells from 1 to 2 wells and extract genomic DNA (*see* **Note 11**).
3. Allow the cells to attach for 24–48 h and then add E8 media. Feed the cells with fresh E8 media every other day (*see* **Note 12**).
4. After 7–10 days dissociate the cells with Gentle Cell Dissociation Reagent for 6–7 min at room temperature. Gently aspirate and add 300 μL of E8 media supplemented with 1 μM Y-27632.
5. Using the P1000 pipette, gently pipette 4–5 times to break down the cell colonies. Transfer 150 μL of the cell suspension into a new well of a 24-well plate. Add 350 μL E8 media in each well and incubate at 37 °C. Extract genomic DNA from the remaining cells (Qiagen, DNeasy Blood & Tissue Kit).
6. For HR clones: proceed to Subheading 3.8. For transposon-excised clones: proceed to Subheading 3.10.

3.8 Junction PCR

1. Design the junction PCR primers to screen for putative genome-editing iPSC clones using the genomic sequence (Subheading 3.5). The primer-binding site should be 100–200 bp outside of the homology arms. We have already optimized several primers that bind inside the ITRs of the

HR vector that can be combined with the sequence-specific primers (*see* **Note 11**).

2. Using the genomic sequence, also design three primer pairs to amplify the wild-type sequence (~300 bp upstream and downstream of the modification site).
3. Set up the junction PCR reactions for each clone by combining the optimized primer pairs in separate reactions (*see* **Note 11**).
4. Identify putative targeted clones by amplification of both the 5′- and 3′-end homology arms. The targeted efficiency is variable (Fig. 4; *see* **Note 13**).
5. After identifying a putative clone, set up a separate PCR reaction using the wild-type primers (Fig. 4, *see* **Note 14**).
6. Verify the PCR amplified products by Sanger sequencing using both the 5′- and 3′-end primers for sequencing.

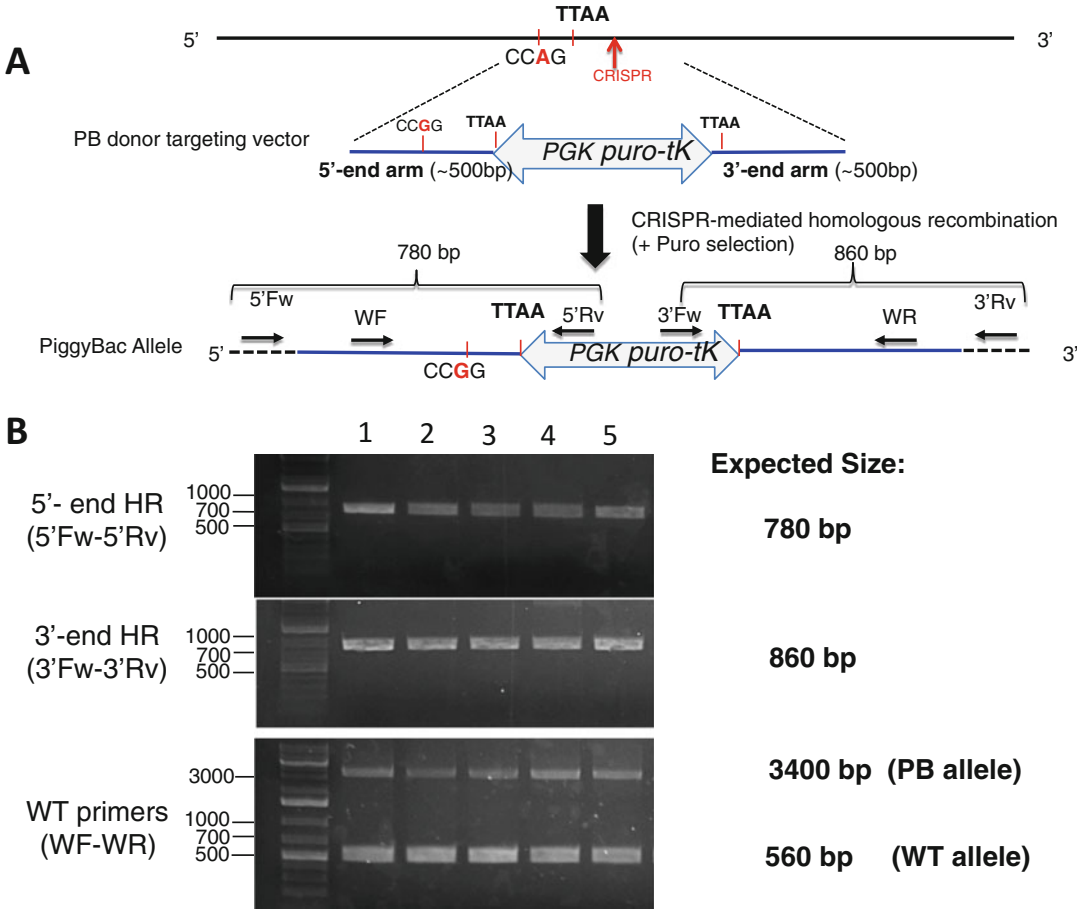

Fig. 4 PCR-based screening of HR clones. (**a**) Schematic of gene editing and primer design. (**b**) An example of PCR results of targeted clones by using 5′-end HR, 3′-end HR and WT primers. Note that all clones have the transposon integrated in the desired locus. Two bands were detected with the WT primer pair (560 bp: WT allele, 3400 bp: *piggyBac* allele). *Dash line*: genomic DNA sequences outside the homology arms

7. After confirmation of a correctly targeted clone, expand the clone and generate frozen stocks.
8. Proceed to Subheading 3.9.

3.9 Excision of the HR Cassette

1. *Day 0*: After expanding the correctly targeted clone, dissociate the cells using Accutase™ at 37 °C for 5 min, and resuspend 1×10^6 cells in 12 mL of E8 media supplemented with 1 μM Y-27632. Plate the iPSCs in a new 6-well plate (2 mL/well; ~1.6×10^5 cells/well).
2. *Day 1*: Change media to E8 (1.75 mL/well). The passaged cells should be 10–25% confluent. Prepare the transfection mix for 1 well of a 6-well plate. Combine 3.75 μL of Lipofectamine® MessengerMAX™diluted in 125 μL of Opti-MEM and 1 μg of excision only *piggyBac*™ transposase mRNA diluted in 125 μL of Opti-MEM (total volume 250 μL). Gently add the transfection mix (250 μL) to 1 well of the 6-well plate. Final volume is 2 mL per well (*see* **Note 15**).
3. *Day 2*: Change media to fresh E8.
4. *Day 3*: Passage each of the transfected wells using Accutase™ into the 6-well plate with E8 media supplemented with 1 μM Y-27632 (1:12 split).
5. *Day 4*: Change media to E8 media supplemented with ganciclovir (1–2 μM) or FIAU (0.1–0.2 μM) for the next 7–8 days (*see* **Note 16**).
6. *Day 12*: Individual colonies should appear. Pick and expand single clones as described in (Subheading 3.7).

3.10 Verification of the Genome-Edited Clones

1. Use the optimized PCR primer pairs (Subheading 3.8) to amplify the homology arms (5′- and 3′-end primers) and wild-type genomic sequence (wild-type primers).
2. PCR products from the 5′- and 3′-ends should be absent in putative clones that the HR cassette has been successfully excised (Fig. 5; *see* **Note 17**).
3. Sequence the amplified PCR products using the wild-type primers. The desired genomic modification and any silent mutations should be detected.

3.11 Off-Target Effects

1. Several methods can be used to predict potential off-target genomic sites.
   (a) Off-target effects in TALENs: We recommend using the PROGNOS tool (http://bao.rice.edu/Research/BioinformaticTools/prognos.html).
   (b) *Off-target in CRISPR/Cas9*: We recommend using the CRISPR Design tool (http://crispr.mit.edu).

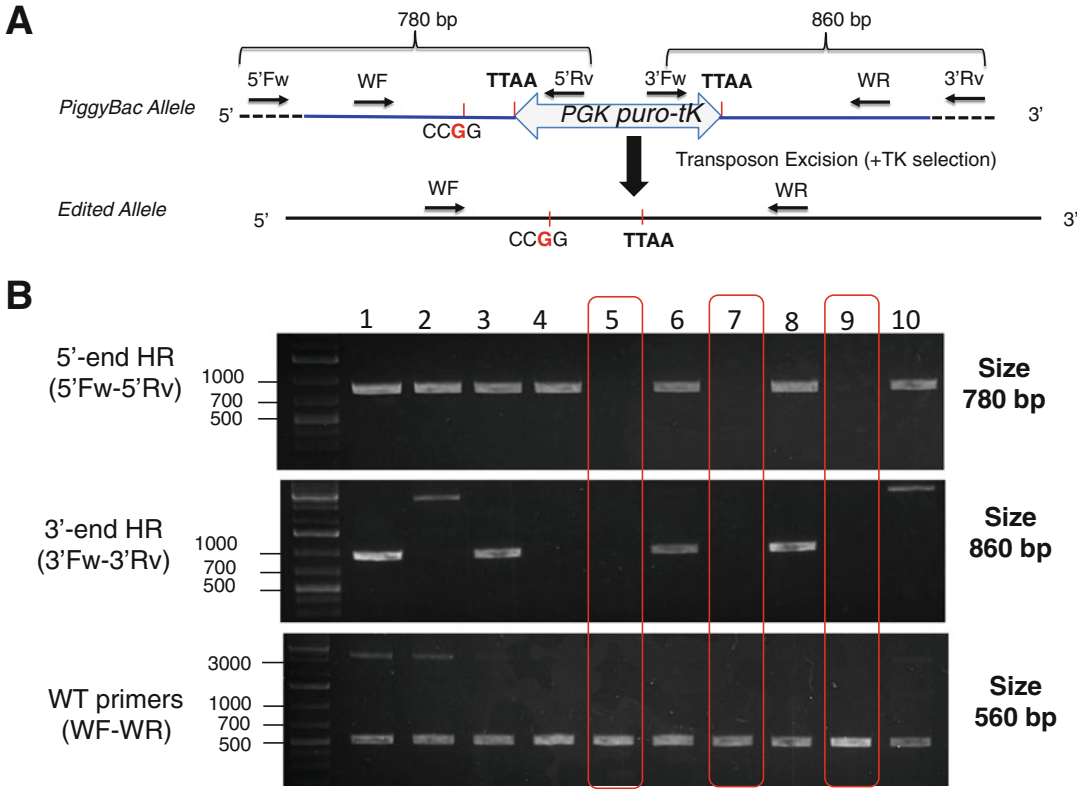

Fig. 5 PCR-based screening of transposon-excised clones. (**a**) Schematic of transposon excision and primer design. (**b**) An example of PCR results of transposon-excised clones by using 5′-end HR, 3′-end HR and WT primers. Successully excised clones are highlighetd (Clones 5, 7 and 9)

2. Whole-exome or whole-genome sequencing may also be performed to comprehensively detect the off-targets caused by the genome editing process.

4 Notes

1. Pre-coating the 24-well plate with 1% gelatin will enhance the attachment of HEK293.
2. Transfection of HEK293 cells is very efficient. The viability of the cells should be >90% and the transfection efficiency should be >80%.
3. Always include the positive and negative controls that are provided with the Surveyor kit (G/C control). The cleavage efficiency varies significanlty among site-specific endonucleases. Choose one endonuclease pair with 5–40% cleavage efficiency as determined by the surveyor assay, and design the HR vector

accordingly. Highly active endonucleases (>40% cleavage efficiency) might be problematic and usually result in frequent NHEJ events.

4. An artificial TTAA sequence can be created by searching for two consecutive amino acids (*see* Table 1).
5. If possible, select a TTAA sequence within the spacer of the TALEN pair or nickase CRISPR/Cas9 binding sites. For conventional CRISPR/Cas9, try to design so that TTAA is inside the CRISPR gRNA. This will ensure that the endonucleases will not cut the recombinant DNA vector without the need of introducing any additional silent mutations.
6. The *piggyBac* Inverted Terminal Repeats (ITRs) each contain unique restriction sites (BsiWI and NsiI) near their ends, which can be used for cloning homology arms. These enzymes are compatible with the MV-PGK-Puro-TK and MV-PGK-Neo-TK vectors. Use these restriction enzymes when possible (*see* Table 2).
7. If a Gene Synthesis service is not available, 5′- and 3′-end inserts can be synthesized by PCR using human genomic DNA as a template and cloning primers that contain compatible restriction enzymes that incorporate the ITR sequences. Subsequently, PCR products can be cloned into a TOPO vector (e.g., StrataClone™ Blunt PCR Cloning Kit), followed by site-direct mutagenesis (e.g., QuikChange II Site-Directed Mutagenesis Kit) to modify the sequences of interest.
8. If the restriction enzymes from the 3′-end insert can cut the 5′-end insert, the cloning of the 3′-end arm should be performed first.

Table 1
Amino acid that can be used to introduce a silent mutation for the TTAA site

	First amino acid	Second amino acid
TT/AA	F, I, L, V	K, N
TTA/A	L	I, K, M, N, T, R, S

Table 2
Preferred restriction cloning enzymes in the *Piggybac* vector

	5′-end	3′-end
5′-end insert	NotI	BsiWI (inside the ITR)
3′-end insert	NsiI (inside the ITR)	AscI

9. The viability of the transfected cells should be more than 80% and the transfection efficiency should be 1–5%.
10. Cells will start to die 48–72 h post-antibiotic selection. Increase the concentration of cells if necessary. The majority of cells should detach by day 5 post-selection, leaving behind 25–100 clones/well of a 6-well plate. Continue the antibiotic selection for 7 days, and then change media back to E8 media.
11. There is no positive control to test the junction PCR primers. The genomic DNA extracted from the transfected cells after antibiotic selection and clone picking is used as a template (~50 ng DNA per reaction) to identify a suitable set of primers that amplify the homology arms. The sequence-specific primers can be combined with the following primers in separate reactions:

    5′-end HR arm primers (*Reverse*):

    (a) R1: 5′-CGTCAATTTTACGCATGATTATCTTTAAC3′

    (b) R2: 5′-GACCGATAAAACACATGCGTCA-3′

    (c) R3: 5′-AGACCGATAAAACACATGCGTCAA-3′

    3′-end HR arm primers (*Forward*):

    (a) F1: 5′-CGACGGATTCGCGCTATTTA-3′

    (b) F2: 5′-GTCCTAAATGCACAGCGACG-3′
12. Individual iPSC clones may contain a fair amount of differentiation after picking. Generally, it is not necessary to remove the differentiated cells prior to passaging. By splitting the cells the overall culture health should improve throughout the early passages. If not, manually pick the undifferentiating iPSC colonies.
13. Although it is difficult to predict, the efficiency of HR ranges from 1/4 to 1/48 of the selected clones. The efficiency is affected mainly by the cutting efficiency of endonucleases and the proximity of the HR vector arms to the cleavage site.
14. Often both alleles are targeted and subsequently cleaved by the endonuclease. However, in most cases, the HR vector repairs only one of the alleles. The other allele could be mutated through the NHEJ process—these clones should be identified and discarded.
15. Although the HR excision is a rare event, typically a single transfection reaction is sufficient to successfully produce a few transposon-excised clones. However, in some instances, the process could be repeated to increase the chances of obtaining a gene-edited iPSC clone.
16. Cells will start to die around 48–72 h post selection. Optimize the concentration of ganciclovir (or FIAU) if cells survive. The majority of cells should detach by day 5–7 post-selection, leaving around 1–20 clones/well of a 6-well plate.

Continue selection for 7 days, and then change media back to normal E8 media. Antibiotic selection of confluent cells is inefficient; therefore, split the confluent cells once again, and then start selection the following day.

17. The *piggyBac* excision efficiency ranges from 10 to 100% after selection.

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Chapter 5

Direct Cardiac Reprogramming as a Novel Therapeutic Strategy for Treatment of Myocardial Infarction

Hong Ma, Li Wang, Jiandong Liu, and Li Qian

Abstract

Direct reprogramming of fibroblasts into induced cardiomyocytes (iCMs) holds great promise as a novel therapy for the treatment of heart failure, a common and morbid disease that is usually caused by irreversible loss of functional cardiomyocytes (CMs). Recently, we and others showed that in a murine model of acute myocardial infarction, delivery of three transcription factors, Gata4, Mef2c, and Tbx5 converted endogenous cardiac fibroblasts into functional iCMs. These iCMs integrated electrically and mechanically with surrounding myocardium, resulting in a reduction in scar size and an improvement in heart function. Our findings suggest that iCM reprogramming may be a means of regenerating functional CMs in vivo for patients with heart disease. However, because relatively little is known about the factors that regulate iCM reprogramming, the applicability of iCM reprogramming is currently limited to the experimental settings in which it has been attempted. Specific hurdles include the relatively low conversion rate of iCMs and the need for reprogramming to occur in the context of acute injury. Therefore, before this treatment can become a viable therapy for human heart disease, the optimal condition for efficient iCM generation must be determined. Here, we provide a detailed protocol for both in vitro and in vivo iCM generation that has been optimized so far in our lab. We hope that this protocol will lay a foundation for future further improvement of iCM generation and provide a platform for mechanistic studies.

Key words Cell reprogramming, Induced cardiomyocyte, Fibroblast, Myocardial infarction, Regeneration

1 Introduction

Heart disease is the leading cause of morbidity and mortality in the developed world [1]. In the USA alone, more than 1.5 million people experience an acute myocardial infarction (AMI) each year, and there are five million AMI survivors who suffer from ischemic cardiomyopathy [1]. Because cardiomyocytes (CMs) in the heart have very limited regenerative potential in response to injury, loss of CMs results in impaired pump function and heart failure. Existing treatments are primarily pharmacological and device-based, and do not address the fundamental problem of CM loss. Indeed, the prevalence of cardiomyopathy is steadily increasing worldwide [1],

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making the identification of novel and effective therapies for this morbid disease an urgent problem in biomedical research.

Unlike the hearts of lower vertebrates [2], the adult human heart has very limited regenerative potential [3, 4]. Although postnatal vertebrate CMs undergo a small amount of cellular renewal, the rate of CM proliferation is very low [3, 5, 6]. One approach to cardiac regeneration has focused on enhancing such proliferative indices genetically or pharmacologically [7–12]. Although whether the magnitude of the beneficial effects would be sufficient to compensate for the functional loss of damaged myocardium awaits confirmation [13], stimulating CM to reenter cell cycle for regenerative purposes is still a vital approach for the treatment of heart disease [7–12]. A second approach to cardiac regeneration has been to derive new CMs for transfer into an injured heart [14–17]. These cells are typically differentiated from multipotent cardiovascular progenitor cells (CPCs) or pluripotent stem cells including both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Although the poor cell survival, low maturation efficiency, limited cell–cell interaction, and functional integration of engrafted cells are major hurdles that remain to be overcome [18, 19], efforts to make this a practical therapy are ongoing. A third newly emerging approach to regenerate an injured heart is to directly convert the resident cardiac fibroblasts (CFs) into functional iCMs, which is discussed in more detail below.

Cardiomyocytes comprise 75% of myocardial mass, but they account for only 30–40% of the total number of cells in the heart. The majority of the remaining cells are CFs, which are responsible for homeostatic maintenance of extracellular matrix (ECM) in the normal heart [20–22]. CFs also respond to ischemia and other injurious stimuli to coordinate chemical and mechanical signals between cellular components in the heart. Upon injury, CFs migrate to the site of injury, proliferate, and contribute to scar formation through fibrosis [22, 23]. Thus, while the biological importance of CFs has long been recognized, their potential as an endogenous resource for cardiac regeneration has been largely neglected until very recently.

Takahashi and Yamanaka's seminal publication in 2006 [24] demonstrating the creation of iPSCs has ushered in a new era of utilizing cellular reprogramming in regenerative medicine. The idea has been leveraged thus far to directly reprogram fibroblasts into various cell types including pancreatic β-cells [25], blood progenitor cells [26], neurons [27, 28], hepatocytes [29, 30], and CMs [31–36]. Ieda et al. first identified that a cocktail of three cardiac transcription factors, Gata4, Tbx5, Mef2C (GMT), is sufficient to convert cultured neonatal and adult CFs into iCMs that displayed most features of functional CMs [32]. More recently, we showed that transduction of GMT can convert resident CFs into iCMs in vivo in an acutely injured murine heart [35]. Furthermore, this conversion resulted in a reduction in scar size and an

improvement in heart function [35]. Subsequently, similar combinations of transcription factors (Tbx5, Mef2c, and Myocardin [34] or GMT plus Hand2 [36]) or microRNAs (miR-1,133,208,499) [33] were used to successfully reprogram CFs into iCMs. The potential of utilizing large pool of endogenous CFs to generate functional iCMs points to an extremely promising avenue to regenerate damaged myocardium in patients. Moreover, this approach circumvents some of the obstacles faced by other approaches including the need to identify a large cellular source and the need for efficient transplantation and integration within the area of injured myocardium.

To further optimize the condition for iCM reprogramming, our lab has recently studied the influence of stoichiometry of G,M,T on iCM reprogramming [37]. We took advantage of the inherent features of the polycistronic system and generated a complete set of polycistronic constructs to include all possible splicing orders of G,M,T in a single mRNA. With this set of unique tools, we found that varying protein stoichiometry of G,M,T resulted in significant differences in iCM reprogramming efficiency and quality. Moreover, we found the optimal stoichiometry to be a relative high level of Mef2c protein expression and low levels of Gata4 and Tbx5 expression (encoded in MGT construct). By addition of an antibiotic selection marker to the best combination MGT, we were able to further enrich transduced fibroblasts with a homogenous gene expression stoichiometry that resulted in an even higher efficiency in generation of mature iCMs [37]. Furthermore, when delivered into a murine infarcted heart, MGT construct resulted in an increased iCM generation compared to using the traditional mix of separate G,M,T viruses [38]. MGT single triplet introduction also led to a further attenuation of cardiac dysfunction and reduction in scar size [38]. Here we describe in details the step-by-step protocol of in vitro and in vivo iCM reprogramming using both the traditional separate G,M,T and our recently developed single triplet MGT system.

2 Materials

2.1 Generation of Retroviruses

1. pMXs-puro retroviral expression vector.
2. Plat-E cell retroviral packaging cell line.
3. Plat-E cell culture medium: Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1× nonessential amino acids, 1 μg/mL puromycin, 10 μg/mL blasticidin, and 100 U/L penicillin/streptomycin.
4. Plat-E cell transfection medium: DMEM supplemented with 10% fetal bovine serum, 1× nonessential amino acids.
5. Coating medium: 0.2% gelatin in PBS.
6. Lipofectamine 2000.

7. Opti-MEM Medium.
8. 0.45 μm syringe filters.
9. Beckman Ultra-Clear centrifuge tube (25 × 89 mm).
10. 1.7 mL microcentrifuge tube.
11. Retro-X qRT-PCR titration kit (Clontech).
12. Polybrene infection reagent.
13. Retrovirus Precipitation Solution (ALSTEM).
14. NIH3T3 cell line.
15. Flow cytometer.

2.2 Generation of Mouse Cardiac Fibroblasts and Cardiac Reprogramming In Vitro

1. αMHC (α-myosin heavy chain)-GFP transgenic mouse.
2. Fluorescent microscope.
3. mFB media: Iscove's Modified Dulbecco's Medium media (IMDM) supplemented with 10% FBS, and 100 U/L penicillin/streptomycin.
4. 0.05% trypsin–EDTA.
5. 40 μm cell strainers.
6. collagenase II.
7. HBSS.
8. MACS kit (Miltenyi Biotec).
9. Anti-Biotin MicroBeads (Miltenyi Biotec).
10. iCM media: 400 mL DMEM supplemented with 100 mL M199 media, and 50 mL FBS.
11. B27 medium: 490 mL PRMI1640 medium supplemented with 10 mL B27 supplement.
12. Cardiac troponin T antibody (Cat# MS-295-PO, Thermo Fisher Scientific).
13. GFP antibody (Cat# A11122, Thermo Fisher Scientific).
14. α-actinin antibody (Cat# A7811, Sigma-Aldrich).
15. Connexin43 antibody (Cat# C6219, Sigma-Aldrich).
16. Mef2c antibody (Cat# ab64644, Abcam).
17. Gata4 antibody (Cat# sc-1237, Santa Cruz Biotechnology).
18. Tbx5 antibody (Cat# sc-17866, Santa Cruz Biotechnology).
19. Alexa Fluor 488 conjugated donkey anti-rabbit IgG (Cat# 711-545-152, Jackson ImmunoResearch Inc).
20. Alexa Fluor 647 conjugated donkey anti-mouse IgG (Cat# 715-605-150, Jackson ImmunoResearch Inc).
21. EVOS FL Auto imaging system.
22. SYBR Green Real-Time PCR Master Mixes.
23. Rhod-3 Calcium Imaging Kit (Thermo Fisher Scientific).

2.3 Mouse Myocardial Infarction Model and Gene Transfer In Vivo

1. periostin-Cre mice.
2. Fsp1-Cre mice.
3. R26R-lacZ mice.
4. Ketamine.
5. Xylazine.
6. MiniVent Type 845 mouse ventilator (Hugo Sachs Elektronik-Harvard Apparatus)
7. 8-0 Prolene suture.
8. Mini-Goldstein Retractor.
9. 50 μL micro syringe.
10. 33 G micro injection needle.
11. 6-0 Prolene suture.

2.4 Evaluation of Transduction Efficiency

1. Cardioplegia buffer: PBS supplemented with 10 mM KCl.
2. Cryosection buffer: PBS supplemented with 0.5% PFA, and 5% sucrose.
3. 15 mL conical centrifuge tube.
4. OCT.
5. Universal Blocking Buffer (Biogenex).
6. RFP antibody (Cat#3993-100, BioVision).
7. PBST: PBS with 0.1% Triton 100.
8. Vectashield with DAPI.
9. Wittenberg Isolation Medium (WIM) (pH 7.4): 116 mM NaCl, 5.4 mM KCl, 6.7 mM $MgCl_2$, 12 mM glucose, 2 mM glutamine, 3.5 mM $NaHCO_3$, 1.5 mM KH_2PO_4, 1.0 mM Na_2HPO_4, 21 mM HEPES, with 1.5 nM insulin, 1× essential vitamins, and 1× essential amino acids.
10. Langendorf digestion medium: Wittenberg Isolation Medium (WIM) supplemented with 0.8 mg/mL collagenase II and 10 mM $CaCl_2$.
11. BD Cytofix/Cytoperm Solution Kit.
12. Fix buffer: 1% PFA/PBS.
13. Thy1 antibody (Cat# 553016, BD Biosciences).
14. Flow cytometer.

2.5 Evaluation of Direct Reprogramming In Vivo

1. 2% Evans blue.
2. Triphenyltetrazolium chloride (TTC).
3. Heart matrices for tissue sampling (Electron Microscopy Sciences).
4. Vevo 2100 High-Resolution Micro-Imaging System with a 40-MHz linear array ultrasound transducer (VisualSonics).

5. Mouse pressure catheter (Millar Instruments).
6. Varian DirectDrive 7 T small-animal scanner.
7. Powerlab system (AD Instruments).
8. Mouse miniature telemetry (Data Sciences International).
9. Mouse ECG.
10. Cx43 antibody (Cat# C6219, Sigma-Aldrich).
11. N-cadherin antibody (Cat# 33-3900, Thermo Fisher Scientific).
12. β-galactosidase antibody (Cat# ab9361, Abcam, Cambridge, MA).
13. Superfusion chamber (Warner Instruments).
14. Ouabain (Sigma-Aldrich).
15. Myocam-S High-Speed Contractility Camera (IonOptix).
16. Video frame grabber (Hauppage).
17. Dextran-conjugated Cascade Blue (MW 10,000).
18. Calcein.
19. Fluo-4.
20. DMSO.
21. PowerLoad Concentrate (Thermo Fisher Scientific).
22. Tyrode's solution.
23. Motion detector (Crescent Electronics).
24. TRIzol.
25. RIPA buffer supplemented with protease and phosphatase inhibitor cocktail.
26. SuperScript III First-Strand synthesis kit.

3 Methods

3.1 Generation of Retroviruses

1. Clone coding regions of mouse Gata4, Mef2c, and Tbx5 respectively into pMXs based retroviral vector [32]. A single polycistronic vector with optimal ratio of Mef2c, Gata4 and Tbx5 significantly increased reprogramming efficiency both in vitro and in vivo [37, 38]. pMXs-dsRed serves as an indicator for evaluation of transduction efficiency. All these vectors can be requested from the lab of Dr. Li Qian.
2. Maintain Plat-E retroviral packaging cell line in culture medium. Split Plat-E cell to 4×10^6 cells per 10-cm dish 1 day before retroviral packaging (*see* **Note 1**).
3. The next day, change with fresh transfection medium at least 1 h prior to transfection. Transfect Plat-E cells with Lipofectamine 2000 when the culture reaches ~80% confluent. Set up the following two mixtures: (1) dilution of 10 μg

packaging vectors in 500 μL Opti-MEM Medium. (2) dilution of 20 μL Lipofectamine 2000 in 500 μL Opti-MEM Medium. Add vector dilution into diluted Lipofectamine 2000 reagent. Mix transfection solution thoroughly by tapping and incubate the mixture for 15 min at room temperature.

4. Add transfection mixture dropwise to the Plat-E cells. Mix it well by moving the plate in back-and-forth and side-to-side motion. Incubate the plate at 37 °C for overnight.
5. After 24 h, replace with fresh prewarmed culture medium without blasticidin and puromycin. Harvest retrovirus containing supernatant at 48 h after transfection. Filter the supernatant with a 0.45 μm cellulose acetate filter (*see* **Note 2**).
6. For virus used for in vitro reprogramming, pellet the virus through incubation with viral precipitation solution. Add 2 mL of the precipitation solution to every 8 mL viral supernatant. Mix gently and precipitate overnight. On the next day, spin the mixture at 1500 × *g* for 30 min at 4 °C. Discard the supernatant and spin again for another 5 min. Gently aspirate the remaining medium. Add 100 μL cold DMEM to resuspend virus from one 10 cm dish. The retrovirus is ready for use (*see* **Note 3**).
7. For virus used for in vivo delivery, pellet the virus through ultracentrifugation. Briefly, transfer the retrovirus containing solution to a Beckman centrifuge tube. Put the tubes into buckets and use SW28 rotor to spin down the virus at 33,000 × *g* at 4 °C for 2 h. Remove the supernatant without disturbing the viral pellet. Wash the pellets once with ice-cold PBS, and combine pellets from 5 to 10 dishes for a higher titer. Resuspend the retroviral pellets with 100 μL of cold DMEM by gently pipetting up and down. Avoid any air bubbles. Aliquot into 1.7 mL Eppendorf tubes and store them at −80 °C.
8. Determine the copy number of the virus by using Retro-X qRT-PCR titration kit.
9. Determine the infectious unit (IFU) of virus. Seed the NIH3T3 cells at 3×10^4/well in 24-well plate. On the second day, transduce the cells with dsRed retrovirus with 5–10-fold serial dilution. After 48 h culture, analyze fluorescence by flow cytometer to determine the infectious unit (IFU). Assume that the fluorescent protein-retrovirus and nonfluorescent protein-retrovirus, which are made in the same batch, have the same viability. In other words, their ratio of IFU/copy number should be the same. Determine the IFU of nonfluorescent protein-retrovirus.
10. Dilute the concentrated virus with DMEM into 1×10^{10} IFU/mL for in vivo gene delivery (*see* **Note 3**).

3.2 Direct Reprogramming In Vitro

Methods of direct reprogramming in vitro described here provide an easy and fast way for purposes of mechanistic studies, high-throughput screening, and potentially clinical applications. Induced cardiomyocytes (iCMs) generated in vitro could be detected at very early time point (d3 for GFP reporter expression and d14 for sarcomeric gene expression). More importantly, those iCMs start spontaneous contraction from 4 weeks and exhibit cardiomyocytes-like electrophysiological features around 6 weeks.

3.2.1 Generation of Mouse Cardiac Fibroblasts from Explant Culture Method

1. Briefly clean neonatal αMHC (α-myosin heavy chain)-GFP transgenic mouse (P1 to P2) with 75% ethanol, followed by decapitation. Make a horizontal incision from under one armpit to the other to dissect the heart out. Move one heart into one well of 24-well plate containing ice cold PBS.
2. Check GFP expression in heart by fluorescent microscope. Choose GFP positive hearts and pool them into a 10 cm dish.
3. Cut the hearts into small pieces less than 1 mm^3 in size using a sterile blade and/or scissors.
4. Transfer every 3–4 minced heart pieces in 2 mL mouse fibroblast (mFB) media to one 10 cm dish and incubate it at 37 °C for 3 h. Add 8 mL mFB media to the settled tissues and continue to culture for 1 week. Change media every 3 days.
5. On day 7, dissociate the explant cells by incubating the cells with 3 mL 0.05% trypsin–EDTA at 37 °C for 5 min. Inactivate trypsin by adding 5 mL mFB media, followed by gently detaching the cells with cell scraper. Collect cells and pass through 40 μm cell strainers to avoid contamination of heart tissue fragments, and then pellet cells by spinning at 200 × *g* for 5 min.
6. Wash cells once with MACS buffer and cells are ready for sorting.

3.2.2 Generation of Mouse Cardiac Fibroblasts from Enzyme Digestion Method

1. Pool GFP positive hearts into 10 cm dish containing 10 mL ice cold PBS. Squeeze ventricles with sterile forceps to remove blood and rinse once with ice-cold DPBS.
2. Trim the hearts to remove other tissues and fat. Cut the heart into four loosely connected pieces.
3. Incubate the heart tissues (from 20 to 30 hearts) in 50 mL Falcon tube with 15 mL warm 0.05% trypsin–EDTA, and incubate at 37 °C for 15 min.
4. Gently aspirate trypsin, and add 10 mL warm type II collagenase (0.5 mg/mL) in HBSS. Vortex the tube on vortexer for 1 min and incubate the tube in 37 °C water bath for 3–5 min. Vortex again for 1 min and let the tissues settle down for 1 min. Collect the supernatant into a new tube containing 10 mL cold mFB medium. Repeat the procedure until all tissue is digested (around 4–5 rounds). At this end, combine all the

collections and filter through 40 μm cell strainer to make single cell suspension. Spin down at 200 × *g* for 5 min, wash once with MACS buffer and finally resuspend in MACS buffer.

5. Optimal: lyse red blood cells. Resuspend cells with 1 mL RBC lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA), keep on ice for 1 min, then add 10 mL MACS buffer and spin down at 200 × *g* for 5 min. Wash one more time with MACS buffer.

3.2.3 Isolation of Thy1.2+ Fibroblasts by MACS (Magnetic-Activated Cell Sorting)

1. Count the viable cell number and add 10 μL Biotin anti-Thy1.2 antibody to every 1×10^7 cells in 90 μL MACS buffer. Incubate the cells with antibody in a refrigerator (2–8 °C) for 30 min.
2. Wash cells once with 10 mL MACS buffer and pellet the cells by spinning at 200 × *g* for 5 min.
3. Add 10 μL anti-Biotin microbeads to 90 μL MACS buffer and use the mixture to resuspend the cells. Incubate the cells with microbeads in a refrigerator (2–8 °C) for 30 min.
4. Wash cells once with 10 mL MACS buffer and centrifuge again at 200 × *g* for 5 min. Then resuspend the cells in 2 mL MACS buffer.
5. While washing the cells, set up a MACS Separator in hood. Insert an LS column to the separator. Apply 3 mL MACS buffer to the column to equilibrate it. Pass the cell suspension through the column, and then wash the column three times with 2 mL MACS buffer each time. After that, take the column off the Separator, add 2 mL MACS buffer to the column, and insert the plunge to flush out the beads-binding cells. Pellet the cells by centrifuge at 200 × *g* for 5 min. Resuspend cells in FB media and they are ready for seeding.

3.2.4 Reprogram Mouse Cardiac Fibroblasts In Vitro

1. Prepare 0.1% gelatin coated 24-well plates. Seed cells into plates at proper density. For fibroblasts generated from explant culture method, the seeding cells density could be around $2–3 \times 10^4$ cells/well of 24-well plate. For fibroblasts generated from enzyme digestion method, the cells density could be around $4–5 \times 10^4$ cells/well of 24-well plate. Culture the cells overnight in mFB media at 37 °C (*see* **Note 4**).
2. On day 0, change culture media to 0.5 mL pre-warmed iCM media containing 4 μg/mL Polybrene. Add 10 μL retrovirus to each well. Incubate cells with virus for 48 h (*see* **Note 5**).
3. On day 2, change virus containing media to 0.5 mL regular iCM media. Change media every 2–3 days.
4. On day 3, change media to iCM media supplemented with 2 μg/mL puromycin for cells transduced with pMx-puro-MGT viruses. Keep it for 3 days. After that, maintain puromycin in

the iCM medium at the concentration of 1 μg/mL for additional 7 days.

5. On day 14, change iCM media to B27 media. Change media every 3 days. Spontaneous beating cell loci may be observed from 3 (cells from enzyme digestion method) or 4 (cells from explant culture method) weeks after viral transduction.

3.2.5 Evaluation of Cardiac Reprogramming In Vitro: Immunocytochemistry (ICC)

1. Cardiac fibroblasts have been isolated from aMHC-GFP transgenic mice where the GFP expression is driven by cardiomyocytes-specific aMHC promoter. This allows the tracking of cells that have been converted from fibroblasts (GFP negative) to iCMs (GFP positive). GFP expression could be monitored under microscope 3 days after viral transduction. Cardiac structure genes, like cardiac troponin T (cTnT) and α-actinin exhibit expression from 1 week and sarcomere structures would appear around 2 weeks.
2. To visualize sarcomere structures in reprogrammed cells, stain the cells with antibodies targeting GFP and cTnT. Prepare the cells by washing three times with ice cold PBS. Fix them with 4% paraformaldehyde/PBS at room temperature for 15–20 min. Wash cells twice with PBS and permeabilized the cells with 0.1% Triton/PBS, followed by washing twice and blocking for 0.5–1 h with 5% BSA/PBS. Then incubate the cells with primary antibodies, anti-GFP and anti-cTnT, in 1% BSA/PBS overnight at 4 °C.
3. On the second day, probe the cells with secondary antibodies, Alexa Fluor 488 conjugated donkey anti-rabbit IgG for GFP and Alexa Fluor 647 conjugated donkey anti-mouse IgG for cTnT. Finally, stain the nuclei with DAPI.
4. Place the plate on EVOS FL Auto imaging system and capture images through the EVOS software. Quantification would be performed by counting GFP+ and/or cTnT+ cell number (reprogrammed cell population) and DAPI+ cell number (total cells) in each field. Reprogramming efficiency is calculated by dividing number of total cells with that of reprogrammed cells.

3.2.6 Evaluation of Cardiac Reprogramming In Vitro: Flow Cytometry (FACS)

1. Dissociate the cells with 0.05% trypsin–EDTA and 96-well deep well plate well to well. Spin at 200 × *g* for 5 min at 4 °C to pellet the cells.
2. Resuspend cells with 100 μL fixation/permeabilization buffer. Treat the cells for 20 min at 4 °C.
3. Wash cell twice with 500 μL 1× wash solution, pellet, and remove supernatant. Thoroughly resuspend each sample with 50 μL primary antibody solution (anti-GFP and anti-cTnT antibodies) and incubate at 4 °C for 30 min.

4. Wash cells once and incubate each sample with 50 μL secondary antibody solution at 4 °C for 30 min.
5. Wash cells once in 1× wash solution. Resuspend cells in 400 μL fixation buffer (DPBS with 1% PFA). Transfer cell into FACS tube with cell strainer cap. Samples are ready for FACS detection.

3.2.7 Evaluation of Cardiac Reprogramming In Vitro: Gene Regulation

1. Lyse cells in TRIzol according to manufacturer's instructions. Either freeze the lysate at −80 °C or proceed immediately to RNA purification.
2. Add 0.2 mL of chloroform, cap sample tubes securely. Shake tube vigorously by hand for 15 s and incubate at RT for 2–3 min.
3. Centrifuge the samples at no more than 12,000 × *g* for 15 min at 4 °C (now the mixture separates into three phases, the upper aqueous phase contains RNAl).
4. Transfer the aqueous phase to a fresh tube and precipitate the RNA by mixing with 0.5 mL of isopropyl alcohol.
5. Incubate samples at RT for 10 min and centrifuge at no more than 12,000 × *g* for 10 min at 4 °C.
6. Remove supernatant, wash RNA pellet with 1 mL of 75% ethanol, mix the sample by vortexing and centrifuge at no more than 7500 × *g* for 5 min at 4 °C (keep it at −20 °C until use).
7. Briefly dry the RNA pellet, dissolve RNA in RNase-free water and incubate for 10 min at 55–60 °C. RNA can also be redissolved in 100% formamide (deionized) and stored at −70 °C.
8. Prepare cDNA using First Strand cDNA synthesis kit according to manufacturer's recommendations.
9. Assess for gene expression using SYBR Green Real-Time PCR Master Mixes together with designed primers. Genes involved in sarcomere structures (Myh6, Myh7, Tnnt2, and Actc1), ion channels (Pln, Slc8a1, and Scn5a), and cell junctions (Gja1, Kcna5, and Cacba1c) could be evaluated. Successful cardiac reprogramming shows significant upregulation of those genes and downregulation of genes represent as fibroblast markers including Col1a1 and Col3a1. GAPDH expression should be detected simultaneously as a housekeeping gene for normalization.

3.2.8 Evaluation of Cardiac Reprogramming In Vitro: Ca^{2+} Oscillation at 4 Weeks

1. Calcium oscillation signals could be detected by using Rhod-3 Calcium Imaging Kit according to the manufacturer's instructions. Reconstitute Rhod-3 in 100 μL of DMSO to yield a stock solution of 10 mM Rhod-3. Store the stock solution desiccated at −20 °C and protected from light in single use aliquots.

2. Reconstitute Probenecid in 1 mL of HBSS to prepare 250 mM stock.
3. Wash cells twice with HBSS buffer.
4. Prepare fresh loading buffer containing Rhod-3 and Probenecid. Add loading buffer to cells immediately and incubate cells in dark at room temperature for 30–60 min.
5. Wash cells twice with HBSS.
6. Add 2 mL incubation buffer (2.5 mM probenecid in HBSS) to cells and incubate cells in dark at RT for 30–60 min.
7. Wash cells once with HBSS. Cells are now ready for live-cell imaging (*see* **Note 6**).

3.3 Direct Reprogramming In Vivo

Direct cardiac reprogramming opens a new avenue for cardiac regeneration and disease modeling. Establishment of an efficient in vivo reprogramming system would be beneficial for in vivo mechanistic studies and ultimately moving this field further toward clinical applications.

3.3.1 Mouse Myocardial Infarction Model and Delivery of Reprogramming Factors

1. Anesthetize mice with 100 mg/kg ketamine plus 10 mg/kg xylazine. Place mice in a supine position on a heating pad at 37 °C.
2. Intubate mice with a 19 G stump needle connected to a MiniVent Type 845 mouse ventilator. Ventilate with 150 μL of stroke volume at 120 breaths per minute. Ventilation is confirmed by chest movement.
3. Perform anterior thoracotomy by cutting the third rib. Open the chest with a Mini-Goldstein retractor. Remove the pericardium and expose the heart (*see* **Note 7**).
4. Ligate the left anterior descending artery (LAD) with an 8-0 prolene suture. The ischemia is confirmed by myocardial blanching (*see* **Note 8**).
5. Mix 10 μL retroviral solution (1×10^8 IFU virus) containing a pooled viruses (Gata4/Mef2c/Tbx5) or a polycistronic MGT virus or a dsRed virus with 10 μL of PBS plus 4 μg/mL polybrene. Inject the mixture into the myocardium through a 50 μL micro syringe incorporated with 33 G injection needle. Single injection with a full dosage is employed along the boundary between the infarct area and border zone based on the blanched infarct area after LAD ligation (*see* **Note 9**).
6. After injection, the chest is closed by suturing muscle layer and skin layer separately with a 6-0 Prolene suture. Let the mouse recover with ventilation on the heating pad.
7. Sham-operated animals will be served as surgical controls and will be subjected to the same procedures as the experimental animals with the exception that the LAD is not ligated.

3.3.2 Evaluation of In Vivo Viral Transduction Efficiency

1. Two days after surgery, use cryosection and immunofluorescent staining to visualize transduction. Isolate all the cells from left ventricle and run FACS to quantify transduction efficiency.
2. Anesthetize mice subjected to MI and dsRed virus injection with 100 mg/kg ketamine plus 10 mg/kg xylazine (*see* **Note 10**).
3. For immunohistochemistry (IHC) on cryosections, open chest to expose the heart. Inject 0.5 mL cardioplegia buffer into left ventricle slowly. Perfuse the heart with 5 mL PBS slowly to remove all the red blood cells. Perfuse the heart with cryosection buffer, then remove the heart and put it into 15 mL conical centrifuge tube containing 10 mL fresh cryosection buffer. Incubate with gentle agitation at 4 °C overnight.
4. On the second day, wash the PFA away from the tissue with a serial dilution of sucrose (5%, 10%, and 20% respectively). Embed the tissue with OCT and freeze in liquid nitrogen.
5. Block sections in Universal Blocking Buffer for 10 min, then stain with RFP primary antibody for 1 h at room temperature. After washing with PBST for three times, incubate section with secondary antibody for 1 h at room temperature followed by washing another three times with PBST. Finally, mount the section with Vectashield with DAPI. Most of $dsRed^+$ cells should present in MI border zone.
6. For FACS, left ventricular cells are isolated after removing heart from chest. Cannulate the heart via aorta and perfuse retrograde with Wittenberg Isolation Medium in a Langendorf apparatus with a constant flow of 3 mL/min at 37 °C for 5 min. Then switch to digestion medium for another 10 min. Dissect left ventricle and mechanically dissociate all the cells. Fix and incubate the cells for RFP/Thy1 staining with BD Cytofix/Cytoperm Solution Kit. Resuspend the cells with Fix buffer and run FACS. Around 4% or even more of cells from the left ventricle should be $dsRed^+$ $Thy1^+$, depending on the viral quantity, quality, and delivery (*see* **Note 11**).

3.3.3 Lineage Tracing of In Vivo Reprogrammed iCMs

1. To explore the mechanisms underlying in vivo direct reprogramming, it is critical to trace the lineage of induced cardiomyocyte (iCM) and characterize the iCM based on their origin.
2. Cross periostin-Cre mice or Fsp1-Cre mice and R26R-lacZ mice to obtain Periostin-Cre:R26R-lacZ or Fsp1-Cre:R26R-lacZ mice to trace the fibroblast-origin iCMs using IHC.
3. To evaluate in vivo reprogramming quality and quantity, cohorts of mice (10–12 mice per group) are sacrificed at 4, 8, and 12 weeks after G/M/T or MGT delivery. Hearts are perfusion-fixed and taken out to be further fixed in 4% PFA or

0.5% PFA for paraffin sections or cryo-sections respectively. Cryosections are used for IHC of cardiac markers αActinin, cTnT, Tropomyocin, αMHC, Connexin43, N-Cadherin, and fibroblast lineage markers Periostin-Cre/Fsp1-Cre; RosaEYFP (anti-GFP) to evaluate the conversion of CFs into iCMs (also *see* Subheading 3.3.6). Paraffin sections are used to determine the scar area using standard Masson-Trichrome staining (Subheading 3.3.4).

4. Cross periostin-Cre mice and R26R-EYFP or R26R-Tomato mice to get Periostin-Cre:R26R-EYFP or Periostin-Cre:R26R-Tomato mice for the following assays such as determining the electrophysiological features (Subheading 3.3.6) and gene expression of iCMs (Subheading 3.3.7) based on EYFP or Tomato labeling.

3.3.4 Evaluation of In Vivo Direct Reprogramming: Determination of Scar Size

1. To evaluate the consistence among different groups, Evans blue/triphenyltetrazolium chloride (TTC) double staining is analyzed 48 h after MI. After anesthetizing and sacrificing the mice, perfuse the hearts retrograde with 2% Evans blue via aorta. All the heart tissue shows in blue except for the area at risk (AAR). Cut left ventricle into several slices with heart matrices for tissue sampling. Incubate the slices in 1.5% TTC for 30 min at 37 °C. Fix with 4% PFA overnight at 4 °C. The infarcted area shows in white, while viable myocardium is in red. Analyze infarct size/AAR size via planimetry with ImageJ software.
2. To determine the scar size, standard Masson-Trichrome staining is applied on hearts 8 weeks after MI. Use ImageJ software to measure the scar area (blue) and viable area (red) on a serial of transverse sections with the first level right below the ligation.

3.3.5 Evaluation of In Vivo Direct Reprogramming: Cardiac Function

1. Echocardiography, hemodynamics, and MRI are analyzed to evaluate the cardiac function at different time points (4, 8, and 12 weeks) after MI with or without delivery of reprogramming factors.
2. Perform echocardiography on anesthetized mice under 1–1.5% isoflurane at a core temperature of ~37 °C with echocardiography machine and probes for mice. Capture parasternal long-axis and short-axis views of left ventricle at a frame rate of 400 Hz. Obtain end-systole or end-diastole defined as the phase in which the smallest or largest area of LV, respectively for ejection fraction assessment. Measure left ventricular end-systolic diameter (LVESD) and left ventricular end-diastolic diameter (LVEDD) from the LV M-mode tracing at the papillary muscle level.

3. Perform hemodynamic assessment by inserting a mouse pressure catheter into the aorta and LV through the right common carotid artery on anesthetized mice under 1–1.5% isoflurane. Connect the transducer to Powerlab system to record heart rate (HR), blood pressure (BP), left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), left ventricular developed pressure (LVDP), +dp/dt and –dp/dt.
4. Perform MRI by a Varian DirectDrive 7 T small-animal scanner on the mice under anaesthetization by inhalation of 2% isoflurane/98% oxygen administered. Insert two ECG leads into the right front and left rear leg for image triggering. After defining the oblique plane of the short axis, use an ECG-triggered two-dimensional gradient echo sequence with an echo time of 2.75 ms, repetition time of 200 ms and a flip angle of 45 °C to obtain nine short-axis images at 12 or 13 phases per cardiac cycle. Each scan consists of 8–9 contiguous slices spanning the left ventricle from apex to base with 1-mm thickness, a matrix size of 128×128, a field of view of 25.6×25.6 mm, and four averages.

3.3.6 Evaluation of In Vivo Direct Reprogramming: Electrophysiology

1. Evaluate the electrophysiology and incorporation of iCMs by miniature telemetry, cell coupling, Ca^{2+} handling/Stimulation, and action potential after direct reprogramming.
2. One of the concerns for cardiac cell therapy is that the cardiomyocyte from exogenous transplant may not incorporate well with the native myocardium, which leads to a potential risk of arrhythmia. To test if direct reprogramming leads to the same concern, ECGs are monitored over the course of iCM conversion. Three days after surgery for a full recovery, implant a miniature telemetry in the abdominal cavity of the mouse. Place and fix two leads to the muscle layer of the neck and xiphoid respectively. Use Dataquest Software to acquire and analyze the ECG data.
3. To determine if iCMs express proteins involved in cell–cell communication, perfuse and fix hearts with 0.5% PFA for frozen section as described in Subheading 3.3.3. After blocking with universal blocking buffer, stain with primary antibody against Connexin43 or N-cadherin or other gap junction proteins with β-galactosidase antibody to localize iCMs.
4. To assess the functional cell–cell junctions, measure the intercellular transmission of excitable Ca^{2+} wave and molecular probes of gap junctional communication by isolating small groups of cells from infarct/border zone, including tomato-labeling iCMs and nonfluorescent CMs. For doing this, shorten the isolation time from 10 to 4 min as described in Subheading 3.3.4.

5. After isolating myocytes, transfer them to a superfusion chamber. Incubated with 2 mM ouabain for 5–10 min to induce a intracellular Ca^{2+} overload. Then, a Ca^{2+} wave activity is revealed under the videomicroscopy.
6. The whole-cell patch-clamp method is used to assess the interconnectivity between iCMs and endogenous CMs. Add a dye pair, 1 mM immobile dextran-conjugated Cascade Blue (MW 10,000) and 5 mM mobile calcein, in standard intracellular solution with 5 mM EGTA, which help to reseal the sarcolemma of the cell receiving molecular dye, for 2 min cytoplasmic loading. Excite blue fluorescence from the immobile indicator at 365 ± 40 nm, and calcein fluorescence at 470 ± 40 nm. Record fluorescent images using IonOptix Myocam-S via a video frame grabber and process imaging with ImageJ software.
7. Prepare the loading solution. Dissolve Fluo-4 in anhydrous DMSO to 5 mM. Mix Fluo-4/DMSO with PowerLoad Concentrate at 1:10. Dilute Fluo-4/DMSO/PowerLoad 1:100 in Tyrode's solution to get a final loading solution for myocytes.
8. Transfer isolated myocytes in loading solution for 30 min at room temperature. Load the cells to a superfusion chamber and de-esterificate for another 20 min. Evoke contraction and Ca^{2+} transient by pulses of stimuli at 0.33 Hz of frequency, 2 ms of duration and 150% of threshold voltage. Record Ca^{2+} transients in batches of ten, averaged numbers are used for statistical analyses. Record resting fluorescence after cessation of pacing, and obtain background light after removing the cells from the field of view at the end of the experiment. Calibrate the Ca^{2+} transients using the pseudo-ratio method, assuming an in situ dissociation constant of 1.1 μM for Fluo-4. Record contractions optically simultaneously with Ca^{2+} transients by illuminating the cell of interest in red light (>665 nm) subsequently directed to a CCD camera. Convert the cell length signals to voltage via a video motion detector.

3.3.7 Evaluation of In Vivo Direct Reprogramming: Gene Regulation

1. As described in Subheading 3.3.4, isolate iCMs from different regions and time points after MI by digestion and FACS (or manual picking if the iCMs are large). Lysate the cells in TRIzol or RIPA buffer to harvest RNA or protein according to manufacturer's recommendations. Prepare cDNA with SuperScript III First-Strand synthesis kit. Assess the gene expression using Taqman system or run western blot to determine protein expression.
2. As described in Subheading 3.3.3, prepare cryosection and evaluate the protein expression of a battery of cardiac or fibroblast markers via immunohistochemistry.

4 Notes

1. Plat-E should be maintained under antibiotics (puromycin and blasticidin) selection. Plat-E of fewer passages (<30 passages) is highly recommended for viral production. The cells need to be about 70–80% confluent when starting transfection to produce an efficient packaging.
2. The quality and quantity of retrovirus are both critical for direct reprogramming. If most (>80%) of the control dsRed-transfected Plat-E cells are red, collect all viral particles. Otherwise, improve the transfection first. Usually the best titer and viable virus can achieve at 48 h after transfection. Some other transfection reagents, such as Nanofect, could also be used to produce virus according to manufactures' indications.
3. Excessive or sufficient virus can reduce the efficiency of reprogramming [39]. All retroviruses should be used freshly and avoid multiple freeze–thaw cycles. A titration is recommended right before viral delivery to ensure there is enough viable virus.
4. The quality and purity of fibroblasts are extremely important. MACS protocol we described here provides a gentle and convenient way to isolate fibroblasts of high-purity and high-viability. The seeding density is also important. Density either too high or too low results in a low percentage of iCMs. If cells are sparsely seeded, they tend to become unhealthy with irregular cell morphology and in large size. It is difficult to convert those senescent-like cells into iCMs. If cells are seeded too densely, the MOI (multiplicity of infection) for viral infection is decreased. Overgrowth of uninfected fibroblasts significantly dilutes the reprogramming events.
5. The amount of virus should be proportionally increased with increased cell number in different culture plates or dishes. We noticed that co-infection of MGT virus with another pMXs vector based retrovirus will decrease the reprogramming efficiency, which possibly results from the fact that cells preferentially uptake more virus of smaller size than MGT virus. If experiments of overexpression or knockdown some genes are needed for reprogramming, we highly recommend using lentivirus for transduction. In this condition, do MGT infections for 24 h, add the lentivirus directly to MGT-containing media, and incubate cells with both MGT and lentivirus for another 24 h.
6. Rhod-3 staining has a high background. Signals start to decrease half hour after final step of washing, especially if incubated at 37 °C. We recommend imaging cells immediately and finish the experiments within an hour.

7. The incision is usually performed in the third intercostal space to avoid extra tissue damage in LAD ligation induced MI model. Instead, we perform the surgery by cutting the third rib which will give a better view for the following viral delivery and is easier to operate [35, 38].
8. The size of infarct region depends on the ligation site. It is important for the evaluation of cardiac function to keep the LAD ligation procedure consistent among mice, which is determined in Subheading 3.3.4.
9. The virus is delivered by a single shot along the margin of infarcted area. Make sure the needle isn't advanced into the left ventricle, causing the viral leakage. The viral solution is being injected while the syringe is withdrawn. When injection finishes, hold the syringe in place for a while to let the viral solution diffuse. Also, a concentrated viral solution is used here to minimize the volume of injection.
10. Retrovirus only infect dividing cells, which are mainly the activated cardiac fibroblasts bordering the infarct zone. Most of adult cardiac fibroblasts are regarded as quiescent cells. Therefore infarcted hearts with dsRed infection are used as negative controls.
11. Single cell CM/iCM isolation and the following assays are used to rule out the false positives from overlaying cells.

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Part III

Gene Delivery Vectors

Chapter 6

Production and Characterization of Vectors Based on the Cardiotropic AAV Serotype 9

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Abstract

Vectors based on adeno-associated virus serotype 9 (AAV9) efficiently transduce cardiomyocytes in both rodents and large animal models upon either systemic or regional vector delivery. In this chapter, we describe the most widely used production and purification method of AAV9. This production approach does not depend on the use of a helpervirus but instead on transient transfection of HEK293T cells with a plasmid containing the recombinant AAV genome and a second plasmid encoding the AAV9 capsid proteins, the AAV Rep proteins and the adenoviral helper functions. The recombinant AAV is then purified by iodixanol density gradient centrifugation. This chapter also describes in detail the characterization and quality control methods required for assuring high quality vector preparations, which is of particular importance for experiments in large animal models.

Key words Adeno-associated virus, Serotype, AAV, AAV9, Cardiotropic, Gene therapy, AAV production, AAV characterization

1 Introduction

Adeno-associated virus (AAV) is a small, non-enveloped virus with a single-stranded DNA genome that is flanked by two inverted terminal repeats (ITRs). It has originally been isolated from an adenoviral preparation [1] and is a "defective" virus in the sense that it requires co-infection with a helpervirus, such as adenovirus or herpesvirus, for productive replication [1].

In part due to its nonpathogenic nature, its limited immunogenicity and its ability to trigger long-term gene expression in postmitotic tissues, even in the absence of genome integration, AAV has been recognized as one of the most promising gene delivery vehicles. Not only has it often become the tool of choice in preclinical animal models, but as of June 2015, 117 clinical trials using AAV have either been completed or are in progress. Moreover, in 2012, treatment of lipoprotein-lipase deficiency has been

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approved for clinical use in Europe, the first approval of a gene therapeutic treatment in the Western World [2].

The collection of AAV serotypes and variants show broad but distinct tissue and cell tropism. For cardiac gene delivery, the AAV serotype 9 (AAV9) has clearly emerged as the most potent serotype, at least in rodents, when delivered systemically [3] and in pigs (personal communication: Dr. Roger Hajjar, Icahn School of Medicine) and possibly in dogs [4, 5] when delivered regionally.

In this chapter, we describe the production and detailed characterization of AAV9 vectors at a scale that is sufficient for both small and large animal experiments.

The AAV vector genome consists of two genes, the Rep gene, which encodes for the Rep proteins, which are involved in DNA replication and encapsidation of the viral DNA into preformed capsids. The viral capsid is composed of 60 copies of the capsid proteins VP1, VP2, and VP3 that are encoded by the Cap gene. Using an alternative reading frame, the Cap gene also encodes the so-called assembly-activating protein (AAP), which facilitates capsid assembly [6].

For recombinant AAV (rAAV) production, the ITRs are the only cis elements required, whereas the Rep proteins, the serotype-specific capsid proteins, AAP and all the helpervirus functions can be provided in trans. One of the drawbacks of AAV is its limited packaging capacity of ~5 kb. But for most applications this is not an issue because the median size of human proteins is 375 amino acids [7].

For small to medium scale AAV production (10^{12}–10^{13} vector genome containing particles), AAV is generally produced by transfection of HEK293 cells. The plasmids used are a so-called cis-plasmid, which contains the transgene (or elements regulating host gene expression, e.g., shRNAs) flanked by the ITRs, and plasmids encoding Rep, Cap, AAP and the necessary (most often adenoviral) helpervirus proteins [8]. In its simplest form, which is the method preferred in our laboratory, Rep, Cap, AAP, and all the adenoviral helper functions are provided on a single plasmid [9].

For AAV9, the virus is harvested 72 h post-transfection from both the cell culture medium as well as the transfected cells. In our laboratory, and many others, the virus is then purified by Iodixanol gradient centrifugation.

Whereas many laboratories only characterize their virus by determining viral titers by qPCR, our experience is that, especially for large animal studies, a more thorough virus characterization that includes SDS-PAGE (to determine virus purity and total viral capsid titers), qPCR (to determine viral genome titers), alkaline agarose gel electrophoresis (to ensure genome integrity and/or viral genome titer) and negative-staining electron microscopy (to determine the percentage of empty particles) is warranted.

2 Materials

2.1 Plasmid Production

1. Cis plasmid containing the rAAV genome to be packaged.
2. pDG9 helper plasmid (AAV9 capsid sequence cloned into the SwaI/ClaI digested pDG (Plasmidfactory, Bielefeld, Germany)).
3. SURE2 (Agilent Technologies, Santa Clara, CA) or Stbl3 (Life Technologies, Norwalk, CT) competent bacteria for transformation with cis-plasmids.
4. QIAfilter Plasmid Maxi Kit (Qiagen, Germantown, Maryland).
5. SmaI (or the isoschizomer XmaI) restriction enzyme.

2.2 Cell Culture

1. HEK 293T/17 cells (ATCC, Manassas, VA).
2. DMEM 4.5 g/l glucose, +L-glutamate + sodium pyruvate.
3. Fetal bovine serum.
4. 10× penicillin/streptomycin.
5. Trypsin 0.025%.
6. PBS without magnesium and calcium.
7. 225 cm^2 tissue culture flask.

2.3 Transfection

1. Vented or non-vented tissue culture triple flask(s).
2. 50 μg cis plasmid and 150 μg helper plasmid per triple flask to be transfected.
3. Linear, 25 kDa polyethylenimine (PEI) (Polysciences Inc. Warrington, PA).
4. 250 ml sterile filter (0.22 μm) bottles.

2.4 Harvesting and Processing

1. 200 ml polypropylene conical centrifuge tubes.
2. 50 ml polypropylene conical tubes.
3. Lysis buffer: 150 mM NaCl, 50 mM Tris–HCl, pH 8.5.
4. Sorvall RC-6+ centrifuge.
5. SH-3000BK, F14-6x-250y, and F13-14x50cy rotors.
6. Universal Nuclease for Cell Lysis (Thermo Fisher, Waltham, MA) (*see* also **Note 1**).
7. Ammonium sulfate.

2.5 Preparation of Ultracentrifugation Gradients

1. Optiprep (60% iodixanol).
2. 5× Optiprep dilution buffer "ODB": 5× PBS, 5 mM magnesium chloride, 12.5 mM potassium chloride.
3. Iodixanol Gradient Layer Solutions (*see* Table 1).
4. Ti70 rotor (Beckman Coulter, Indianapolis, IN).

Table 1
Components to prepare iodixanol gradient layer solutions sufficient for one complete (8 tubes) Ti70 rotor

Iodixanol gradient layer (%)	Optiprep (ml)	5 M NaCl (ml)	5× ODB (ml)	Water (ml)	Total for 1 full rotor (ml)
15	16.1	12.8	12.8	22.5	64.2
25	18.0	0	8.6	16.5	43.1
40	23.5	0	7.0	4.7	35.2
60	35.2	0	0	0	35.2

5. OptiSeal polypropylene 26×77 mm ultracentrifuge tubes (37.4 ml, Beckman Coulter, Indianapolis, IN).
6. 5 ml syringe and 18 gauge needles.

2.6 Dialysis

1. 10 mm flat width, 12–14 kDa MWCO, regenerated cellulose dialysis Tubing (Spectrum Labs, Piscataway, NJ).
2. Dialysis tubing closures.
3. Lactated Ringer's Solution.
4. 0.22 μm sterile syringe filter.
5. Large sterile bottle or container.
6. Orbital shaker.

2.7 Quantitative Real Time PCR

1. Real time PCR machine.
2. Real time PCR tubes and caps compatible with specific machine.
3. Real time PCR Master Mix SYBR Advantage (Clontech, Mountain View, CA).
4. Forward and reverse primers that anneal perfectly to both the reference standard and the AAV sample to be quantified (*see* also **Note 2**).
5. AAV2 Reference Standard Material (ATCC VR1616, Manassas, VA) or calibrated "home-made" AAV reference standard (*see* **Note 3**).

2.8 Capsid Particle Content and Purity

1. Equipment and reagents for SDS-PAGE gel electrophoresis.
2. 6× SDS-PAGE loading buffer: 0.8 g SDS, 5 ml 1 M Tris–HCl pH 6.8, 5 ml glycerol, 5 mg bromophenol blue. Store in aliquots at −20 °C. After thawing, add 50 μl beta-mercaptoethanol per 1 ml of 6× loading buffer immediately prior to adding the buffer to the viral or BSA samples.
3. BSA protein standard (Pierce, Thermo Fisher, Waltham, MA).
4. Pre-Soak buffer: 50% methanol, 10% acetic acid.

5. Staining buffer: 50% methanol, 10% acetic acid, 0.003% Coomassie Brilliant Blue R-250.
6. Destaining buffer: 40% methanol, 8% acetic acid.
7. Odyssey infrared scanner (Li-Cor, Lincoln, NE).

2.9 Electron Microscopy

1. Carbon Coated Copper EM Grids.
2. Dumont inverse, anti-capillary tweezers.
3. Uranyl acetate 2% solution.
4. Chromatography paper.
5. Transmission electron microscope.

2.10 Alkaline Gel

1. "Submarine style" electrophoresis apparatus and power supply.
2. Electrophoresis grade agarose.
3. 50× Alkaline Gel Buffer: 2.5 M sodium hydroxide, 50 mM EDTA.
4. Alkaline Gel loading dye: 4× Alkaline Gel Buffer, 1.2% SDS, 20% Glycerol, 0.01% Xylene Cyanol.
5. DNA Mass Ladder.
6. Gel Red (Biotium, Fremont, CA) or equivalent dye (e.g., SYBR Gold, (Thermo Fisher, Waltham, MA)) that allows the sensitive detection of *single stranded* DNA.

3 Methods

3.1 Plasmid Cloning and Production

1. To prevent the potential loss of intact ITRs all our cis plasmids are cloned and maintained in either SURE2 or Stbl3 cells and grown at 30–32 °C (*see* **Note 4**). pDG9 can be maintained in a regular *E. coli* strain such as DH5α and can be grown at 37 °C. We routinely purify our plasmids with QIAFilter Plasmid Maxi Kit. For the production of the cis plasmid, one 2 l Erlenmeyer flask with 600 ml Luria–Bertani medium should produce enough plasmid for ten triple flasks. Since the amount of pDG9 needed for the same number of triple flasks is three times the amount of the cis plasmid, we grow the bacterial cultures in three 2 l flasks with 600 ml Luria-Bertani medium each.
2. To confirm that the majority of the cis-plasmid contains two intact ITRs we digest the cis plasmid with SmaI (or XmaI) restriction enzyme and analyze the digested DNA on an agarose gel.

3.2 Cell Maintenance (See Note 5)

1. HEK293T are maintained in DMEM supplemented with 10% FBS and 1× Penicillin/Streptomycin ("medium") for all growth and passaging steps unless otherwise noted.

For continuous culture, the cells are grown to approximately 70–80% confluency and then split 1:10 or 1:20 to be ready to be split again 3 days or 4 days later, respectively.

2. To split cells from a 225 cm^2 flask, gently remove the culture medium, add 20 ml PBS without Mg/Ca to cover the surface and gently rock the flask to completely cover the cells. Remove the PBS and add 5 ml 0.025% Trypsin to the side of the flask, not directly onto the cells. Spread over the cells by gently "rocking" the flask several times. Incubate at 37 °C for 1–2 min. Then rock the flask to completely dislodge the cells. Pipet 25 ml media onto the cells and pipet up and down to prepare a homogenous cell suspension. Add 50 ml of a 1:10 or 1:20 dilution of cell suspension in fresh medium to a new flask (*see* also **Note 6**).

3.3 Transfection

1. To prepare the PEI solution for transfection add 250 mg PEI powder to 200 ml sterile, deionized water and stir with magnetic stir bar. Adjust pH to 1.9 by addition of 10 N hydrochloric acid. Stir overnight to assure complete dissolution of the PEI. The next day, add dropwise 1 N sodium hydroxide to adjust the pH to 4.5. Then add water to 250 ml and sterile filter with a 0.22 μm filter bottle. Store aliquots at −80 °C. Once thawed, the solutions can be stored at 4 °C for up to 2 months.
2. The day before transfection, detach cells with Trypsin and split either ~1:4 or 1:6 (*see* **Note 7**) into triple flasks. One confluent 225 cm^2 flask is sufficient to seed two triple flasks (total area 1000 cm^2) at a 1:4 dilution. Prior to seeding the triple flasks, remove a sufficient amount of cells to seed a 225 cm^2 flask for the continuation of cell passaging.
3. The morning after seeding the triple flasks, check the flasks for cell confluency. If ~70% confluent, proceed to transfection immediately. If the confluency is less than ~70%, perform the transfection later in the day.
4. For each triple flask to be transfected, warm 90 ml DMEM (2% FBS, 1× Pen/Strep) to 37 °C.
5. Prepare the transfection mix by adding in the following order to 20 ml room temperature DMEM (no FBS, no Pen/Strep): 50 μg cis plasmid and 150 μg pDG9 (briefly vortex) and 700 μl PEI solution (pH 4.5). Vortex for 10–20 s and incubate at room temperature for 15 min.
6. Add the transfection mix to the pre-warmed 2% FBS medium and mix by swirling.
7. Gently remove the media from the cells in the triple flasks and replace with transfection mix media.
8. Grow the transfected rAAV producing cells for 3 days, then harvest.

3.4 Harvesting and Processing

3.4.1 Preparation of Cell Lysate

1. Detach the cells by tapping the flask vigorously and transfer the solution to a sterile 200 ml conical flask. Pellet the cells by centrifugation at 1000 × *g* for 15 min in a Sorvall RC-6+ centrifuge and SH-3000BK swinging bucket rotor.
2. Collect the cell culture supernatant in a sterile bottle for later processing.
3. Gently add 10–15 ml PBS to the side of the conical tube. Swirl gently to dislodge, but not break apart, the pellet. Transfer the pellet to a 50 ml conical tube. Multiple pellets can be combined into one 50 ml tube for further processing. If cells remain in the large conical, use the PBS to rinse the cells off the large tube and transfer to the small tube.
4. Pellet the cells by centrifugation in a Sorvall RC-6+ centrifuge and SH-3000BK rotor at 1000 × *g* for 10 min. Pour the supernatant into the bottle containing the cell culture supernatant (*see* **Note 8**). Proceed with media to **step 1** of Subheading 3.4.2. At this step, the cell culture supernatant can be frozen at −80 °C to be processed later.
5. Resuspend the cell pellet in 10 ml lysis buffer (*see* **Note 9**).
6. To lyse the cells, the cell suspension is repeatedly frozen and thawed. The cell suspension can either be frozen by putting it into a −80 °C freezer or on dry ice. The cell suspension is thawed in a 37 °C water bath followed by brief vortexing. This freeze/thaw cycle is repeated twice. Aggregation of cellular debris during the freeze–thaw cycles is normal. Any of the freezing steps can be used as a stopping point.
7. To digest genomic DNA and non-encapsidated viral DNA and cellular RNA add 2 μl (10 U/μl) of Universal Nuclease to the thawed, crude lysate and incubate for 30 min at room temperature (*see* also **Note 1**).
8. Centrifuge the crude lysate in a Sorvall RC-6+ centrifuge and F13-14x50cy fixed angle rotor at 5000 × *g* for 20 min and transfer the supernatant to a new tube (discard the pellet). The new tube is ready for Iodixanol gradient ultracentrifugation. It can be stored at −80 °C at this point.

3.4.2 Processing of the Cell Culture Supernatant

1. If the cell culture supernatant was frozen (Subheading 3.2), thaw it at 37 °C, but do not incubate longer than necessary. Per 100 ml of supernatant add 31.3 g ammonium sulfate and shake for 1–2 min to dissolve completely the ammonium sulfate. Incubate the mixture on ice for at least 30 min (*see* **Note 10**).
2. To pellet the virus precipitate, centrifuge in a Sorvall RC-6+ centrifuge and an F14-6x250y fixed angle rotor at 8300 × *g* for 30 min. Because the pellet may be loose, pour off the supernatant as soon as possible after centrifugation. THE SUPERNATANT CANNOT BE STERILIZED WITH

BLEACH. INSTEAD, THE SUPERNATANT MUST BE AUTOCLAVED AND THEN BE DISCARDED. Ammonium Sulfate is incompatible with bleach, and treatment of the supernatant with bleach would result in the production of TOXIC GASES AND EXPLOSIVE COMPOUNDS.

3. The pellet will accumulate on the side of the centrifuge tube. The pellet is resuspended with a pipette in 10 ml Lysis Buffer (*see* **Note 9**). The resuspension is complete when there is no remaining pellet on the wall of the tube and the solution is homogeneous without any remaining "chunks".
4. Add 2 μl (20 U) of Universal Nuclease for Cell Lysis (*see* **Note 1**) and incubate 30 min at room temperature (or according to manufacturer's instructions if using an alternative nuclease). The virus suspension is now ready for iodixanol gradient purification of the virus.

3.5 Preparation of Ultracentrifugation Gradients

3.5.1 First Gradient

1. Pipet 9.5 ml AAV cell lysate or virus suspension from cell culture supernatant into OptiSeal ultracentrifuge tubes. Try not to introduce bubbles.
2. Insert a Pasteur pipet into the tube with the tip centered at the bottom (*see* **Note 11**).
3. To form the gradient, underlay the viral solution in this order by slowly adding to the top of the Pasteur pipette: (a) 7.3 ml 15% iodixanol, (b) 5 ml 25% iodixanol, (c) 4 ml 40% iodixanol, and (d) 4 ml 60% iodixanol. Before adding the next solution, allow all of the previous solution to reach the bottom of the tube.
4. Carefully remove the Pasteur pipet without disturbing the gradient.
5. If at this point the tube is not completely filled, add any leftover AAV crude lysate, virus suspension from the cell culture supernatant or lysis buffer to the top of the tube to fill the tube up to the neck. Any bubbles must be removed prior to centrifugation to prevent the collapse of the tubes during centrifugation. To remove potential bubbles slightly squeeze the tube until the bubbles rise above the neck of the tube and remove them with a pipet. Seal the tube by firmly inserting the plastic stopper.
6. Use a permanent marker to draw a line at the 40–60% interface. The interfaces are easy to see before centrifugation but more difficult to visualize after centrifugation.
7. Centrifuge at (350,333 × *g*) in a Ti70 rotor for 1 h (*see* **Note 12**).
8. The majority of AAV particles will be located at the 60–40% iodixanol interface. The virus can be collected by inserting an 18-gauge needle (connected to a 5 ml syringe) just below the

interface and slowly withdrawing 3 ml (These 3 ml will consist of approximately the top 1 ml of the 60% and the bottom 2 ml of the 40% iodixanol solution). Alternatively, if no second gradient is performed to concentrate further the virus, it is preferable to collect fractions from the bottom (*see* below: Subheading 3.5.2, **steps 5** and **6**). Using this approach will yield virus preparation with less empty particles (*see* Fig. 2).

9. Determine the AAV titer/yield by qPCR (*see* below Subheading 3.7). If the viral titers are too low, combine the virus from multiple tubes and proceed to second gradient.

3.5.2 Second Gradient

1. For a second round of iodixanol gradient purification (*see* **Note 13**), dilute the harvested AAV/iodixanol solution in 1× ODB such that the percentage of iodixanol is lower than 25%. For this calculation, the AAV/iodixanol harvested from the first gradient is assumed to be 47% (1 ml 60% + 2 ml 40%).
2. Add 22 ml of the AAV/iodixanol dilution into an OptiSeal ultracentrifuge tube.
3. This solution is then underlaid sequentially with 4 ml 40% iodixanol and 60% iodixanol using the technique described above.
4. Centrifuge the tubes at 69,000 rpm (350,333 × *g*) in a Ti70 rotor for 2 h.
5. Collect 1.25 ml fractions from the bottom of the tube by creating a needle hole at the bottom of the tube, and letting the contents drip into Eppendorf tubes pre-marked at the 1.25 ml level. This can be done by covering the top opening of the tube with a finger to create a vacuum to prevent flow, then releasing the finger when the appropriate fraction tube is placed under the bottom hole.
6. Run an alkaline gel of the fractions to identify the fractions containing the majority of full-length viral genomes and combine those fractions.

3.6 Dialysis

1. Soak dialysis tubing in lactated Ringer's solution overnight.
2. The next morning, squeeze out any lactated Ringer's solution left in the tubing, clamp one side of the tubing with a dialysis clip, pipet the AAV/Iodixanol into the tubing, and clamp the other end with a dialysis clip. To allow the easy recovery of the virus, leave at least 2 in. of tubing hanging outside one of the clamped ends of the tubing.
3. Place the closed dialysis tubing in a large sterile bottle with at least 100 dialysate volumes of lactated Ringer's solution.
4. Agitate the dialysis solution gently for 1–2 h on an orbital shaker platform, and replace the lactated Ringer's solution.

5. Agitate the dialysis solution for an additional 4–5 h, and replace with fresh lactated Ringer's solution.
6. Shake gently overnight. Remove the clamp on the side with additional tubing and collect the contents of the tubing in a 50 ml tube. Filter through a 0.22 μm sterile syringe filter. Aliquot and store at −80 °C (*see* **Note 14**).

AAV Vector Titration and Quality Control (See **Note 15**)

3.7 Viral Titer Determination by Quantitative Real-Time PCR (qPCR)

1. Prepare a 2× master mix of reagents (2× SYBR Green, primers, water, ROX passive reference dye) that will be enough for duplicates of the following samples: four dilutions of standards (*see* **Note 2**), two dilutions of AAV sample, and one no-template control. Add 8 μl of master mix and 2 μl virus dilutions to each tube for a final reaction volume of 10 μl.
2. Thaw an aliquot of the ATCC AAV reference virus (or a well characterized in-house produced AAV standard) as well as an aliquot of the AAV to be quantified (*see* **Note 3**).
3. Prepare a standard curve of the reference virus by diluting the virus stock: 1:1000; 1:10,000; 1:100,000; and 1:1,000,000.
4. Dilute the AAV sample 1:10,000 and 1:100,000.
5. Perform the PCR according to the manufacturer's instructions for the PCR reagents and instrument manufacturer's instructions.
6. Calculate the viral titer based on the standard curve and dilution of virus sample using the qPCR machine software (*see* **Note 3**).

3.8 Assessment of the Purity of Vector Preparations and Determination of Total Viral Capsid Titers

1. Prepare 10% polyacrylamide gels and buffers and assemble apparatus for running SDS-PAGE.
2. Dilute BSA standard solution to concentrations of 200, 100, 50, 25 ng/μl, then mix 10 μl of each with 20 μl water and 6 μl 6× SDS-PAGE Loading Buffer.
3. Mix 30 μl of AAV sample with 6 μl 6× SDS-PAGE Loading Buffer.
4. Heat the viral samples at 95 °C for 5 min and the BSA standards at 95 °C for 3 min (BSA partially degrades at boiling times longer than 3 min).
5. Load the samples into the wells and run at 125 V until the dye line reaches the bottom of the gel, usually 2–3 h.
6. Remove the gel from the electrophoresis plate assembly and soak in Pre-Staining Solution for 10 min while gently agitating on a rocking platform.
7. Immerse the gel in Staining Solution and rock gently overnight in an airtight container.

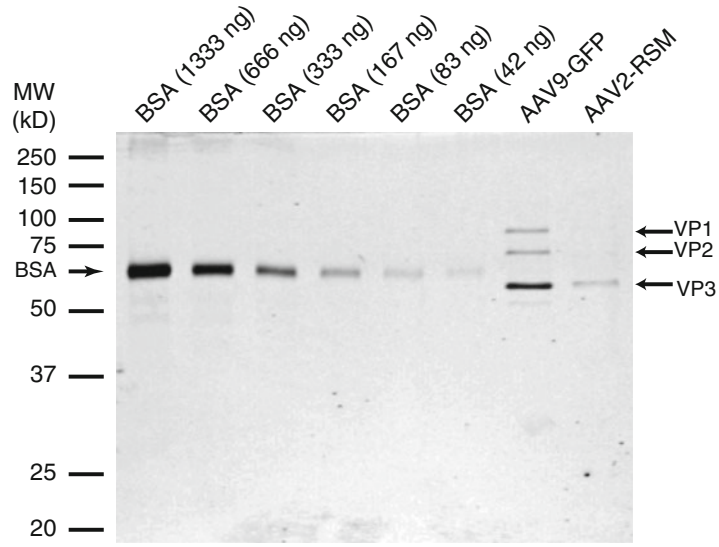

Fig. 1 Representative gel for assessment of capsid content and purity. From *left* to *right*: BioRad Precision Plus Dual Color Ladder, BSA 1,333 ng, BSA 666 ng, BSA 333 ng, BSA 167 ng, BSA 83 ng, BSA 42 ng, AAV9-GFP (1.32x10 vg), AAV2-RSM (2.41x10 vg)

8. The following day, replace the staining solution with destaining solution and add a rolled up paper towel to one side of the container. Gently rock until the destaining solution and/or the paper towel becomes soaked with stain. Then replace the spent destaining solution with fresh destaining solution and clean paper towel. Repeat until the gel shows little to no background staining (*see* Fig. 1).
9. Wash the gel by gently rocking it for 5 min in water. Repeat this wash step once with fresh water.
10. Scan the gel on the LI-COR Odyssey Infrared Scanner or equivalent fluorescence scanner. If no fluorescence scanner is available, the gel can be scanned with a regular scanner, although this appears to modestly reduce the accuracy of the assay.
11. Quantify the relative intensity of the BSA and AAV VP3 bands with the LI-COR software or freely available Image J (http://imagej.nih.gov/ij/).
12. Plot the band intensity (arbitrary units [au]) vs. ng of BSA loaded. Use a linear regression to obtain an equation that correlates ng of protein to band intensity. Use this equation to calculate the ng of protein in the VP3 band of the AAV sample.
13. The total viral particle (vp) titer can be calculated with the following formula: $\text{vp}/\mu\text{l} = \text{ng VP3}/4.987 \times 10^{-9}/\mu\text{l}$ AAV sample loaded per lane (*see* **Note 16**).

3.9 Determination of Percent Genome Containing AAV Particles by Electron Microscopy

1. Pick up an EM grid with a Dumont Anti-Capillary Reverse (self-closing) tweezers and set down on the bench with the shiny side of the grid facing up.
2. Pipet 5 µl of AAV sample onto the grid and allow it to dry by evaporation. This may take 30–60 min.
3. Wash the grid by pipetting, drop by drop, about 200 µl water onto the grid.
4. Wick excess water by slowly placing chromatography paper vertically next to the grid.
5. Pipet 5 µl 2% uranyl acetate solution onto the grid. Incubate for 5 min (*see* **Note 17**), and then wick off as above. Let the grid dry.
6. Visualize AAV particles with a transmission electron microscope at 50,000-fold magnification. Viral capsid containing a viral genome will appear as homogeneously white hexagons, while empty capsids show as hexagons with a white rim but a dark center (*see* Fig. 2).
7. Randomly count at least 100 particles to determine approximate percentage of genome-containing vs. empty AAV particles.

3.10 Alkaline Gel Electrophoresis to Determine Vector Genome Integrity (and Viral Genome Titers)

1. Prepare the alkaline agarose gel by adding 1 g agarose to 98 ml water and microwave to dissolve. Let the solution cool until it can be handled without gloves, then add 2 ml 50× Alkaline Electrophoresis Buffer, swirl to mix, pour to cast the gel.
2. Place the gel into an electrophoresis apparatus, fill with 1× Alkaline Running Buffer, and place everything in a cold room.
3. Dilute DNA ladder (500 ng) to 25 µl with water.
4. Mix 25 µl AAV sample or diluted DNA ladder with 8.5 µl 4× alkaline sample loading buffer, heat to 95 °C for 3 min, then cool on ice prior to loading on the gel.
5. Load the gel and run overnight at 20 V in the cold room using a dedicated pow er supply (*see* **Note 18**).

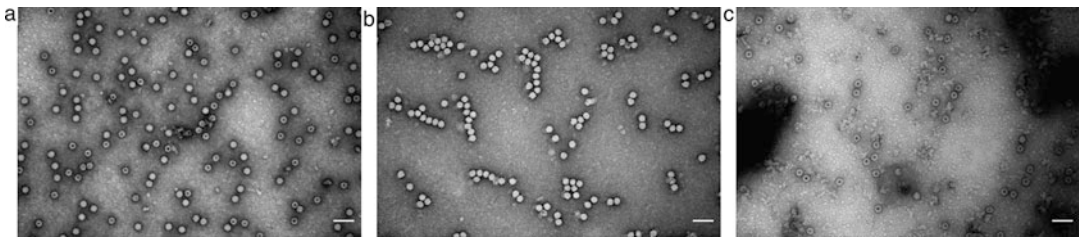

Fig. 2 Electron microscopy images of AAV. (**a**) Virus collected after the first ultracentrifugation gradient; (**b**) Fraction 4 collected from the second ultracentrifugation gradient; (**c**) Fraction 7 collected from the second ultracentrifugation gradient. *Viral capsids containing a viral genome appear as homogeneously white hexagons, while empty capsids show as hexagons with a white rim but a dark center* (**a**) ~60% genome containing particles, (**b**) >98% genome containing particles, (**c**) ~5% genome containing particles

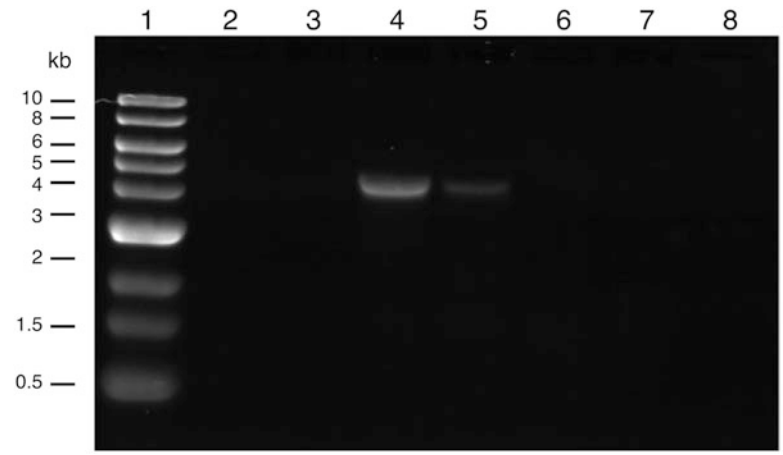

Fig. 3 Alkaline gel of fractions collected from an iodixanol gradient. *Lane 1*: 1 kb DNA ladder (500 ng). *Lanes 2–8*, 20 μl of fractions 2–8 of a first iodixanol gradient of AAV9-LMNA. The capsid protein content in fractions 5 and 6 is higher than in fraction 4, indicating that fraction 5 and especially fraction 6 contain empty capsids (SDS-PAGE, not shown)

6. Remove the gel from the electrophoresis apparatus, place the gel in a container and cover it with 0.1 M Tris–HCl pH 8.5 and rock gently for 1 h.
7. Discard the buffer and replace with 4× GelRed or SYBR Gold in 0.1 M NaCl and rock protected from light for 2 h.
8. Rinse the gel briefly in tap water.
9. Visualize the gel with a UV transilluminator and capture a digital picture (*see* Fig. 3 and **Note 19**).

4 Notes

1. Alternative nucleases that digest double DNA, single-stranded DNA and RNA can also be used, for instance benzonase (Sigma-Aldrich, St. Louis, MO), to achieve the same result. If benzonase is used, add 2 μl 2.5 M $MgCl_2$ and 2 μl benzonase (1500 U/μl) to 10 ml crude lysate and incubate 1 h at 37 °C.
2. Extensively characterized, AAV2 and AAV8 reference standard material can be obtained from ATCC (cat. nos. VR-1616 and VR-1816, respectively). The broad use of these reference standards should facilitate the reproducibility of titer determinations among different laboratories. For economic reasons, it might be preferable to employ the ATCC standard viruses to prepare a thoroughly characterized in house reference virus preparation. It needs to be pointed out; however, unless ITR-

specific primers are used [12], the reference standards can only be used for the titration of AAVs that share other common sequence elements with the AAV standards.

3. If an in house reference standard is used that is single-stranded, and the virus to be titered is self-complementary, the final titer should be divided by 2. In this context, it also needs to be pointed out that the accurate titer determination of double-stranded AAVs has additional pitfalls, *see* [13].
4. For further information on designing and constructing cis plasmids, *see* Gray [10].
5. HEK293T cells are a human cell line, and all work must be performed in accordance with Biosafety Level 2 (BSL2) regulations. This includes the use of a BSL2 laminar flow tissue culture hood. Adeno-associated viruses are BSL1, but all work with AAV should be performed under sterile conditions.
6. 293T cells can be passaged for about 1–2 months after thawing the initial vial, as long as they are regularly split at least twice a week.
7. Split cells depending on confluency. If the flask is very confluent, split 1:6, if less confluent 1:4. The goal is to achieve approximately 70% cell confluency the next day.
8. Because this PBS pellet wash solution can contain AAV, we routinely combine it with the cell culture supernatant.
9. The maximum volume of crude lysate that can be loaded in a single ultracentrifugation tube is 10 ml. However, the cell pellets from up to three triple flasks can be combined and resuspended in 10 ml lysis buffer. After freeze/thaw and clearing of the lysate the combined lysate can be loaded into a single tube. Similarly, it is possible to resuspend the pellets of precipitated AAV from cell culture supernatant of up to three triple flasks.
10. Although incubation on ice for as little as 30 min is sufficient for virus precipitation, keeping the ammonium sulfate/cell culture supernatant mixture for several days at 4 °C will not affect the quality of the virus. However, once the virus precipitate has been spun down, the pellet should be resuspended and loaded onto a gradient the same day. Resuspending the pellet and freezing for a later gradient is not recommended as aggregation appears upon thawing.
11. The creation of multiple iodixanol gradients can be facilitated by using a multichannel peristaltic pump (available, for instance, from Watson-Marlow; Paramus, NJ) to assemble several gradients simultaneously. With such a system the different iodixanol solutions can be delivered to multiple tubes at the same time, which reduces the number of necessary pipetting steps. Using this method, up to four centrifugations

(with Ti70 rotors, holding 8 tubes each) can be performed in 1 day.

12. If a different rotor is used, the speed and run times have to be adjusted using the *k*-factors of the Ti70 rotor the 69,000 rpm ($k = 44.9$) and the respective *k*-factor of the alternate rotor at a given speed. A convenient tool for this calculation can be found at: https://www.beckmancoulter.com
13. Calculate whether the AAV titer is sufficiently high for the planned experiments. Especially for in vivo experiments, when small injection volumes are necessary, it might be necessary to concentrate further the virus. In addition to concentrating the virus, if the virus was collected as a single fraction during the first gradient purification, this second ultracentrifugation gradient also allows the removal of empty capsids that may have been collected together with the genome-containing AAV particles from the first gradient.
14. For long-term storage, AAV is preferably stored at −80 °C. However, AAVs are extraordinarily stable; recent reports have shown no loss of activity after 1 week of storage at room temperature [11]. This can have important implications for the shipping of AAVs. In particular, the quality of the virus is likely unaffected if all dry ice has evaporated due to unanticipated delays in the delivery of the virus. Because of the danger of damaging the AAV genome, never expose AAV to UV light (such as in a bio-safety cabinet).
15. The quality of AAVs purchased from academic vector cores or commercial vendors can vary significantly. This is especially true for double-stranded AAVs or AAVs with a genome size approaching the maximum packaging capacity of the AAV capsid. Therefore, we recommend a thorough in-house characterization before performing any experiments.
16. The calculation of capsid concentration is based on the molecular weight of VP3 (calculated from its amino acid composition), which is 60,063 Da. Because each capsid contains 50 VP3 subunits, the combined molecular weight of VP3 subunits in a single capsid is approximately 3 MDa, and each capsid contains 4.9868×10^{-18} g of VP3 [14]. Dividing the amount of VP3 (in g) by 4.9868×10^{-18} g will yield the number of viral particles.
17. The incubation time is critical. Shorter incubation times will cause empty viral particles to appear as particles with encapsidated genomes.
18. Moving the power supply frequently from the cold room to room temperature will damage the power supply due to water condensation.

19. Alkaline gel electrophoresis can also be used to determine AAV titers. For this, a dilution series of a mass DNA ladder, or dilutions of a linear DNA fragment of known concentration and, preferably, of similar size to the AAV genome (e.g., the cis plasmid digested with SmaI if the only SmaI sites are in the viral ITRs) will be loaded onto the same gel as the AAV samples to be quantified. Quantification can then be achieved with the freely available ImageJ (http://imagej.nih.gov/ij/) or similar program. This method is especially useful if the AAV samples to be quantified do not share sequence elements with the reference standard and for double-stranded AAVs.

Acknowledgments

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Chapter 7

Cell-Based Measurement of Neutralizing Antibodies Against Adeno-Associated Virus (AAV)

Andreas Jungmann, Oliver Müller, and Kleopatra Rapti

Abstract

In recent years gene therapy using adeno-associated viral (AAV) vectors to treat cardiac disease has seen an unprecedented surge, owing to its safety, low immunogenicity relative to other vectors and high and long-term transduction efficiency. This field has also been hampered by the presence of preexisting neutralizing antibodies, not only in patients participating in clinical trials but also in preclinical large animal models. These conflicting circumstances have generated the need for a simple, efficient, and fast assay to screen subjects for the presence of neutralizing antibodies, or lack thereof, in order for them to be included in gene therapy trials.

Key words Neutralizing antibody, Assay, AAV vectors, In vitro, Human and animal serum

1 Introduction

Cardiovascular diseases are the leading cause of morbidity and mortality in the western countries [1]. Adeno-associated viral (AAV) gene therapy [2–4] is a promising alternative to current therapies, such as pharmacological treatments. However, AAVs are naturally occurring viruses, meaning that both humans [5–7] and animals [8–11] used in biomedical research carry neutralizing antibodies (nAb) against the serotypes. Animals or candidates carrying these antibodies cannot benefit from AAV based gene therapies, as the AAVs are neutralized before they reach their target, such as the heart or the liver [12, 13]. Several approaches have been developed to evade such neutralizing effects; however, the current strategy is to screen animals or patients and exclude them from further studies [13]. The latter necessitates the development of fast, efficient, and cost-effective methodologies to screen for them. A summary of screens for neutralizing activity conducted in humans and large animals can be found in Table 1. As shown, for the most of the studies the nAb assay was used to assess seroprevalence of antibodies against AAVs. Attention should be brought to the variability in

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Table 1
Overview of major seroprevalence studies in humans and large animals

Publication	Assay	Serotype/% of population with indicated nAb titers (cutoff value: ≥50% neutralized)	Serum tested	Comments (Processing of serum, material used for dilution, transgene)
[7]	nAb	AAV1/59.5% (≥1/2) Increase with age and geographical variation	Serum from healthy and patients with chronic systolic heart failure (Indicated are heart failure patients)	-, Culture Media (FBS), GFP reporter gene
[23]	nAb	AAV1/0% (1/5) AAV2/8% (1/5) AAV5/100% (1/5) AAV6/8% (1/5) AAV8/0% (1/5) AAV1/100% (1/5) AAV2/0% (1/5) AAV5/0% (1/5) AAV6/100% (1/5) AAV8/0% (1/5) AAV1/47% (1/5) AAV2/30% (1/5) AAV5/100% (1/5) AAV6/6% (1/5) AAV8/35% (1/5)	Horse Serum Dog serum Pig serum	Heat inactivated serum, DMEM without FBS, b-galacotsiadase
[24]	nAb	AAV1/36.5% (1/14) AAV2/35.3% (1/14) AAV5/37.6% (1/14) AAV8/32.9% (1/14) AAV9/36.5% (1/14)	Human serum from subjects of different ages, healthy vs hemophilia patients (Indicated are healthy subjects)	-, FBS, b-Galactosidase transgene
[25]	nAb	AAV2/96.6% (>1/10); 89.4% (>1/80) AAV5/40.2% (>1/10); 8.6% (>1/80) AAV8/=82% (>1/10)	Human serum from subjects originating from different countries + HIV infected individuals (Indicated are healthy Chinese human subjects)	Heat inactivated serum, DMEM + 10% FBS, luciferase
[26]	nAb, ELISA	AAV2/1/100–1/316 AAV8/1/1–1/31 AAV9/1/3–1/100	Sheep serum	Heat inactivated serum, Heat inactivated FBS, luciferase
[27]	nAb	AAV2/22.1% (≥1/20) AAV8/15.7% (≥1/20)	Human serum from subjects of different ages	Heat inactivated plasma, DMEM without FBS, b-galactosidase

(continued)

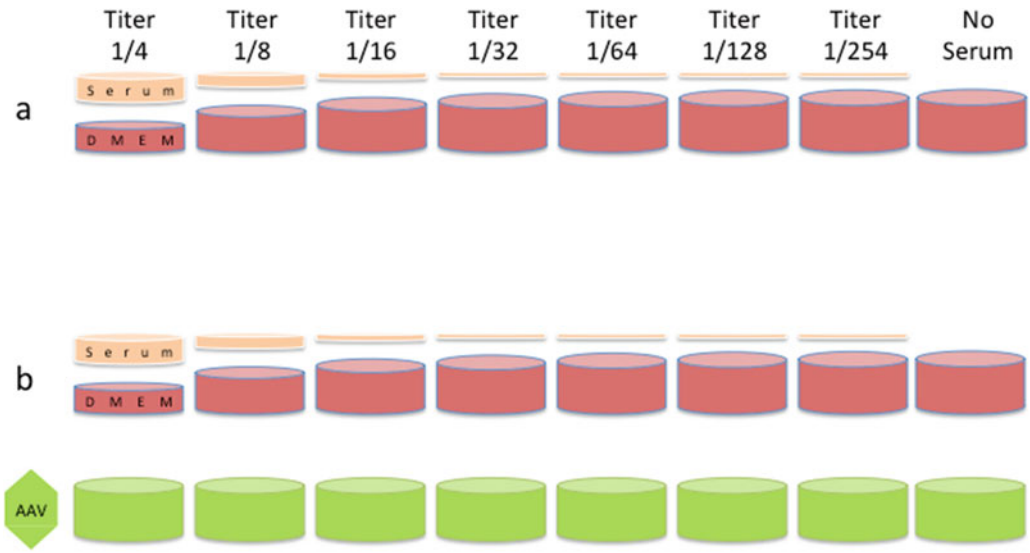
Table 1
(continued)

Publication	Assay	Serotype/% of population with indicated nAb titers (cutoff value: ≥50% neutralized)	Serum tested	Comments (Processing of serum, material used for dilution, transgene)
[5]	nAb, ELISA	AAV1/67% (1/20) AAV2/72% (1/20) AAV5/40% (1/20) AAV6/46% (1/20) AAV8/38% (1/20) AAV9/47% (1/20)	Human serum (Heat inactivated) (donors between 25 and 64 years of age)	Heat inactivated serum, 10% FCS, luciferase
[6]	nAb	AAV1/20–43% (1/20) AAV2/28–56% (1/20) AAV7/12–29% (1/20) AAV8/14–31% (1/20) AAVrh32.33/3% (1/20)	Human serum from healthy volunteers from four different continents	Heat inactivated serum DMEM without FBS (cells supplemented 2 h later with FBS), b-galactosidase
[28]	nAb, ELISA	AAV2/37.5% (<1/3.1)	Human serum from normal subjects and subjects injected intramuscularly or intravascularly with AAV vectors (Indicated are healthy subjects)	-, Heat inactivated normal mouse serum, b-galactosidase
[29]	nAb	AAV2/10–30% (1/20) AAV5/4–20% (1/20) AAV6/6–30% (1/20)	Human serum healthy vs Cystic fibrosis adults and children	-, DMEM + 2% FBS, human placental alkaline phosphatase (AP)
[30]	nAb	tgAAVCF (AAV2)/1/20–1/2560	Human serum from patients older than 15 years of age, all lung disease	-, WT Adenovirus 5 and then WT AAV2 infected 293 cells, hybridization to immobilized DNA
[31]	ELISA	AAV2/50% (1/50–1/400)	Human serum	-, ELISA blocking buffer

the way one interprets seropositive samples in the population, in other words the cutoff value used to determine the percentage of the population that is positive for nAb against an AAV serotype. It is also noteworthy that serum is the preferred tissue. Immunoglobulins can also diffuse into extravascular tissues, but serum collection is easy, and highly indicative of the humoral immune status [14].

The methods proposed in most studies to measure AAV neutralizing ability of serum, or else the nAb titers, are (1) the nAb assay, in which one tests the ability of serum to inhibit transduction of cells by AAVs carrying usually luciferase as a reporter gene, followed by (2) ELISA and in one study (3) a native dot blot analysis. ELISA-based assays [15] are easy and provide extensive information regarding the immunoglobulins binding to viruses, also with regard to class categorization (IgG, IgM, IgA, IgE), but antibody binding does not necessarily reflect neutralization of transduction. It is also of note, that there are additional factors in the blood that could also affect AAV transduction efficiency, such as galectin 3 binding protein [16] and C-reactive protein [17]. In vivo assays provide useful information in a more relevant setting, but they are complicated, time consuming, costly and have ethical concerns. The method that provides the most useful insights, is cost effective and can be upscaled for screening of multiple samples concurrently is the cell-based nAb assay [8, 18]. The principle is simple: several dilutions of serum (the lower the dilution, for example 1/4, the higher the amount of serum, and therefore the higher the putative amount of antibodies) are mixed with equal amount of AAVs (quantified in viral genomes) and the gene transduction is measured (Fig. 1). The mixes of the different serum dilutions with AAVs, and also a no-serum control, are used to transduce cells. If the serum contains more antibodies against AAVs, then the higher amount of antibodies present in the lower dilutions of serum (for example 1/4) will bind and thus inactivate more AAVs leading to lower transduction output by the reporter gene, in comparison to the higher dilutions (for example 1/256). The no-serum control provides the maximum transduction efficiency, to which the samples are to be compared. The output is usually measured as percent transduction efficiency compared to the no-serum control. A typical curve of a serum with relatively abundant antibodies appears as a linear increase of the % transduction levels as the dilution factor increases (for example 1/4–1/256) (Fig. 1d). A typical curve of a serum that does not contain antibodies is almost flat, with even the lower serum dilution samples exhibiting 100% transduction levels, compared to no serum control (Fig. 1d). The nAb titer is determined as the highest dilution, in which more than 50% of inhibition is observed for the first time.

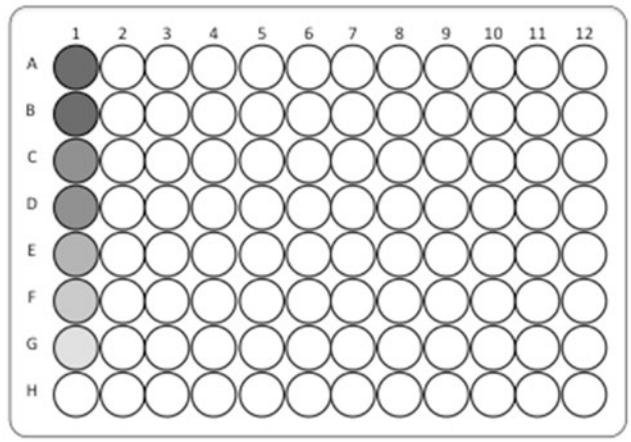
Fig. 1 Overview of the neutralizing antibody assay. (**a**) Schematic depiction of the test serum dilutions in DMEM, P/S. (**b**) Schematic depiction of the dilutions in the "Serum Mix Plate," after the addition of AAV. (**c**) Schematic depiction of the "Serum Mix Plate" and the "Cell Plate," as described in the protocol (in the form of a 96 well plate). "Cell Plate" has triplets for assessing the variability. (**d**) Sample Neutralization Assays superimposed by the schematic depictions of the serum/antibody dilutions mixed with AAV. The graphs represent the serum dilutions and the no serum control on the *x*-axis and the percent of transduction compared to no-serum control on the *y*-axis. The *left* graph depicts an nAb assay using serum without neutralizing antibodies, the *middle* graph depicts an nAb assay using a serum with moderate levels of neutralizing antibodies and the *right* graph depicts an nAb assay using serum with high levels of neutralizing antibodies, where even the smallest amount of serum (dilution 1/256) leads to a more than 50% inhibition of transduction compared to no serum control



c

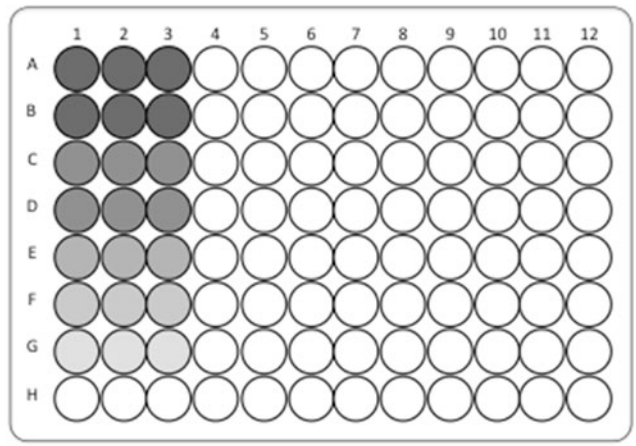
Serum Mix Plate (SMP)

	Column 1
A Titer 1/4	20 µl Serum + 20 µl DMEM, P/S, 40 µl DMEM, P/S, AAV
B Titer 1/8	10 µl Serum + 30 µl DMEM, P/S, 40 µl DMEM, P/S, AAV
C Titer 1/16	5 µl Serum + 35 µl DMEM, P/S, 40 µl DMEM, P/S, AAV
D Titer 1/32	2.5 µl Serum + 37.5 µl DMEM, P/S, 40 µl DMEM, P/S, AAV
E Titer 1/68	1.25 µl Serum + 38.75 µl DMEM, P/S, 40 µl DMEM, P/S, AAV
F Titer 1/128	0.625 µl Serum + 39.375 µl DMEM, P/S, 40 µl DMEM, P/S, AAV
G Titer 1/256	0.3125 µl Serum + 39.6875 µl DMEM, P/S, 40 µl DMEM, P/S, AAV
H No Serum	40 µl DMEM, P/S, 40 µl DMEM, P/S, AAV



Cell Plate

	Column 1 through 3
A Titer 1/4	20 µl from A1 in SMP
B Titer 1/8	20 µl from B1 in SMP
C Titer 1/16	20 µl from B1 in SMP
D Titer 1/32	20 µl from B1 in SMP
E Titer 1/68	20 µl from B1 in SMP
F Titer 1/128	20 µl from B1 in SMP
G Titer 1/256	20 µl from B1 in SMP
H No Serum	20 µl from B1 in SMP



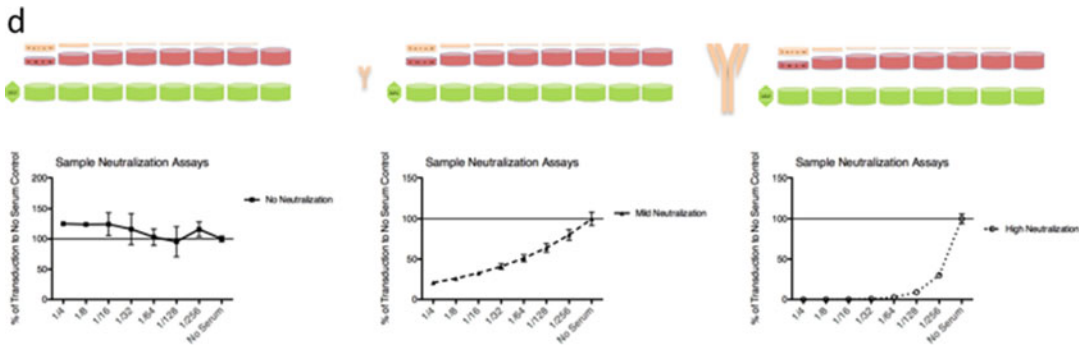


Fig. 1 (continued)

2 Materials

Prepare all solutions using ultrapure water (resistivity of 18.2 MΩ cm at 25 °C).

2.1 nAb Assay

1. 293T Cell Line.
2. 96-well plates.
3. V-Bottom 96-well plate (V96 Microwell plate with Lid).
4. T75 or T175 Culture flask.
5. Neubauer chamber.
6. GIBCO® Dulbecco's Modified Eagle Medium (DMEM).
7. L-glutamine (200 mM) (L-Glu).
8. Penicillin/streptomycin (P/S).
9. Fetal bovine serum (FBS).
10. Complete Medium: 1× DMEM, 10% FBS, 2 mM L-Glu, 1% P/S.
11. DMEM, P/S: 1× DMEM, 1% P/S.
12. PBS 10×.
13. PBS 1× (PBS 10× diluted 1:10 in MilliQ Water at room temperature).
14. Trypsin–EDTA 0.25%.
15. Trypsin–EDTA 0.05%: (Dilute trypsin–EDTA 0.25% 1:5 in DMEM) (Store at 4 °C).

2.2 Luciferase Assay

1. Luminometer Plates.
2. Luminoskan™ AscentMicroplate Luminometer (Ascent Software Version 2.6) (Ascent).

3. 1 mg/ml luciferin•potassium salt 10×: 10 mg luciferin•potassium salt in 10 ml MilliQ water. Prepare on ice, protected from light. Prepare 1 ml aliquots and store at −20 °C protected from light.
4. 5 mM coenzyme A (CoA) 100×: 25 mg CoA, free acid, in 6.1 ml of MilliQ water. Prepare on ice. Prepare 100 µl aliquots and store in −20 °C. Do not freeze thaw more than 3–4 times.
5. 500 mM $MgSO_4$ 100×: 600 mg $MgSO_4$ in 1 ml MilliQ water. Prepare 100 µl aliquots and store in −20 °C.
6. 1 M tricine HCl stock solution: 18 g tricine HCl in 100 ml water. Adjust pH to 7.8 with NaOH. Filter-sterilize and store at room temperature.
7. 1 M 1,4-dithio-D,L-threitol (DTT): 1.54 g DTT in 10 ml MilliQ water. Prepare 105 µl aliquots and store in −20 °C. Do not freeze thaw more than 3–4 times.
8. 0.5 M ethylenediaminetetraacetic acid (EDTA): 18.61 g EDTA in 80 ml MilliQ water. Add NaOH to adjust pH to 8.0. Test pH using pH strips until EDTA is dissolved. Filter-sterilize and store at room temperature.
9. Assay buffer 10×: 12.5 ml of 1 M tricine HCL stock solution, 500 µl of 0.5 M EDTA solution, 138 mg ATP, 50 mg BSA in 50 ml MilliQ Water (250 mM tricine•HCl pH 7.80, 5 mM EDTA (pH 8.0), 5 mM adenosine triphosphate (ATP), 10 mg/ml bovine serum albumin (BSA)). Dissolve; prepare 1 ml aliquots and store at −20 °C.
10. 1 M Tris•HCl: 12.1 g Tris•HCl in 80 ml MilliQ Water. Adjust pH to 8.0 with HCl and fill with MilliQ water till 100 ml. Filter-sterilize and store at room temperature.
11. 5 M NaCl: 29.2 g NaCl powder in 80 ml MilliQ Water. Fill with MilliQ water to 100 ml. Filter-sterilize and store at room temperature.
12. 2× Lysis Buffer Stock Solution: 1 ml 1 M Tris•HCl pH 8.00, 3.32 ml 5 M NaCl, 1 ml NP40 to a final volume of 100 ml with MilliQ Water (10 mM Tris•HCl pH 8.00, 150 mM NaCl, 1 % NP40). Store at room temperature.
13. 2× Lysis Buffer: 7.5 ml 2× Lysis Buffer Stock Solution, 75 µl 1 M DTT. Prepare on ice, fresh before the assay.
14. Reaction Mixture: 1 ml Assay Buffer 10×, 0.1 ml 500 mM $MgSO_4$ 100×, 0.1 ml 5 mM coenzyme A (CoA) 100×, 1 ml 1 mg/ml luciferin·potassium salt 10×, 29.4 µl 1 M DTT in 7.77 ml MilliQ Water. Prepare on ice, fresh before the assay, protect from light.

3 Methods

Carry out all procedures at room temperature and keep all solutions on ice.

3.1 nAb Assay

For the assay, two plates are needed: one "Cell Plate" (normal cell culture treated 96 well plate) and one "Serum Mix Plate" (V-Bottom 96 well plate). In the latter varying serum dilutions will be mixed with constant amount of AAVs. These will then be transferred to the "Cell Plate." Column 1 of "Serum Mix Plate" contains enough solution for quadruplets (the assay is in triplicate) and will be transferred in three Columns of the "Cell Plate" (Columns 1–3) (Fig. 1c).

3.1.1 Day 0: Preparation of "Cell Plate" (See Note 1)

1. Take the flask with 293T cells (usually a confluent T75 is enough for one 96-well plate) from the incubator and place it under the hood.
2. Remove complete medium and add 10 ml 1× PBS. Wash the cells and remove medium.
3. Add 1.5 ml of trypsin–EDTA 0.05 % and incubate at 37 °C for 3–5 min, until the cells detach. Add 8.5 ml of complete medium and count the cells in a Neubauer chamber.
4. Transfer 1×10^7 cells (*see* Note 2) to a falcon tube and centrifuge at $300 \times g$ for 5 min. Remove all supernatant carefully and resuspend in 5 ml DMEM, P/S (*see* Note 3).
5. Using a multichannel pipette, transfer 50 µl of cell suspension to each well of a 96-well plate. This corresponds to 1×10^5 cells/well (*see* Note 2). This will be the "Cell Plate."
6. Transfer plate to the incubator (37 °C, 5 % CO_2) and incubate for 4–5 h.

3.1.2 Day 0: Preparation of "Serum Mix Plate" (See Notes 4–6)—Part I: Serum Mix (Fig. 1a, b)

Bring all materials under the hood: V-bottom 96-well plate, DMEM, P/S, test serum, multichannel pipette, and AAVs (the virus preferably on ice, although it is quite stable at room temperature for short periods of time) (*see* **Notes 4** and **5**). The dilutions of the serum will be done on the vertical axis that is from row A to row G, and row H will have the no-serum control. Every column represents one assay, for example one AAV serotype with one test serum. One can test multiple AAVs with multiple sera at the same time. In this protocol we will describe only one assay for simplicity purposes, although several assays can be performed in parallel (four columns in a "Serum Mix Plate" can be used for a whole (12 columns) "Cell Plate"). The final volume per well in the "Serum Mix Plate" will be 80 µl, which corresponds to quadruplet (4 × 20 µl). 20 µl will be transferred to each of 3 wells in the "Cell Plate" for a triplicate, leaving some serum-AAV mix in the "Serum Mix Plate" to account for pipetting losses. In the "Serum Mix Plate" half of this volume (40 µl of 80 µl) will be serum dilutions and the other

half will be the AAV solution (40 µl of 80 µl). For a visual description of the assay, please refer to Fig. 1.

1. Using the multichannel pipette, pipette 40 µl of DMEM, P/S to each well in column 1.
2. To well A1 (row A) pipet 40 µl of test serum and mix by pipetting three times. The volume now is 80 µl.
3. Transfer 40 µl from well A1 to well B1 (row B) and mix by pipetting three times. A1 contains now 40 µl: 20 µl of test serum and 20 µl of DMEM, P/S. The volume in B1 now is 80 µl.
4. Transfer 40 µl from well B1 to well C1 (row C) and mix by pipetting three times. B1 contains now 40 µl: 10 µl of test serum and 30 µl of DMEM, P/S. The volume in C1 now is 80 µl. Continue until row G. From well G1 remove 40 µl of the last 80 µl and discard.

3.1.3 Preparation of "Serum Mix Plate" (See Notes 4 and 5): Part II: Virus Addition and Incubation (Fig. 1b)

Each well of the "Cell Plate" contains 1×10^5 cells. In order to achieve an infection of 1000 vg/cell (*see* **Note 2**), one needs to add 1×10^8 vg/well. Each well of the "Serum Mix Plate" contains a mix for quadruplet infection (3× for each well of the "Cell Plate" and one extra), which corresponds to 4×10^8 vg/well. In total, for column 1 one needs 3.2×10^9 vg ($8 \times 4 \times 10^8$) in a volume of 320 µl DMEM, P/S (8×40 µl).

1. Prepare AAV-Luc mix in DMEM, P/S: 3.2×10^9 vg in 320 µl of DMEM, P/S in a 1.5 ml tube. Mix well by pipetting and inverting the tube.
2. Transfer 40 µl of the mix to each well of column 1 in "Serum Mix Plate" and mix by pipetting.
3. Transfer "Serum Mix Plate" to the incubator and incubate for 30 min at 37 °C.

3.1.4 Transfer of Test Serum/AAV Mix to Cell Plate (Fig. 1c)

1. Place both "Serum Mix Plate" and "Cell Plate" in the sterile hood.
2. Using the multichannel pipette, take 20 µl from each well of column 1 of "Serum Mix Plate" and transfer it to column 1 of "Cell Plate." In order to minimize damage to the cells, do not pipette directly onto the cells, but release the drop on the side of the wall of the well and let it drop. Swirl gently to mix. Repeat for columns 2 and 3 of "Cell Plate."
3. Transfer "Cell Plate" to the incubator and incubate at 37 °C with 5 % CO_2 for 2 days (*see* Note 7).

3.1.5 Day 2: Luciferase Assay (See Notes 8–12)

1. Prepare working solutions.
2. Remove the plate from the incubator. The rest of the procedure can be done on the working bench.

3. Lysis: Using a multichannel pipette, transfer 70 µl of 2× Lysis Buffer to each well and mix by pipetting. Incubate at room temperature with gentle rocking for 10 min.
4. Mix with substrate: transfer 20 µl of cell lysate to the luminometer plate using the multichannel pipette. Transfer 100 µl of reaction mixture and mix (*see* Note 8).
5. Read plate in the Luminoskan™ Ascent Microplate Luminometer.

3.2 Analysis of nAb Assay Data

3.2.1 Calculation of Mean Value and Standard Deviation of "Percent of Transduction to No-Serum Control" (See Notes 13 and 14)

1. Percent of Transduction to no-Serum Control, mean value: (average of RLU (Relative Luciferase Units) values of sample – average of RLU values of negative control)/(average of RLU values of no-serum control – average of RLU values of negative control) × 100.
2. Percent of Transduction to no-Serum Control, standard deviation: (standard deviation of: RLU values of each sample – average RLU value of negative control)/(average of RLU values of no-serum control – average of RLU values of negative control) × 100.

3.2.2 Determination of the nAb Titer

1. The titer is determined as the highest serum dilution in which more than 50% of inhibition is observed, in other words the first titer in which the percent of transduction to no-serum control is less than 50% (*see* Notes 13 and 14).

4 Notes

1. Cell line: Several lines can be used for this assay, such as 293T, C2C12, HeLa, CHO, Huh7, HepG2 etc. The cell line does not affect the output of the assay [18], as the assay is based on comparison to an internal control. Most laboratories use typically the 293T cell line, therefore the described experiments are performed with 293T. Even though some serotypes, such as AAV8 and 9, inefficiently transduce this cell line, the RLUs (Relative Luciferase Units) provide a sufficient range of values for conclusive results. One could choose another line, but one should also consider the cost and labor intensity.
2. Cell number: the cell number can greatly influence the transduction output in terms of RLUs (Fig. 2). It is recommended to use high numbers, so as to achieve confluence of 90%. This maximizes the number of cells available to be transduced, thereby reducing the viral genomes/cell needed for a reasonable output. The lower the viral genomes/cell ratio is, the higher the sensitivity of the assay [18].

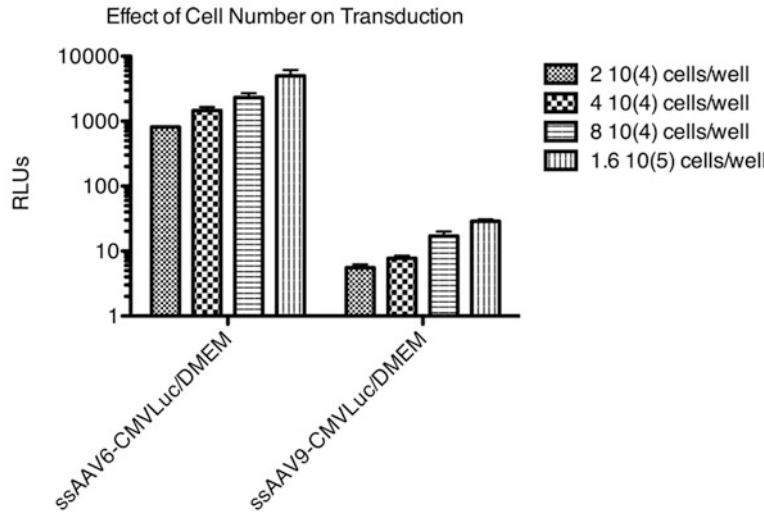


Fig. 2 Effect of cell number on transduction efficiency of AAV6 and AAV9: Serial dilutions of 293T cells were plated and were infected with 1×10^8 vg of each serotype. The luciferase measurements were performed 24 h later. There is a positive correlation between cell number and luciferase output, as measured in RLUs

3. The DMEM, P/S solution has to be pre-warmed at 37 °C. The cells are plated without serum, as serum (FBS, pooled mouse serum [8]) can affect transduction of different serotypes (Fig. 3). Since there is no serum in the medium, the cells are relatively sensitive. In addition, since they will also be used within hours, plating efficiency needs to be maximized. It is therefore recommended to pre-warm the DMEM, P/S for at least 30 min. It is also recommended to make aliquots of 15 ml in 15 ml Falcon tubes for single use. This way the medium is minimally exposed to air and thus preserved better (the pH value is not altered).
4. Virus purification: It is important that the AAV content is as high as possible in full (viral genome containing) capsids. The iodixanol purification methodology provides a simple and efficient system for the purification of AAVs from cell debris, non-assembled capsids and non-packaged DNA and for the enrichment of full as opposed to empty capsids. However, attention should be paid to the separation of full from empty capsids, as higher empty capsid content leads to an underestimation of the nAb titer. It is therefore advised to collect fractions from the iodixanol gradients that are expected to contain the highest encapsidated viral genome content. It should be pointed out that at the 25–15 % iodixanol layers one will detect non-packaged DNA, but not AAV viral capsid packaged DNA (Fig. 4) [19, 20].

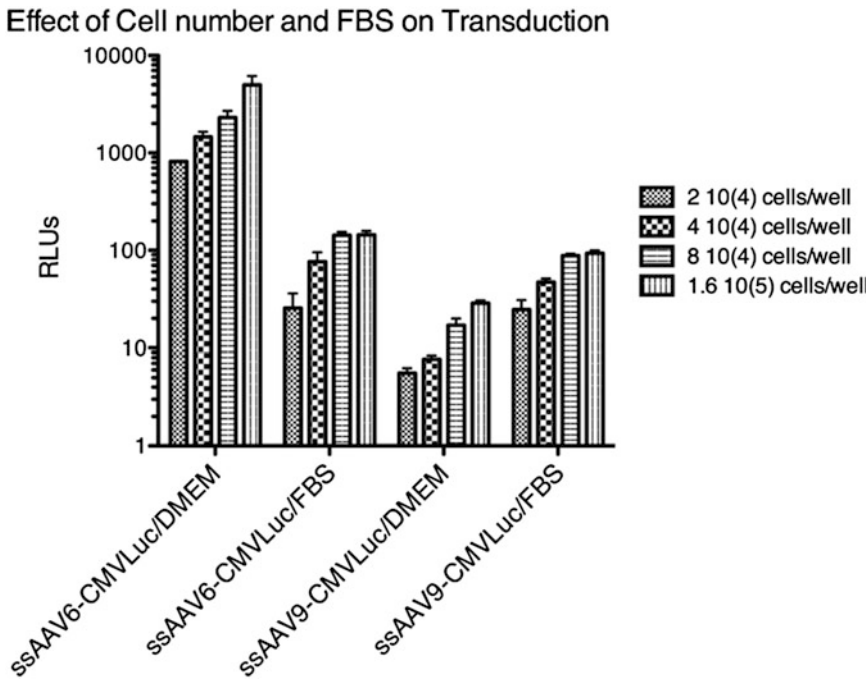


Fig. 3 FBS inhibits transduction by AAV6 but enhances transduction by AAV9: Serial dilutions of 293T cells were plated and were infected with 1×10^8 vg of each serotype in the absence or presence of 20 μl of FBS. The luciferase measurements were performed 24 h later

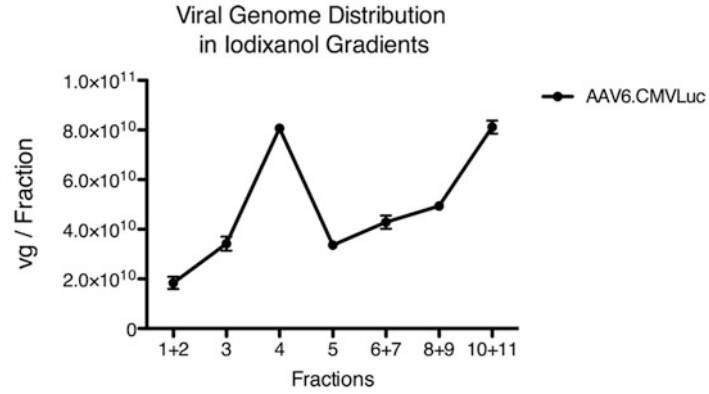


Fig. 4 Viral genome distribution of AAV6 in iodixanol gradients. The cell lysate of 293T cells transfected for the production of AAV6 (double plasmid transfection as described before [8]) was loaded on an iodixanol gradient (2 ml 15 %, 2 ml 25 %, 2 ml 40 %, and 2 ml 60 %[8]). Fractions of 0.5 ml were collected starting from 60 % towards 25 %. The majority of the viral genomes was concentrated in fraction 4 (corresponds to the top part of 60 % fraction) and it was collected for experiments. Fractions 5 and higher [19, 20] contain higher amounts of empty capsids and should be avoided. Fractions 10 or higher contain unpackaged viral genomes and plasmids and should be avoided (*see* **Note 4**)

5. Transgene: The choice of transgene is critical for the assay, as it can influence the sensitivity [18]. Luciferase (preferably under the control of a strong promoter, such as CMV) is the best/suggested system of choice, as it is highly sensitive, it has a high dynamic range (up to 5 orders of magnitude in most luminometers), and it is easy to measure (30 min to prepare and read an entire plate).
6. Heat inactivation of test serum: several reports propose the heat inactivation of the complement components of the serum. Deposition of activated complement components on the viral capsid results in enhanced uptake by specific cell types, such as CD4+ [21]. However, the cell lines typically used in the assays described herein are not affected by complement activation and heat inactivation of serum has no effect on the output of the assay (data not shown and [18]).
7. Transduction efficiency: some serotypes may not transduce certain cell types efficiently. However, the appropriate cell lines, for example cardiac myocytes for the cardiotropic AAVs, may be costly and time consuming. There are several technical improvements that have been explored by individual researchers.
   (a) Days of incubation: incubation of 2 days would give sufficient time for"slow" transducing serotypes, such as AAV9, to maximize the output expression. One should consider however the effect on cell viability due to prolonged culture.
   (b) The simplest way to perform the assay is to dilute the test serum in DMEM, P/S. The first row (A) has the highest content of test serum, which is then gradually reduced up to row G, whereas the no-serum control contains no serum. Serum and the factors therein affect, however, cell viability and function, which are necessary for viral transduction. This practically means that the transduction efficiency, which is reflected by the luciferase activity levels, will be higher in the wells with test serum compared to no-serum control, assuming of course that the serum does not contain antibodies (the wells in row A would have transduction efficiencies of more than 100%). The test serum from a specific species can also be diluted in serum from the same species, but attention should be paid to the fact that the serum may contain antibodies [8]. There are commercial kits to remove antibodies from serum. Another alternative is also commercially available immunoglobulin depleted serum (Immunoglobulins (all) Depleted Serum, SF505-2, BBI Solutions). However this does not preclude the presence of other factors that affect transduction efficiency [16, 17]. All these alternatives should be tested before the assay is performed.

(c) Addition of FBS 24 h after infection for incubation times longer than 2 days: an alternative to increase the transduction efficiency is to increase the incubation time to more than 2 days, as there are several rate limiting steps in AAV transduction [22]. Addition of FBS at 10–15% several hours after infection, so that the transduction efficiency is not affected as seen with AAV6 (Fig. 3), can also improve the output of the assay. Even though, as shown in Fig. 5, the mean RLU values with (24 h DMEM, P/S, addition of FBS to 15% for another 24 or 48 h) and without FBS at 48 h after infections are not affected, the variation in the wells, as indicated by the high standard deviation of the no-FBS sample, is reduced. That is because the viability of the cells in the no-FBS condition reduces with time, so in some of the wells the luciferase output was already decreasing at the time of the measurement. As shown in Fig. 5, incubation of the cells for longer times (3 days) decreases the efficiency of the assay.

8. Preparation of luciferase substrate: the luciferase substrate has a short half-life, which should be determined experimentally for each laboratory. Typically it is around 30 min. The plate reading requires about 5 min, so the order with which the luciferase substrate is pipetted into the luminometer plate is critical. It is recommended to pipette row A to row H and follow this order when setting up the luminometer.

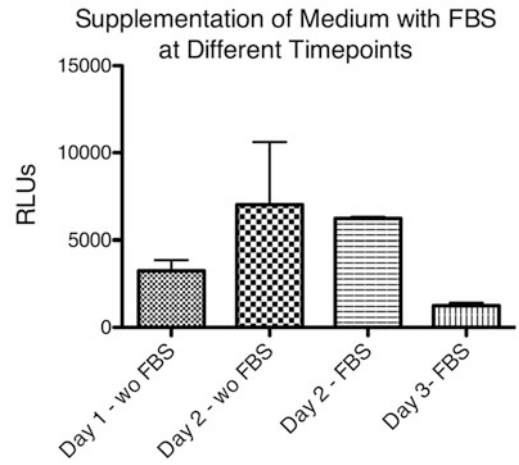


Fig. 5 Supplementation of medium with FBS 24 h after infection improves assay output. Cells were infected and 24 h later FBS was added and measurements were taken at 24, 48, and 72 h post infection (Day 0 of assay). Addition of FBS seems to stabilize the cells, which results in lower variability in luciferase assay output. However, luciferase assay output is reduced after 3 days in culture

9. Determination of dynamic range of relative luciferase units: before using a new luminometer, one needs to determine the dynamic range. To do so, one can simply use commercially available luciferase enzyme. Alternatively, simply transfecting 293T cells with different amounts of a plasmid carrying the CMV-Luc cassette should suffice. The higher range of the assay is determined, when lysates from cells transfected with several sequentially increasing amounts of DNA produce the same RLUs, which means a plateau has been reached. The lower range can be determined using no-transfection control.
10. Positive and negative controls: the no-serum control serves as a positive control, where the lack of serum with nAbs would produce maximum AAV transduction efficiency, high expression of luciferase in the cells, and RLU values in the assay. As a negative control, non-infected control cells are used. Both positive and negative controls should be included in every plate. If the transduction efficiency of a serotype typically exceeds the negative control by at least 3 orders of magnitude, then one can omit negative control. Additional controls for the assay can be used, particularly when first determining the sensitivity of the assay, such as pooled human serum (available through multiple vendors) or IVIG (intravenous immunoglobulins). These reagents are guaranteed to inhibit transduction, at least at the higher titer dilution and therefore should provide useful information to the inexperienced researcher.
11. Luciferase assay substrate: there are commercially available kits that are simpler to use, and probably more stable and with longer half-life compared to the system described here. However, for screening of multiple sera, the cost rises proportionally. Since this system is approximately 20–40-fold more economical, it is recommended to use this when planning to screen multiple samples.
12. Luminescence reader: there are various commercially available readers, with varying dynamic ranges. It is recommended to determine this range, prior to the experiments. In order for the assays to be comparable, the results are expressed as percent of transduction compared to no-serum control.
13. Range of serum dilutions: depending on the availability of serum and the level of inhibition (it is quite often that even a dilution of 1/256 will completely inhibit transduction) a range of higher serum dilutions can be chosen, such as 1/16 through 1/1024. In order to achieve this dilution, simply add 5 μl of test serum to 35 μl of DMEM, P/S at well A1 of "Serum Mix Plate" (Subheading 3.1.2, step 1) and continue with the dilutions from that point on.

14. Titer determination: if the transduction of the critical titer dilution is very close to the 50% (for example 49%), it is suggested to repeat the assay. In general, there is inter-assay variability. That is, repeating the assay with the same serum will sometimes not produce the same titer, but a neighboring one. It is also recommended to repeat the assays twice for sera with titers in the range of interest or cutoff.

Acknowledgment

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Chapter 8

Synthesis of Modified mRNA for Myocardial Delivery

Jason Kondrat, Nishat Sultana, and Lior Zangi

Abstract

Cardiac gene therapy shows tremendous promise in combating the growing problem of heart disease. Modified mRNA (modRNA) is a novel gene delivery system used in vitro or in vivo to achieve transient expression of therapeutic proteins in a heterogeneous population of cells. Incorporation of specific modified nucleosides enables modRNA to be translated efficiently without triggering antiviral and innate immune responses. ModRNA has been shown to be effective at delivering short-term robust gene expression to the heart and its use in the field of cardiac gene therapy is expanding. Here, we describe a stepwise protocol for the synthesis of modRNA for in vivo myocardial delivery.

Key words Modified mRNA, Myocardial infarction, Myocardial delivery, Gene therapy, In vitro transcription

1 Introduction

The field of Cardiac gene therapy is expanding, yet its use in the clinical setting is limited. Currently the most widely used method for targeting gene expression to the heart is through viral vectors, particularly the adeno-associated virus (AAV) vector [1–3]. Viral gene therapy shows promise yet its applications are limited due to its length of expression and inability to regulate gene expression in a quantifiable dose manner [1–3]. Modified mRNA (modRNA) is a novel and effective gene delivery method that provides short-term (1–2 weeks), titratable gene expression for use both in vitro or in vivo [4–9].

The use of unmodified exogenous RNA as a gene delivery method is ineffective due to its instability outside the cell and the strong innate immune response it elicits when transfected into cells [10, 11]. However, Kariko et al. discovered that the substitution of uridine and cytidine with pseudouridine and 5-methylcytidine, respectively, drastically reduced the immune response elicited from exogenous RNA [11, 12]. Investigation into the mechanism revealed that the nucleoside substitutions resulted in a conformational change in the RNA that caused reduced response

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by toll like receptors 3, 7, and 8 (TLR3, TLR7, TLR 8), and retinoic acid-inducible gene 1 (RIG-1) [13]. A further decrease in RIG-1 response from modRNA was seen upon removal of the 5′ triphosphates [4, 10]. In order to increase stability and translational efficiency, a 3′-O-Me-m7G(5′)ppp(5′)G Anti Reverse Cap Analog (ARCA) cap is substituted at the 5′ end of the RNA molecule [4, 5, 10].

The synthesis of modRNA for in vivo use involves four stages: DNA template creation containing the desired transcript, in vitro transcription (IVT), 5′ phosphate removal with Antarctic phosphatase, and precipitation with 5 M ammonium acetate salt. Investigation into the use of modRNA for experimental and clinical purposes is growing rapidly. Daily transfection with modRNA encoding reprogramming factors OCT4, SOX2, MYC, and KLF4 were successful at reprogramming human fibroblasts back to pluripotency [5, 8]. Additionally, modRNA has been shown to be capable of directing cell fate in vitro by using MyoD modRNA that resulted in the conversion of fibroblasts to skeletal muscle cells [2]. ModRNA has also shown promise in directing cell fate in vivo. We used VEGF-A modRNA to direct heart progenitor cells to induce vascularization in the heart [4]. The expanding use of modRNA technology in vivo and its potential use in the field of cardiac gene therapy motivated us to generate a step-wise, streamlined protocol for the effective synthesis of modRNA for in vivo use.

2 Materials

All solutions should be made in nuclease-free water unless otherwise specified. All materials used in this protocol should be nuclease free.

Equipment

1. PCR thermocycler.
2. Microfuge.
3. Vortex mixer.
4. Thermomixer (eppendorf).
5. NanoDrop.
6. Nuclease-free water.
7. 15 ml nuclease-free conical tubes.
8. Nuclease-free strip PCR tubes.
9. Ethanol (100 and 70%).
10. 2 ml Ambion Elution Tubes.

Primers for Tail PCR

Forward Primer: 5′-TTG GAC CCT CGT ACA GAA GCT AAT ACG-3′

Reverse Primer: 5′-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TCT TCC TAC TCA GGC TTT ATT CAA AGA CCA-3′

2.1 Construction of DNA Template for In Vitro Transcription Using pTEMPIz Plasmid

1. T4 polynucleotide kinase enzyme.
2. 100 mM ATP.
3. 2× KAPA HiFi HotStart ReadyMix PCR master mix.
4. *AleI* enzyme.
5. *AfeI enzyme*.
6. Antarctic phosphatase enzyme.
7. T4 DNA ligase enzyme.
8. One Shot® ccdB Survival™ 2T1 Phage-Resistant (T1R) cells.
9. QIAquick® gel extraction kit.
10. QIAquick® PCR purification kit.
11. One shot chemically competent *E. coli*.
12. QIAprep® spin Miniprep kit.
13. 10× Phosphorylation Buffer.

2.2 Synthesis of Linear DNA Template with a Poly T Tail for IVT Reaction

1. 2× KAPA HiFi HotStart ReadyMix PCR master mix.
2. Primer Solution: 1 μM each of forward and reverse primer.
3. *DpnI* enzyme.
4. QIAquick® PCR purification kit.

2.3 In Vitro Transcription Reaction

1. Ambion T7 Megascript® Kit (Life Technologies Cat#: am1334-5).
2. GTP 75 mM solution (provided in Megascript® kit).
3. ATP 75 mM solution (provided in Megascript® kit).
4. Pseudouridine-5′-triphosphate 100 mM solution.
5. 5-methylcytidine-5′-triphosphate 100 mM solution.
6. Trilink Biotechnologies Anti Reverse Cap Analog, 3′-O-Me-m^7G(5′)ppp(5′)G 10 μmol (Cat #: N-7003).
7. T7 TURBO DNase enzyme (provided in Megascript® kit).
8. Ambion MEGAclear™ Transcription Clean-Up kit (Life Technologies; cat#: AM1908).

2.4 RNA Phosphatase Treatment

1. Antarctic phosphatase enzyme.

2.5 RNA Precipitation

1. 5 M ammonium acetate salt solution (Provided in AmbionMEGAclear™ Kit).
2. Elution Buffer (Provided in AmbionMEGAclear™ Kit).

2.6 Preparation for modRNA Injection

1. Lipofectamine®RNAiMAXtransfection reagent (Thermofisher Cat#: 13778150).
2. OptiMEM Reduced Serum Medium, no phenol red.
3. Ultra-Fine insulin syringe needle 31 g 8 mm.

3 Methods

Carry out all procedures at room temperature, in a non-sterile environment unless otherwise specified. All materials used should be nuclease free.

3.1 Construction of DNA Template for In Vitro Transcription Using pTEMPlz Plasmid

1. The DNA template for IVT is constructed by cloning 5′- and 3′-UTRs into pZErO-2, a vector backbone with resistance to ampicillin, to generate pTEMPlz. pTEMPlz is a cloning vector into which an ORF of interest can be inserted between the UTR's (Fig. 1).
2. The 5′- and 3′-UTRs are synthesized de novo by synthetic oligos. The synthesized UTR's are annealed together and amplified using forward and reverse primers. To provide an

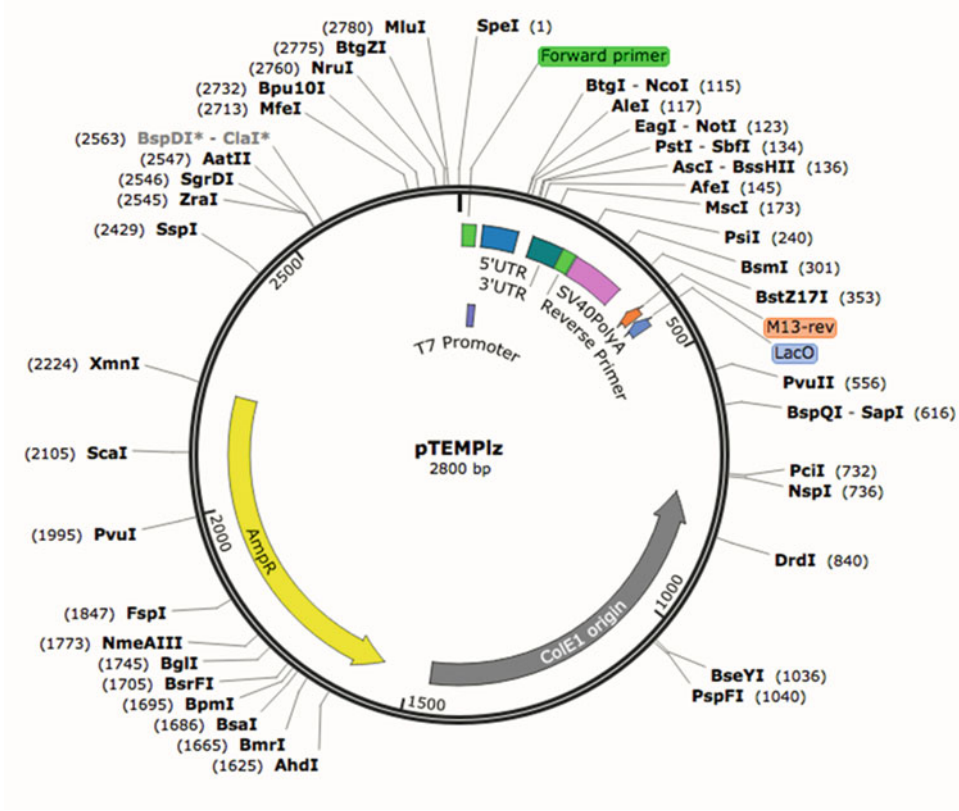


Fig. 1 Plasmid map of pTEMPlz

entry site for the ORF, *AleI* and *AfeI* restriction sites are introduced in between 5′ and 3′ UTRs (*see* **Note 1**).

3. The PCR-amplified fragment and pZErO-2 vector with ampicillin resistance are digested with *HindIII* and *NotI*, and ligated together to create pTEMPlz (*see* **Note 2**).
4. Before insertion into pTEMPlz, the ORF is amplified by using phosphorylated forward and reverse primer pair for the gene of interest. Phosphorylation of the primers is done using the T4 polynucleotide kinase enzyme according to reaction below:

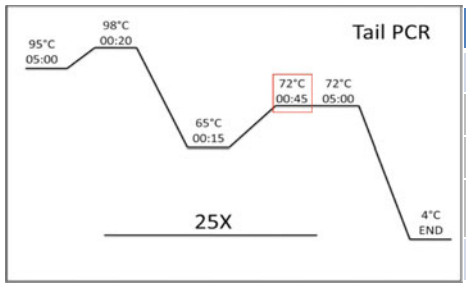
10× Phosphorylation Buffer	5 µl
Forward primer (100 µM)	3 µl
Reverse Primer (100 µM)	3 µl
100 mM ATP	0.5 µl
T4 polynucleotide kinase	10 U
Nuclease-free water	50 µl

Incubate reaction at 37 °C for 1 h.

5. To inactivate enzyme, incubate reaction at 65 °C for 20 min. Dilute reaction to 300 µl by adding 250 µl of water giving final 1 µM primer mixture.
6. Amplification of the ORF of interest is done using the PCR reaction below:

Primer mix (1 µM) from above	10 µl
Template DNA	1–100 ng
Water	50 µl
HiFi HotStart ready mix (2×)	25 µl

Run mixture in Thermocycler with settings according to Fig. 2.



Step	Temp	Time	# of cycles
Initial denature	95°C	5 min	1
Denaturation	98°C	20 sec	25
Primer anneal	65°C	15 sec	
Extension	72°C	30 sec/kb ORF insert	
Final extension	72°C	5 min	1

Fig. 2 PCR setting for synthesizing DNA tailed template. Highlighted in *red* is the elongation step that must be set based on the size of the sequence insert. Elongation step requires 30 s per KB of ORF insert. PCR setting is based of manufacturer instructions from 2× KAPA HiFi HotStart ReadyMix kit

7. Isolate the amplified target using QIAquick® gel extraction kit.
8. Before insertion of ORF, pTEMPlz is linearized and dephosphorylated. To linearize plasmid, digest pTEMPlz with *AleI* and *AfeI* according to the reaction below:

pTEMPlz Plasmid DNA	2 μg
Nuclease-free water	30 μl
10× Buffer 4	3 μl
AleI	5 U
AfeI	5 U

Incubate in thermomixer for 1 h at 37 °C. Purify digest using QIAquick® PCR purification kit and elute in 30 μl of elution buffer.

9. Dephosphorylate linearized pTEMPlz according to reaction below:

Linearized plasmid from **step 1**	30 μl
10× antarctic phosphatase buffer	5 μl
Antarctic phosphatase	5 U
Nuclease-free water	50 μl

Incubate reaction at 37 °C for 1 h. Inactivate enzyme by incubating at 65 °C for 15 min.

10. Isolate linearized and dephosphorylated plasmid using QIAquick® gel extraction kit. Determine the quantity of pTEMPlz product using NanoDrop. Plasmid can be stored in −20 °C for future use.
11. Perform blunt end ligation of ORF of interest into pTEMPlz according to the reaction below:

Linearized dephosphorylated TEMPlz plasmid	50 ng
Amplified ORF	Threefold molar excess
10× T4 DNA ligase buffer	2 μl
T4 DNA ligase	4 U
Nuclease-free water	20 μl

Mix reagents and incubate overnight on melting ice at room temp or at 16 °C. Negative control ligation reaction might be necessary to monitor self-ligation of plasmid.

12. Perform transformation of plasmid with competent cells and grow on an ampicillin agar plate.

13. To isolate positive clones with correct orientation perform colony PCR. Extract between 8 and 10 colonies from ampicillin agar plate with a pipette tip. Stab individual tips in 200 µl of Luria Broth (LB) and rinse several times in 75 µl of TE buffer under pH 8.0, and incubate tips in 37 °C in a shaker. Tubes are then boiled for 5 min to lyse bacteria and spin to pellet debris. Take 2 µl of supernatant and perform colony PCR using forward primer and gene specific reverse primer. Run PCR sample on 1 % agarose gel to identify clones with positive orientation.
14. Culture 200 µl of LB with correct orientation clones in larger volume of LB overnight in a 37 °C shaker and extract using QIAprep® spin Miniprep kit.
15. Determine the quantity of plasmid product using NanoDrop and dilute to a concentration between 1 and 5 ng/µl.

3.2 Synthesis of Tailed DNA Template

1. Create a 1600 µl PCR master solution according to the reaction below:

Plasmid solution (1–5 ng/µl) (*see* **Note 3**)	400 µl
Primer solution (1 µM primers)	400 µl
2× KAPA HiFi HotStart ReadyMix	800 µl

   Aliquot 50 µl of PCR master solution into 32 separate PCR tubes.
2. Run PCR using the thermo cycler (setting listed in Fig. 2). Please note that length of elongation step will vary depending on DNA polymerase used and ORF length (*see* **Note 4**).
3. To digest methylated plasmid DNA, combine product into one eppendorf tube and digest with 30 µl of *DpnI*.
4. Purify the PCR using QIAquick PCR Purification Kit (Qiagen cat #: 28106) and elute final product in nuclease-free water.
5. Measure concentration of tailed product using NanoDrop machine and adjust concentration using nuclease-free water to 100–200 ng/µl.
6. For quality control analysis, check purity of Tailed DNA template product on a 1 % agarose gel together with the original DNA plasmid (Fig. 3a).

3.3 In Vitro Transcription (IVT) Reaction (1 ml Reaction Volume)

1. Prepare a custom NTP's in one eppendorf tube according to Table 1.
2. Mix reagents for IVT reaction in the following order into one eppendorf tube:
   (a) 400 µl of custom NTP's from Table 1.
   (b) 400 µl of the DNA tailed template (200 ng/µl).

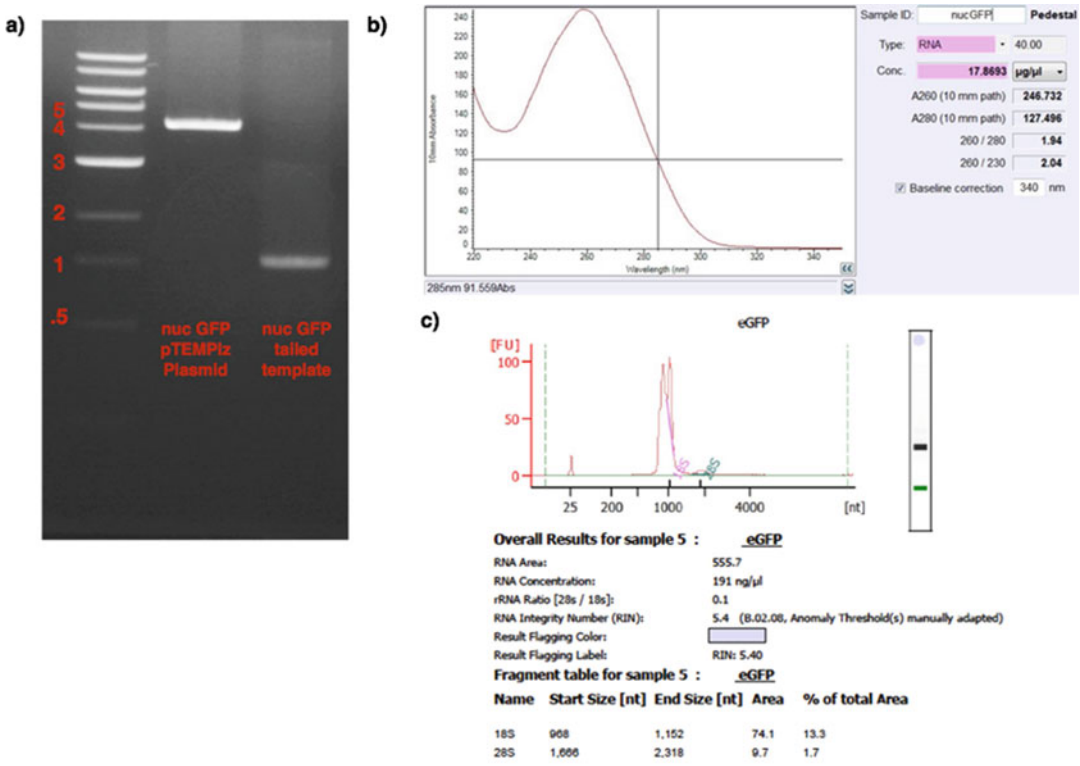


Fig. 3 Quality Control analysis for modRNA synthesis. (**a**) 1 % agarose gel determining correct size of the plasmid pTEMPlz with ORF insert and tailed DNA template for IVT. (**b**) Ideal NanoDrop result of final modRNA product. Ideal concentration is between 15 and 20 μg/μl. 260/280 values closer to 2 indicate purity. (**c**) Bioanalyzer result for quality control of synthesized modRNA

Table 1
Instructions for custom NTP master solution preparation. Add all NTPs and water into one eppendorf vial

Nucleotide	Stock concentration (mM)/stock amount (μmol)	How much to add (μl)	Final concentration (mM)
ARCA	10 μmol	Use entire Trilink vial	6
GTP	75 mM	36 μl from Ambion kit	1.5
ATP	75 mM	183 μl from Ambion kit	7.5
5-methylcytidine	100 mM	138 μl from Trilink vial	7.5
Pseudouridine	100 mM	138 μl from Trilink vial	7.5
Nuclease-free water	N/A	205 μl from Ambion kit	–

(c) Vortex 10× Reaction Buffer from the T7 megascript kit to dissolve any precipitate and add 100 µl.

(d) Add 100 µl of T7 Enzyme. This will give you a 1 ml IVT reaction.

(e) Mix thoroughly and incubate in thermomixer at 37 °C for 4–6 h.

3. Add 30 µl of T7 Turbo DNase and mix gently. Incubate at 37 °C in Thermomixer for 15–20 min to halt the reaction (*see* **Note 5**).
4. Purify reaction using Ambion MEGAclear™ Transcription Clean-Up kit and elute each tube three times with 50 µl of 95 °C elution buffer to obtain 150 µl of RNA product in each tube.
5. Combine the RNA mixture from each tube into one eppendorf tube.

3.4 RNA Phosphatase Treatment

1. Add nuclease-free water to the RNA you extracted to obtain a 1.5 ml solution.
2. Add 150 µl of Antarctic Phosphatase Buffer (10×) and 150 µl of Antarctic Phosphatase enzyme.
3. Mix thoroughly and incubate in thermomixer at 37 °C for 1 h.

3.5 RNA Precipitation Using Ammonium Acetate

1. Transfer the 1800 µl RNA solution to a 15 ml conical tube.
2. Add 180 µl of 5 M Ammonium Acetate and mix thoroughly.
3. Add 5200 µl of cold (−20 °C) 100% ethanol to solution and aliquot into 3–4 2 ml eppendorf tubes.
4. Let tubes stand in −20 °C overnight.
5. Centrifuge tubes at 10,000 rpm for 30 min at 4 °C. Carefully discard supernatant.
6. Dissolve each pellet in 500 µl of 70% ethanol. Consolidate modRNA ethanol solutions from each tube into 1 eppendorf tube.
7. Centrifuge tube at 10,000 rpm for 30 min at 4 °C.
8. Gently pour out and discard supernatant, and using a Kimwipes, gently clean the inside of the tube. Take care not to disturb the pellet. Invert tube and let stand for no more than 2 min to air-dry pellet.
9. Using a pipette, gently take out any small drops of ethanol left around the pellet.
10. Resuspend pellet using 45–50 µl of elution buffer.
11. Leave modRNA in elution buffer for 5 min then gently pipette till the pellet is dissolved.
12. RNA solution can now be used in vivo, stored in −20 °C for up to 6 months, or −80 °C for 5 years.

3.6 ModRNA Yield

1. Measure concentration using NanoDrop machine (Fig. 3b). The ratio of A_{260}/A_{280} should be greater than 1.8 with values closer to 2.0 indicating higher purity. Depending on yield, concentration should be close to 20 μg/μl.
2. For better quality control analysis take a 1 μl sample from the final modRNA solution and dilute it into 100 μl of nuclease-free water. Analyze sample using a bioanalyzer machine (Fig. 3c).

3.7 Preparation of modRNA for Myocardial Injection in Mice

1. Combine 40 μl of RNAiMax and 5 μl of OptiMEM in one eppendorf tube and vortex. Let mixture sit for 10 min at room temperature.
2. In another eppendorf tube combine 150–200 μg (*see* **Note 6**) of modRNA and 5 μl of OptiMEM. Spin down to eliminate liquid on the sides of the tube.
3. After letting the RNAiMAX and OptiMEM mixture sit for 10 min at room temperature, add the liquid from the tube with the modRNA mixture to the tube with the RNAiMAX mixture (*see* **Note 7**).
4. Let combined mixture stand for 15 min at room temperature. Extract mixture into the 31 G insulin syringe and inject into mouse myocardium (Example of result shown in Fig. 4).

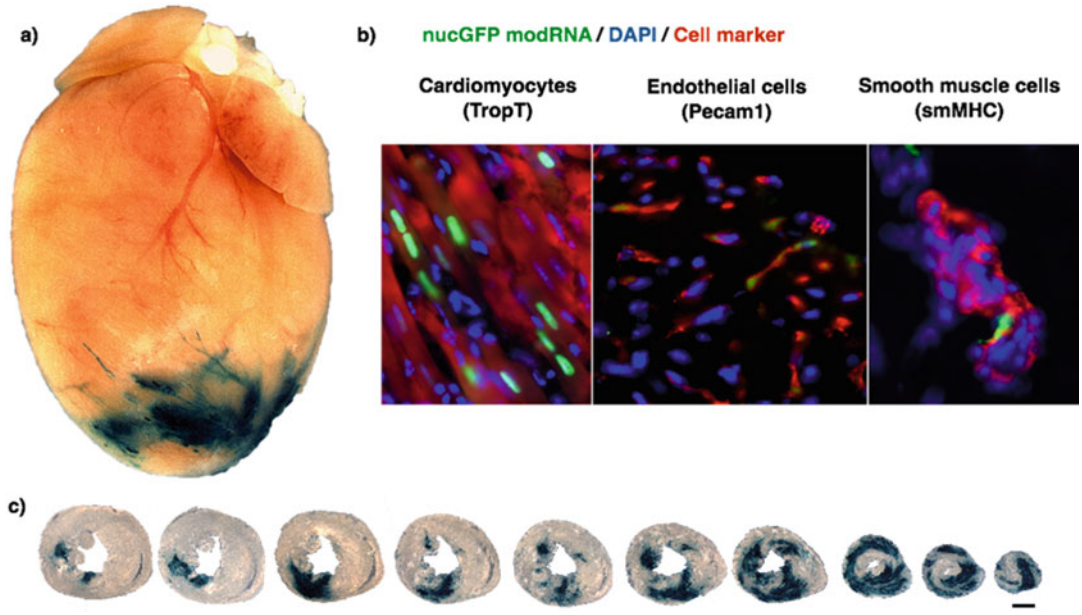


Fig. 4 (**a**) Whole heart view of mouse heart injected in vivo with modRNA encoded with LacZ gene. 24 h after injection, the mouse was sacrificed, the heart was fixed with 4 % PFA, and stained with x-gal. (**b**) immunostaining of mouse heart injected in vivo with modRNA encoded with nuclear GFP. (*left*) Cardiomyocytes (TropT: *Red*), Endothelial cells (Pecam1: *Red*) and smooth muscle cells (smMHC: *Red*) positive for nuclear GFP (*Green*). (DAPI: *Blue*). (**c**) X- cross section of Rosa26 LacZ mouse heart injected with modRNA encoded with Cre Recombinase. Transfected cells with Cre Recombinase can be stained with x-gal resulting in *dark blue* color

4 Notes

1. The adenine nucleotide (A) of the first codon (ATG) should be omitted from forward primer sequence as it is provided by the *AleI* site.
2. pTEMPlz plasmid and derivatives should be propagated in bacterial strain resistant to the ccdB gene product such as One Shot® ccdB Survival™ 2T1 Phage-Resistant (T1R) cells.
3. Transformation of plasmid in competent cells such as One shot® TOP10 chemically competent *E. coli* might be necessary to attain a proper amount of DNA plasmid needed to create enough tailed DNA template for the IVT reaction.
4. If using 2× KAPA HiFi HotStart ReadyMix, elongation step of Thermocycler should be set at a ratio of 30 s per Kb of ORF length.
5. At this point uncleaned modified mRNA can be stored in −80 °C up to 2 weeks to be cleaned at a later date.
6. If concentration is 20 μg/μl, then add 10 μl.
7. It is important to add the modRNA mixture to the RNAiMAX mixture and not the other way around.

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Chapter 9

Exosomes-Based Gene Therapy for MicroRNA Delivery

Prabhu Mathiyalagan and Susmita Sahoo

Abstract

Despite recent advances in scientific knowledge and clinical practice, cardiovascular disease management and treatment remain a major burden. While several treatment strategies using drugs and surgeries are being developed for cardiovascular manifestations, gene-based therapies hold significant promise. Recent findings from our laboratory unveiled a novel mechanism that exosomes, secreted nanovesicles from stem cells, mediate cardiac repair via transferring their unique repertoire of microRNAs (miRNA) to recipient cells in the heart. Exosomes, unlike other vectors for gene delivery, present unique advantages such that exosomes are a cell-free natural system for ferrying RNA between cells, robust exosomal membrane can protect the RNA/gene of interest from digestion, and exosomes are rapidly taken up by target cells making them a more efficient vehicle for gene delivery. Here, we describe a stepwise protocol developed in our laboratory for generating exosomes from human $CD34^+$ stem cells that carry exogenously applied Cy3 dye-labeled pre-miR miRNA precursors. We demonstrate that human $CD34^+$ stem cell exosomes can rigorously enter into recipient cells and deliver Cy3 dye-labeled pre-miR miRNA precursors to regulate gene expression. Identification of key molecular targets to treat disease conditions is the foremost critical step and the novel approach presented here to generate exosomes carrying exogenous genetic information offers a valuable clinical tool for more effective treatment strategies.

Key words Gene therapy, $CD34^+$ stem cells, Exosomes, Microvesicles, MicroRNA

1 Introduction

Almost after three decades since first report, exosomes are now recognized as vital mediators of cell–cell communication. Exosomes are extracellular vesicles of endocytic origin secreted by almost every cell type in humans and typically range in 30–100 nm size [1]. Exosomes carry macromolecules including lipids, proteins, and nucleic acids (mainly RNA) and their composition reflects their parent cell [2]. In the past few years, the exosomes field has grown exponentially both in the aspect of fundamental cell biology as well as pathologies involving cancer and cardiovascular diseases [3]. Indeed, secreted exosomes containing biologically active macromolecules can selectively bind to cell surface receptors presented by recipient cells. Upon receptor binding, exosomes are thought to transduce specific

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intracellular signaling thereby inducing physiological changes in recipient cells [4]. Another mechanism by which exosomes mediate biological information is through direct transfer of intra-exosomal content such as mRNA and microRNA (miRNA or miR) inside recipient cells by fusion with cell membrane [5]. Herein, in-depth understanding of exosomes-mediated biological information transfer is a critical step towards gaining deeper insights to exosomes function in both physiology and pathophysiology.

MiRNAs are critical regulators of gene expression at the post-transcriptional level in higher eukaryotes [6]. Regulation of miRNA expression is tightly coordinated in a developmental and tissue-specific fashion [7]. Perturbations to miRNA expression lead to severe developmental anomalies [8]. Several miRNAs have been implicated to have role in chronic diseases and conditions such as cancer, heart and neurodegenerative diseases [9]. Consequently, miRNAs are regarded as potential pharmacological candidates for therapeutic treatment as well as for biomarker discoveries in heart failure [10]. For instance, expression of miRNA is deregulated under pathological heart conditions and a major pharmacological interest is to restore endogenous miRNA expression either through the use of miRNA "mimics" or "inhibitors" under disease states [11, 12]. However, a major limitation is the lack of efficient cell-specific gene delivery system as well as off-target toxicity effects. Adeno-associated virus (AAV) is one of the efficient delivery methods for gene therapy that has been widely in practice for long time. In the treatment of heart failure, AAV-mediated gene delivery for genes implicated in cardiac contractility has significant clinical value [13]. A major limitation in using viral vectors is the existence of endogenous neutralizing antibodies in human sera. In addition, viral-mediated vectors are prone to entering of undesired cell and tissue types when introduced intravenously or intramuscularly. To successfully achieve high efficient delivery of key genes for therapeutics, choice of reliable gene delivery method is the foremost critical step. Our approach to overcome such limitations is through modulation of exosomes by targeted approach as a reliable gene delivery system for the expression and function of miRNAs in recipient cells. Indeed, exosomes offer unique advantage over several gene delivery systems for at least two reasons. One of that is exosomes are natural carriers of biological information in the form of miRNA and can deliver miRNA content to specific cell types via receptor-mediated binding. Secondly, exosomes are rapidly taken up by recipient cells, therefore minimal off-target effects are exhibited through systemic circulation.

Recent discoveries highlight that exosomes selectively carry miRNAs in abundance and mediate crosstalk between different cell types by direct transfer of miRNA in to recipient cells [14]. For example, human $CD34^{+}$ stem cell-derived exosomes carry miRNAs that are implicated in proangiogenesis and ischemic tissue repair [15].

This natural exosomes-mediated communication can be harnessed to deliver exogenous genetic material to further enhance the success and outcomes of gene therapy [16]. In this Chapter, we will demonstrate how exosomes can be modulated using exogenous Cy3 dye-labeled pre-miR miRNA precursors, which are miRNA mimics that will enable direct transfer of pre-miR miRNA precursors into recipient cells via exosomes uptake (Fig. 1). Throughout this protocol, we use Cy3 dye-labeled pre-miR miRNA precursors

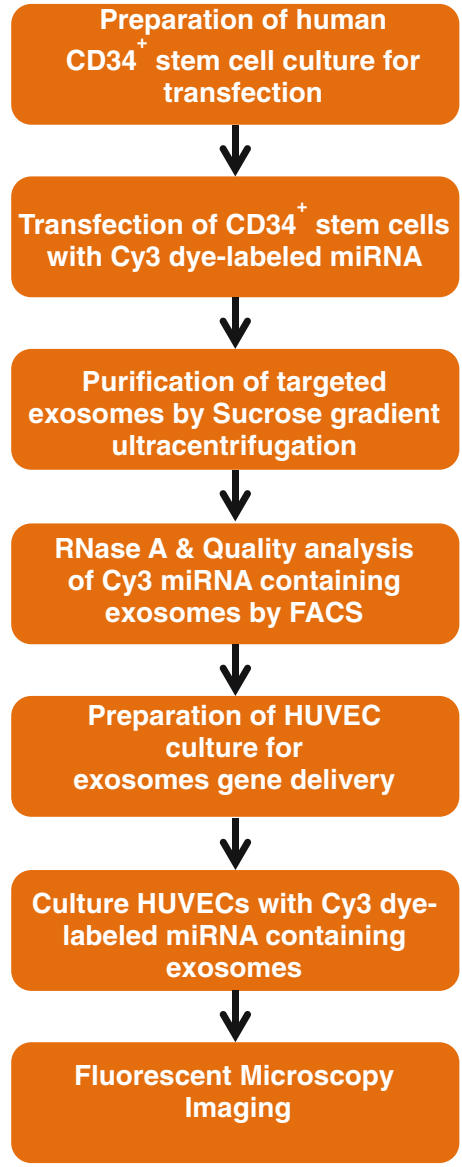

Fig. 1 Workflow depicting critical stages involved in exosomes-mediated miRNA gene delivery

to monitor exosomes uptake in real time using fluorescent imaging technique. We further demonstrate using flow cytometry analysis that human $CD34^+$ stem cell-secreted exosomes predominantly carry Cy3 dye-labeled pre-miR miRNA precursors. We then show using fluorescent imaging that human umbilical vein endothelial cells (HUVEC) specifically uptake $CD34^+$ stem cell exosomes containing Cy3 dye-labeled pre-miR miRNA precursors.

2 Materials

All aqueous solutions, buffers, reagents, H_2O, tips, and pipettes used in this protocol are molecular biology grade, sterile, and RNase/DNase-free certified.

2.1 General Laboratory Materials

- 15 and 50 ml polypropylene conical tubes.
- 10 and 25 ml serological pipettes.
- RNase/DNase-free pipettes and tips.
- Sterile non-latex gloves.
- Sterile RNase/DNase-free 1.5 ml eppendorf tubes.
- RNaseZap Wipes.
- 70% ethanol.
- Alcohol wipes.

2.2 Cell Culture

- Human $CD34^+$ stem cells isolated from peripheral blood of GCSF-mobilized individuals.
- GFP-expressing human umbilical vein endothelial cells (HUVEC).
- X-VIVO 10 medium.
- Human serum albumin.
- Human recombinant FLT-3 ligand protein (FLT-3L).
- Human recombinant stem cell factor protein (SCF).
- Human recombinant vascular endothelial growth factor (VEGF).
- Phosphate buffered saline (1×) without Ca^{2+} and Mg^{2+}.
- T25 and T75 cell culture flasks (cell culture treated).
- 24-well culture plates.
- Trypan blue.
- Hemocytometer.
- EBM2 media with MV SingleQuots.

2.3 Cy3 miRNA Transfection, Exosomes Isolation, and HUVEC Uptake

- Cy3 dye-labeled pre-miR miRNA precursors.
- Opti-MEM Medium.
- Lipofectamine RNAiMAX Reagent.

- RNase A enzyme.
- RNase inhibitor.
- Polycarbonate ultracentrifuge tubes with aluminum cap assembly for the Type 70 Ti rotor.
- Bottlebrush.
- Soap.
- 4 μm latex Beads.
- FACS buffer (1× sterile PBS, 0.5–1% BSA or 5–10% FBS, 2 mM EDTA).
- Protease- and nuclease-free sucrose.
- Tris base.
- D_2O.
- 10 N HCL.
- Sterile 0.22 μm filter.
- 16-well chambered cover glass for cell culture.

2.4 Equipment

Cell culture incubator capable of 37 °C, 5% CO_2 and 85% relative humidity.

pH meter.

Inverted cell culture microscope.

Confocal microscope.

Small bench top low-speed centrifuge.

High-speed centrifuge.

Ultracentrifuge with Type 70 rotor.

Laminar flow hood for sterile techniques.

Flow cytometer.

Dynamic light scattering equipment for nanoparticle size characterization.

3 Methods

Always work under sterile conditions inside the laminar flow hood. All of the described steps in this protocol including Human $CD34^+$ stem cell culture, Lipofectamine-mediated transfection of Cy3 dye-labeled pre-miR miRNA precursors, exosomes isolation and exosomes uptake by HUVECs require sterile cell culture practice. Use of sterile, RNase/DNase-free eppendorf tubes and polypropylene conical tubes are highly recommended as miRNA is subject to degradation by ubiquitously present RNase enzyme and will interfere with transfection efficiency.

Work in a laminar flow hood designated as RNase-free zone and always wipe with ethanol and RNaseZap wipes at regular intervals in between performing steps.

3.1 Human CD34+ Stem Cell Culture

1. Warm X-VIVO 10 medium in a 37 °C tissue culture water bath.
2. Dissolve each tube of FLT-3L, SCF and VEGF in XVIVO (1 ml or as required) for preparing complete X-VIVO 10 medium on ice.
3. Prepare X-VIVO 10 medium (complete) for CD34+ stem cell culture by adding

Human Serum Albumin	0.25 %
FLT-3L	100 ng/ml
SCF	100 ng/ml
VEGF	20 ng/ml

4. It is strongly recommended that fresh complete media is prepared on the day when the experiment is run.
5. Prepare cells for counting and establish an estimated number of total CD34+ stem cells using trypan blue cell staining in a hemocytometer under microscope.
6. In a T75 flask, plate human CD34+ stem cells at a cell density of 250,000–300,000 cells/ml using complete X-VIVO 10 medium. A minimum of 2×10^6 cells is required for exosomes preparation.
7. Culture cells in a cell culture incubator capable of 37 °C, 5 % CO_2 and 85 % relative humidity for 24–48 h to achieve 70–80 % confluence.

3.2 Lipofectamine-Mediated Transfection of Cy3 Dye-Labeled pre-miR miRNA Precursors in Human CD34+ Stem Cells

Cy3 dye-labeled pre-miR miRNA precursors must be protected from light. Ensure that the tubes and cell culture flasks containing Cy3 dye-labeled pre-miR miRNA precursors are protected from light. Excessive exposure to light can interfere with the quality of fluorescent imaging analysis.

1. On the day of transfection, collect media containing human CD34+ stem cells. Ensure maximal cell recovery by gently flushing new complete X-VIVO media into T75 flask and collect remaining cells.
2. Centrifuge the media containing CD34+ stem cells in 15 ml polypropylene conical tubes at $400 \times g$ for 7 min. Discard the supernatant. This will remove the cell debris and exosomes secreted during this period of cell culture.

3. To the cell pellet, add freshly prepared 1–2 ml X-VIVO 10 (complete) media and mix gently.
4. In a sterile 24-well culture plate, dilute Lipofectamine by mixing gently 150 µl Opti-MEM medium with 9 µl Lipofectamine in one well. Prepare this mix in at least 8 wells of a 24-well culture plate.
5. In a new well, dilute Cy3-labeled pre-miR miRNA precursors in Opti-MEM medium by mixing gently 3 µl Cy3 pre-miR (10 µM stock) with 150 µl Opti-MEM medium. Prepare this mix separately in at least 8 wells.
6. Transfer diluted Lipofectamine (~159 µl) to each well containing Cy3 pre-miR (~153 µl) and mix gently.
7. Incubate the miRNA-lipid complex in room temperature for 5 min.
8. To each well containing Lipofectamine and Cy3 pre-miR mix, add 1 ml of X-VIVO medium containing 250,000–300,000 human $CD34^+$ stem cells. Swirl the 24-well plate gently to mix contents.
9. Allow reverse transfection to occur by leaving cells in incubator for at least 24 h at 37 °C.
10. After 24 h of transfection, collect cells in a 15 ml sterile polypropylene conical tube.
11. Centrifuge 50 ml polypropylene conical tubes containing $CD34^+$ stem cells at $400 \times g$ for 7 min. Discard the supernatant. This will remove the free Cy3 dye-labeled pre-miR miRNA precursors, non-transfected lipid-miRNA complexes, cell debris and exosomes secreted during this period of cell culture.
12. To the cell pellet, add freshly prepared X-VIVO 10 (complete) media and mix gently.
13. Plate these cells in a fresh T25 flask at a cell density of 250,000–300,000 cells/ml using complete X-VIVO 10 medium.
14. Leave the transfected $CD34^+$ stem cells in culture for up to 30–36 h in an incubator at 37 °C, 5% CO_2 and 85% relative humidity for Cy3 miRNA secretion via exosomes.
15. At this stage, Cy3-transfected cells can be visualized using fluorescent microscopy (Fig. 2). The transfection efficiency is generally 60–80%.

3.3 Isolation and Purification of Cy3 miRNA-Containing Exosomes from $CD34^+$ Stem Cells

1. The day before exosomes isolation, wash the bottom of polycarbonate ultracentrifuge tubes inside with hot water and soap using a bottlebrush.
2. Place washed glass ultracentrifuge tubes inside laminar flow chamber to maintain sterile condition inside the tubes.

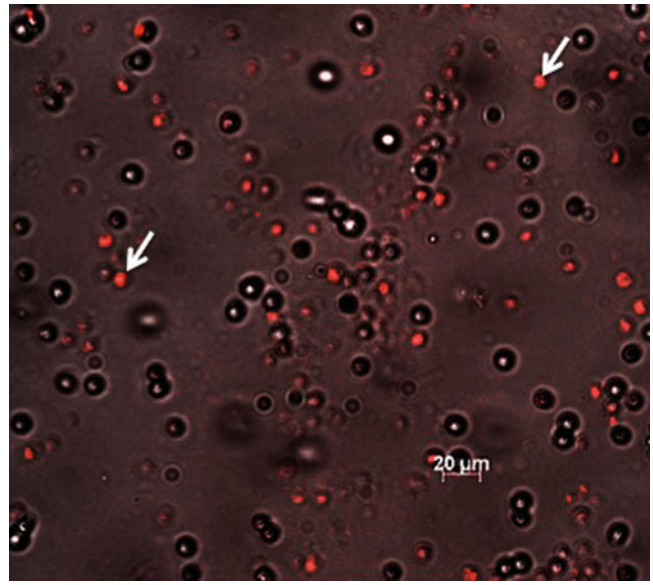

Fig. 2 Fluorescent microscopy imaging of Lipofectamine-mediated transfection of Cy3 dye-labeled pre-miR miRNA precursors in human $CD34^+$ stem cells. Live confocal microscopy imaging at 20× magnification showing efficient transfection of Cy3-labeled pre-miR miRNA precursors in human $CD34^+$ stem cells. Cells that appear orange indicate Cy3 dye-transfected human $CD34^+$ stem cells (*white arrows*)

3. Spray 5 ml of 70% ethanol alongside the inner walls of ultracentrifuge tubes and close the lid. Shake and invert several times to wash all sides of the tube as well as the lid.
4. Aspirate out ethanol and leave the tubes open overnight inside laminar flow chamber to let residual ethanol evaporate.
5. On the day of exosomes isolation, wash polycarbonate ultracentrifuge tubes using 5 ml of sterile PBS.
6. Turn on the ultracentrifuge (with the Ti70 rotor inside) and chill to 4 °C with the "vacuum" mode. This will take approximately 2–3 h.
7. Set ultracentrifuge to 100,000 × *g*, time 60–75 min.
8. Following 30–36 h of Cy3-transfected human $CD34^+$ stem cell culture, collect transfected cells and conditioned media together using serological pipette in a fresh 15 ml polypropylene conical tube.
9. In a small bench top low-speed centrifuge, place 15 ml polypropylene conical tube containing transfected $CD34^+$ stem cells and conditioned media and centrifuge at 500 × *g* for 10 min at 4 °C to separate cells from the conditioned media.
10. Carefully transfer the supernatant containing conditioned media to a fresh 15 ml polypropylene conical tube and leave on ice. Freeze the cell pellet at −80 °C or culture in fresh X-VIVO 10 (complete) media if required for further analysis.

11. Centrifuge the supernatant containing exosomes at 2000 × *g* for 15 min at 4 °C to remove cell fragments and debris [17].
12. In a high-speed centrifuge, place the conical tubes containing conditioned media and centrifuge at 14,000 × *g* for 20 min. This will pellet large cell debris, apoptotic bodies and other undesired extracellular vesicles leaving the soluble exosomes in the supernatant. Carefully transfer the supernatant to a fresh conical tube.
13. Prepare Tris/sucrose/D_2O solution as follows

    30 g protease- and nuclease-free sucrose.

    2.4 g Tris base.

    50 ml D_2O.

    Adjust pH to 7.4 with 10 N HCL drops.

    Adjust volume to 100 ml with D_2O.

    Sterilize by passing through 0.22 μm filter.

    Store up to 2 months at 4 °C.
14. Load 4 ml of ice-cold Tris/sucrose/D_2O solution at the bottom of an ultracentrifuge tube. This will make a cushion/floatation of exosomes.
15. Layer the supernatant containing exosomes from step 12 (of this section) on top of the sucrose cushion. Layering must be done gently without disturbing the sucrose cushion interface. Bring the volume up to the neck of the tube. Balance tubes by adding ice-cold PBS (w/o Ca^{2+} and Mg^{2+}). Tighten the lids (*see* **Note 1**).
16. Ultracentrifuge the tubes at 100,000 × *g* for 60–75 min at 4 °C.
17. Remove supernatant and collect ~4 ml sucrose cushion, which now contains exosomes. Only collect the sucrose cushion and do not contaminate with pellet, if there is any.
18. Transfer the sucrose (~4 ml) to a fresh sterile polycarbonate ultracentrifuge tube. Bring the volume up to the neck of the tube using ice cold PBS (w/o Ca^{2+} and Mg^{2+}). Balance tubes by adding ice-cold PBS. Mix well. The mass of the tubes should be within 0.03 g. Ensure the tubes are precisely balanced using a bench top weighing balance.
19. Ultracentrifuge the tubes at 100,000 × *g* for 60–75 min at 4 °C.
20. The exosomes pellet is extremely fragile and easily disturbed by minimal physical distractions and can lead to significant loss in exosomes quantity. Therefore utmost care must be taken when aspirating the supernatant leaving the exosomes pellet intact.
21. Aspirate supernatant and resuspend pellet in 50–100 μl ice-cold PBS and carefully transfer to fresh sterile RNase/DNase-free eppendorf tubes.
22. Exosomes can be frozen for future experiments or can be used immediately for downstream analysis.

3.4 Exosomes Quality Analysis and Preparation for Gene Delivery

1. To avoid precipitation of undesired RNA molecules sticking on to exosomal outer membranes, it is recommended to perform RNase A digestion of isolated exosomes sample.
2. Add RNase A enzyme to exosomal preparation at a 100 μg/ml final concentration and mix gently.
3. Incubate at 37 °C for 15 min.
4. To avoid RNase A carryover throughout further steps, addition of RNase inhibitor is recommended.
5. Add RNase Inhibitor to the mixture 1 U/ml and incubate at 37 °C for 10 min.
6. In a fresh ultracentrifuge tube, load 4 ml of ice-cold Tris/sucrose/D_2O solution at the bottom of an ultracentrifuge tube.
7. Carefully layer the RNase A treated exosomes sample on top of the sucrose cushion. Bring the volume up to the neck of the tube. Balance tubes by adding ice-cold PBS (w/o Ca^{2+} and Mg^{2+}). Tighten the lids.
8. Ultracentrifuge the tubes at 100,000 × *g* for 60–75 min at 4 °C.
9. Remove supernatant and collect ~4 ml sucrose cushion, which now contains exosomes. Only collect the sucrose cushion and do not contaminate with pellet, if there is any.
10. Transfer the sucrose cushion (~4 ml) to a fresh sterile ultracentrifuge tube. Bring the volume up to the neck of the tube. Balance tubes by adding ice-cold PBS (w/o Ca^{2+} and Mg^{2+}). Mix well.
11. Ultracentrifuge the tubes at 100,000 × *g* for 60–75 min at 4 °C.
12. Aspirate supernatant and resuspend pellet in 50–100 μl ice-cold PBS and carefully transfer to fresh sterile RNase/DNase-free eppendorf tubes. To determine the intactness of exosomes post-RNase treatment, dynamic light scattering (DLS) analysis can be performed (*see* **Notes 2** and **3**).
13. In a fresh sterile RNase/DNase-free eppendorf tube, aliquot required volume of 4 μm latex beads (4×10^7 beads/ml). It is recommended to use 200,000 beads per 1×10^6 cells.
14. Wash beads at least two times using FACS buffer (500 μl) and pellet beads each time by centrifugation at 8000 × *g* for 4 min.
15. Resuspend beads in 100 μl FACS buffer and transfer to a fresh eppendorf tube.
16. To the washed beads, add exosomes prepared from 2×10^6 $CD34^+$ stem cells and mix gently.
17. Incubate at room temperature for at least 30 min. Without rocking.
18. Transfer beads + exosomes mixture to a laboratory rocker and incubate for additional 2 h with rocking (*see* **Note 4**).

19. Centrifuge bead-tagged exosomes at 8000 × *g* for 4 min.
20. Wash the bead-tagged exosomes pellet by adding 500 µl FACS buffer and centrifugation at 8000 × *g* for 4 min.
21. Resuspend the pellet (beads + exosomes) in 200 µl FACS buffer.
22. Keep the beads + exosomes mixture on ice.
23. Perform flow cytometry (*see* **Note 5**).

    Note that we recommend always including controls such as (1) Beads only, (2) beads + unstained exosomes, and (3) beads + Cy3 miR-containing exosomes. All these are processed simultaneously and treated identically.
24. FACS analysis will ensure the quality of exosomes purification as well as the enrichment of Cy3-tagged exosomes for downstream gene delivery protocols.
25. As shown in Fig. 3, we confirm efficient transfection of Cy3-labeled pre-miR miRNA precursors to human CD34⁺ stem cells and release of Cy3 miRNA via exosomes secretion. This ensures that CD34⁺ stem cell-derived exosomes can be used for targeted gene delivery.

3.5 Direct Transfer of Cy3 Dye-Labeled pre-miR miRNA Precursors by Human CD34⁺ Stem Cell-Derived Exosomes

1. To monitor Cy3-tagged exosomes uptake by fluorescent microscopy, we used HUVEC cell line that constantly express Green Fluorescent Protein (GFP).
2. Care should be taken to minimize exposure of GFP-tagged HUVEC cells to light throughout the procedure.
3. Culture GFP-expressing HUVECs in a T75 flask using EBM2 media until it reaches 70–80 % confluence.

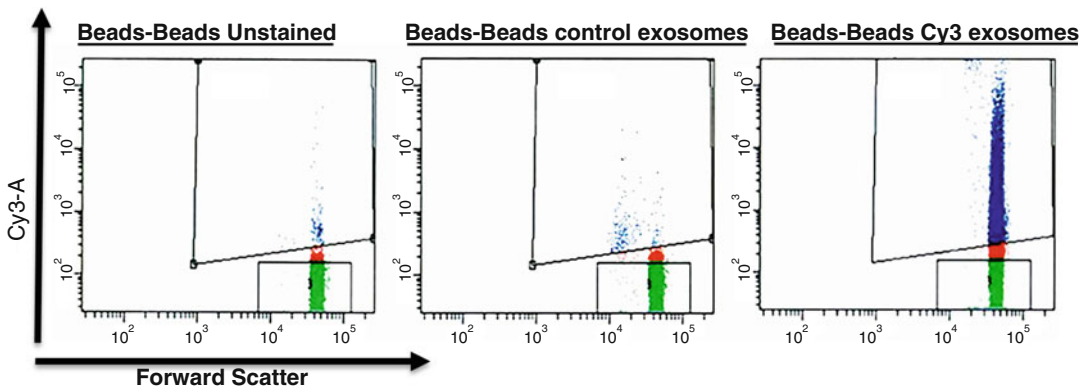

Fig. 3 Fluorescent activated cell sorting analysis of targeted human CD34⁺ stem cell exosomes containing Cy3 dye-labeled pre-miR miRNA precursors. CD34⁺ stem cell derived exosomes were prepared by sucrose gradient centrifugation and coated on to 4 µm latex beads followed by visualization by flow cytometry. (**a**) Beads-beads control (**b**) Beads-beads control exosomes (non-Cy3) (**c**) Beads-beads Cy3 exosomes

4. Prepare a sterile 16-well chambered cover glass for exosomes miRNA delivery.
5. Upon 70–80 % confluence, trypsinize GFP-expressing HUVEC cells and perform cell counting by trypan blue staining.
6. Carefully transfer $2–3 \times 10^4$ GFP-expressing HUVEC cells to 16-well chambered cover glass.
7. Normalize exosomes volume to initial $CD34^+$ stem cell number, i.e., it is recommended to use exosomes from 1×10^6 $CD34^+$ stem cells per $2–3 \times 10^4$ GFP-expressing HUVEC cells. For example, if exosomes were prepared from 3×10^6 $CD34^+$ stem cells, use one-third of exosomes sample to transfect $2–3 \times 10^4$ GFP-expressing HUVEC cells in a 16-well chambered cover glass.
8. Cover the 16-well chambered cover glass.
9. Place the 16-well chambered cover glass in a dark cell culture incubator capable of 37 °C, 5 % CO_2 and 85 % relative humidity.
10. Culture HUVECs in presence of exosomes for 12–24 h.
11. Exosomal uptake by HUVECs can be visualized live under a fluorescent/confocal microscope (Fig. 4).

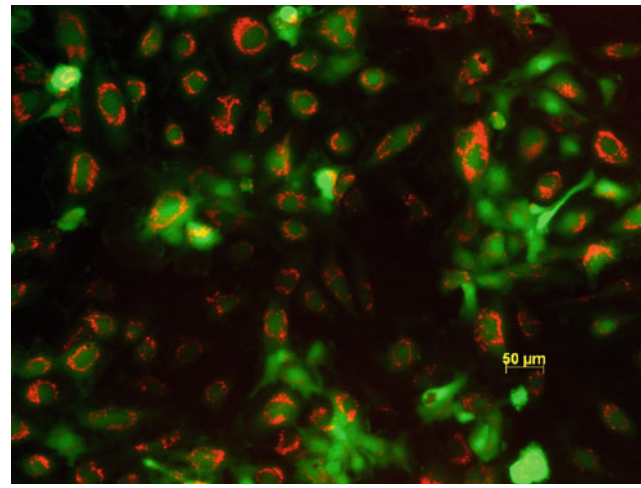

Fig. 4 In vitro monitoring of human $CD34^+$ stem cell derived exosomes-mediated delivery of Cy3 dye-labeled pre-miR miRNA precursors in GFP-tagged HUVECs. Visualization of human $CD34^+$ stem cell-derived exosomes inside HUVECs. *Red* color is representative of Cy3 miRNA containing exosomes after entering HUVEC cells. HUVEC cells expressing green fluorescent protein

4 Notes

1. Layering of exosomes sample on top of sucrose cushion needs caution when performing. A minimal disruption to exosomes-sucrose interface can significantly affect exosomes quality and quantity after ultracentrifugation. Such disruptions can introduce unwanted protein and large lipid complexes as contaminants in exosomes preparation thereby affecting exosomes purity.
2. If the exosomes sample is limited, exosomes sample used for dynamic light scattering analysis can be reused for downstream procedures such as RNA/protein/flow cytometry analysis.
3. During DLS analysis, if there is a significant proportion of particle size greater than 500 nm present, performing an additional sucrose-based gradient ultracentrifugation step is recommended to further deplete contaminating larger particles.
4. When incubating 4 μm beads with exosomes for uptake experiments with HUVECs, rocking should be performed with high speed at room temperature. This is typically 50–60 RPM.
5. When performing flow cytometry, it is also recommended to include a positive control by incubating 4 μm beads with Cy3 miRNA only. This control will assist in flow cytometry gating.

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Chapter 10

Lipidoid mRNA Nanoparticles for Myocardial Delivery in Rodents

Irene C. Turnbull, Ahmed A. Eltoukhy, Daniel G. Anderson, and Kevin D. Costa

Abstract

An area of active research in the field of cardiac gene therapy aims to achieve high transfection efficiency without eliciting immune or inflammatory reactions. Nanomedicine offers an attractive alternative to traditional viral delivery vehicles because nanoparticle technology can enable safer and more controlled delivery of therapeutic agents. Here we describe the use of lipidoid nanoparticles for delivery of modified mRNA (modRNA) to the myocardium in vivo, with a focus on rodent models that represent a first step toward preclinical studies. Three major procedures are discussed in this chapter: (1) preparation of lipid modRNA nanoparticles, (2) intramyocardial delivery of the lipid modRNA nanoparticles by direct injection with an open chest technique in rats, and (3) intracoronary delivery of the lipid modRNA nanoparticles with open chest and temporary aortic cross clamping in rats.

Key words Lipid nanoparticles, Modified mRNA, Lipidoid, Intramyocardial injection, Intracoronary injection

1 Introduction

Nanotechnology is a multidisciplinary field that uses principles from chemistry, biology, physics and engineering to design, fabricate and study nanoparticle structures, defined as having at least one dimension in the 1–300 nm range. For biomedical applications, nanoparticles made using a variety of formulations, including polymers or lipid-based components, have been used to encapsulate drugs, DNA and RNA [1, 2]. Lipid nanoparticles are attractive as nonviral transfection agents, allowing delivery of genetic material with temporally controlled release at high transfection rates. DNA and RNA carried in lipid nanoparticles also exhibit lower degradation rates and higher likelihood of cellular uptake compared to free nucleotides in solution [3]. In addition, the use of synthetic modified mRNA (modRNA) incorporating pseudouridine instead of uridine results in a lower degree of

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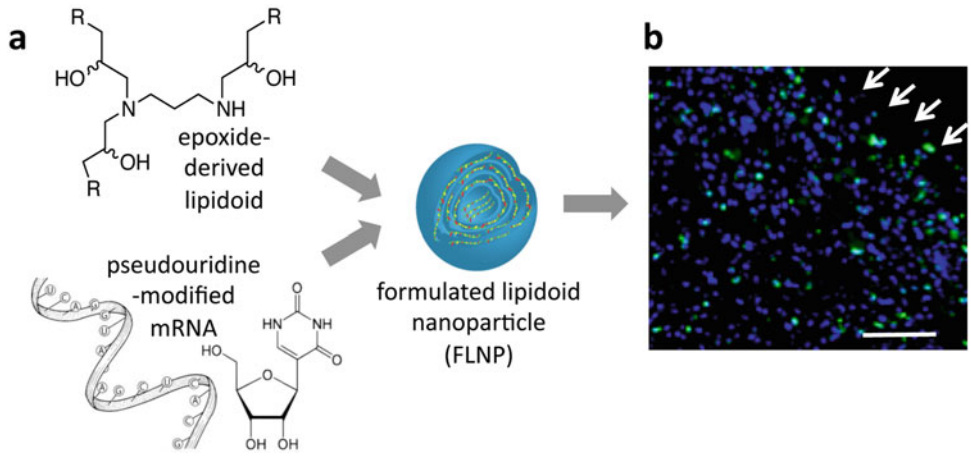

Fig. 1 Schematic of preparation of formulated lipidoid nanoparticles (FLNP) for myocardial delivery of modRNA. (**a**) An epoxide-derived lipidoid mixed in ethanol with stabilizers (DSPC, cholesterol, PEG-DMG), is added to pseudouridine-modified mRNA in citrate buffer, resulting in synthesis of FLNPs. (**b**) Representative immunofluorescence microscopy image of rat heart (10 μm cryosection) 20 h after intramyocardial injection of FLNP/modRNA (10 μg of eGFP modRNA), showing GFP positive cells (*green*) and nuclei stained with DAPI (*blue*). *Arrows* point to the epicardium. Scale bar = 100 μm

translation inhibition [4]. We have recently combined lipid-based nanoparticles and mod mRNA technologies, and demonstrated highly efficient delivery and expression in myocardium of small and large animal models (Fig. 1) [5].

To help make this technology accessible to other investigators, herein we describe lipid-based nanoparticles containing modRNA using a custom formulation consisting of an epoxide-derived lipidoid (mixed in ethanol with the stabilizers DSPC, cholesterol, and PEG-DMG), added to modified mRNA (dissolved in citrate buffer) resulting in synthesis of formulated lipid nanoparticles (FLNP) via nanoprecipitation. The nanoparticles are then purified by dialysis at 4 °C for 3 h. After the dialysis period, this synthesis process renders formulated lipidoid/modRNA nanoparticles (FLNP/modRNA) that can be maintained at 4 °C and remain stable for at least 14 days from the time of synthesis; this stability is clearly advantageous for future preclinical studies, since it allows for storage and transport of the nanoparticle solution to the facility where it would be required.

We also describe two different routes of delivery to the heart, which are applicable for rat in vivo studies: (1) intramyocardial injection using a common open chest technique, and (2) intracoronary injection using temporary aortic cross clamping in an open-chest preparation. Both have proven to be effective delivery methods for virus-associated gene therapies to the heart [6], and recently we showed their use for delivery of FLNP/ enhanced green fluorescent protein (eGFP) modRNA [5]. There are variations of the cross-clamping technique, ranging from clamping the

aorta alone, aorta and pulmonary artery, and superior and inferior vena cava [6–11]. Of particular interest is the intracoronary delivery route, for although it requires an invasive procedure for small animal applications as described here, it serves to evaluate a method of delivery that involves only a minimally invasive procedure by percutaneous catheter-based intracoronary delivery in large animals and humans, which is appropriate in preclinical and translational studies.

2 Materials

2.1 Formulated Lipidoid mRNA Nanoparticles

1. Lipids (*see* **Note 1**). Prepare 10 mg/ml ethanol solutions of the four lipids listed below:
   (a) Epoxide-derived lipidoid reagent, termed C14-113 lipidoid (MW 541), as synthesized in Love et al. [12]. Store at 4 °C.
   (b) Distearoyl phosphatidylcholine (DSPC) (MW 790.15) (Avanti Polar Lipids). Store at −20 °C.
   (c) Cholesterol (MW 386.66). Store at −20 °C.
   (d) Polyethylene glycol (PEG)-lipid conjugate (PEG-DMG) (MW 2555) (14:0 PEG2000 PE) (Avanti Polar Lipids). Store at −20 °C.
2. eGFP modRNA (1 mg/ml in water) (*see* **Note 2**).
3. 10 mM sodium citrate buffer (pH 3.0), sterile-filtered.
4. 1× phosphate-buffered saline (PBS, pH 7.4), sterile-filtered.
5. Ethanol 100%.
6. Deionized water.
7. Microcentrifuge tubes (sterile, DNAse/RNAse free).
8. Barrier pipette tips (sterile, DNAse/RNAse free).
9. Slide-A-Lyzer Dialysis Cassette 20K MWCO.
10. Sterile disposable syringes (1 ml).
11. Hypodermic needles (18 G–21 G, at least 1.5″ long).
12. Large glass beaker (4 l).
13. Stir bar.
14. Stir plate (placed in cold room at 4 °C).
15. Aluminum foil (to cover glass beaker during dialysis).
16. Permanent ink marker.

2.2 Intramyocardial Delivery of Lipid mRNA Nanoparticles

1. Drugs: ketamine, xylazine, buprenorphine.
2. Electric razor (animal hair clipper).
3. 16 G or 18 G IV catheter.
4. Mechanical ventilator.

5. Heating pad.
6. Adhesive tape (1–2″ wide).
7. Gooseneck lamp-microscope illuminator.
8. Stereo microscope.
9. Surgical instruments (sterile): Micro-Adson forceps, Hemostat clamps, Extra Fine Bonn scissors, Alm retractor, Castroviejo needle holder, Halsey needle holder, scalpel blade and handle.
10. Sutures: Silk 6-0 (tapered needle) and 4-0, Nylon 5-0.
11. Gauze pads (2″ × 2″).
12. Cotton-tipped applicators.

2.3 Intracoronary Delivery of Lipid mRNA Nanoparticles

Same as in Subheading 2.2 above, and in addition include the following:

1. 24 G × ¾″ IV catheter.
2. Atraumatic clamp.

3 Methods

3.1 Calculation of Required Materials

Calculate the required quantities of each component, following **steps 1–6**.

1. Establish the total amount of μg of mRNA that you plan to use for injection(s). Based on that, the total formulation volume (μl) is calculated for a final mRNA concentration of 0.1 mg/ml. *Total Formulation Volume* (μl) = {*total mRNA to be used* (μg)}/{*final mRNA concentration* (mg/ml)}.
2. Next, calculate the "required" volume of the organic lipid mixture (μl) and the aqueous mRNA mixture volume (μl) using the following formulas (based on the 1:3 volumetric mixing ratio of the organic lipid mixture to aqueous mRNA solution):
   Required Volume of Organic Lipid Mixture (μl) = {*Total Formulation Volume* (μl)} × (1/4).
   Aqueous mRNA Mixture Volume (μl) = {*Total Formulation Volume* (μl)} × (3/4).
3. Calculate the required volume of each of the organic lipid mixture components: C14-113 lipidoid, DSPC, Cholesterol, and PEG-DMG at a working molar ratio of 50:10:38.5:1.5 and a working concentration of 4 mg/ml, 1.168 mg/ml, 2.201 mg/ml, and 0.567 mg/ml respectively; starting with a stock concentration of 10 mg/ml for each of the components (*see* **step 1** of Subheading 2.1). Substitute the component in square brackets by lipidoid, DSPC, Cholesterol, or PEG-DMG.
   Total required volume of [*lipidoid*] (μl) = {*working concentration of* [*lipidoid*] (mg/ml)} × {*Total Required Volume of Organic Lipid Mixture* (μl)}/{*stock concentration of* [*lipidoid*] (mg/ml)}.

4. Calculate the required ethanol volume in which the lipidoid components will be diluted.
   Required ethanol volume (μl) = {*Required Volume of Organic Lipid Mixture* (μl)} − {(*required volume of lipidoid* μl) + (*required volume of DSPC* μl) + (*required volume of Cholesterol* μl) + (*required volume of PEG-DMG* μl)}.
5. Calculate "scale up" volumes of each organic lipid mixture component. It is important to always make more organic lipid mixture than what is required so that at the time of synthesis sufficient lipid solution is available (*see* **Note 3**). A scale-up factor of 2 is generally adequate; it can be adjusted as needed.
   Scale up volume of [*lipidoid*] (μl) = {*Total required volume of* [*lipidoid*] (μl)} × {*scale up factor*}.
   The above calculations serve to specify the minimum stock quantities required from each of the components of the organic lipid mixture.
6. Based on a 10:1 mass ratio of C14-113 lipidoid to mRNA, and the 1:3 volumetric mixing ratio of the organic lipid mixture to aqueous mRNA solution, dilute the stock modRNA in citrate buffer as needed. A stock concentration of 1 mg/ml is ideal; if substantially below this concentration, mRNA can be concentrated by the standard protocol of nucleic acid precipitation and centrifugation (using an RNAse-free mixture of sodium acetate buffer and ethanol). For a working concentration of 4 mg/ml of lipidoid, a working mRNA concentration of 0.133 mg/ml is needed, that is, 4 mg/ml lipidoid/(10 mg lipidoid/1 mg mRNA) × (1 ml lipidoid/3 ml mRNA). Using the given stock mRNA concentration, the aqueous mRNA mixture volume, and the working mRNA concentration, calculate the stock mRNA volume and citrate buffer volume required for the dilution as follows.
   Stock mRNA volume (μl) = {*Aqueous mRNA Mixture volume* (μl)} × {*Working mRNA concentration* (mg/ml)}/{*Stock mRNA concentration* (mg/ml)}.
   Citrate buffer volume (μl) = {*Aqueous mRNA Mixture volume* (μl)} − {*Stock mRNA volume* (μl)}.
   To organize the equations and easily perform the calculations each time they are needed, we recommend creating an electronic spreadsheet using the values and formulas shown in Fig. 2. An example of the results from these calculations is shown in Fig. 3.

3.2 Preparation of Formulated Lipidoid modRNA Nanoparticles

1. Bring all stocks of the required components to room temperature (lipidoid, DSPC, PEG, and cholesterol) and make sure they are well dissolved prior to use (*see* **Note 4**).
2. Prepare the organic lipid mixture. In a microcentrifuge tube, first add the "scaled up" volume of ethanol, then add the "scaled up" volumes of each of the four lipid components in the following order: lipidoid, DSPC, cholesterol, and PEG-DMG. Mix by pipetting several times after adding each component.

	Final mRNA concentration (mg/ml)				0.1		
	Working lipidoid concentration (mg/ml)				4		
1)	Total mRNA to be used (µg)				1) Indicate the total amount of µg of mRNA that you plan to use for injection/s.		
1)	Total Formulation volume (µl)				1) Total mRNA to be used (µg) ÷ Final mRNA concentration (mg/ml)		
2)	Required volume of Organic Lipid Mixture (µl)				2) Total formulation volume (µl) ÷ **4**		
2)	Aqueous mRNA Mixture Volume (µl)				2) Total formulation volume (µl) x **0.75**		
3)	**Organic Lipid Mixture**				3)	5)	
	Organic lipids	Stocks mg/mL	MW	Working mixture Molar ratio	mg/mL	Required Volume (µl)	Scale up Volume (µl)
	C14-113B	**10**	**541**	**50.0**	**4**	working lipidoid (mg/ml) x total required volume (µl) ÷ lipidoid stock (mg/ml)	Lipidoid required volume (µl) x lipid scale-up factor
	DSPC	**10**	**790.15**	**10.0**	**1.168**	working DSPC (mg/ml) x total required volume (µl) ÷ DSPC stock (mg/ml)	DSPC required volume (µl) x lipid scale-up factor
	Cholesterol	**10**	**386.66**	**38.5**	**2.201**	working Cholesterol (mg/ml) x total required volume (µl) ÷ Cholesterol stock (mg/ml)	Cholesterol required volume (µl) x lipid scale-up factor
	PEG-DMG	**10**	**2555**	**1.5**	**0.567**	working PEG (mg/ml) x total required volume (µl) ÷ PEG stock (mg/ml)	PEG required volume (µl) x lipid scale-up factor
4)	Ethanol (µl) (Required and scale up, respectively)					Total required volume (µl) - (lipidoid, DSPC, Cholesterol, PEG required volumes (µl)	Ethanol required volume (µl) x lipid scale-up factor
	Total volume (µl) (Required and scale up, respectively)					Total formulation volume (µl) ÷ **4**	Total required volume (µl) x lipid scale-up factor
5)	Lipid scale-up factor factor					2	
6)	Stock mRNA concentration (mg/ml)					6) Indicate concentration of stock mRNA	
	Working mRNA concentration (mg/ml)					**0.13**	
	Aqueous mRNA Mixture						
	Aqueous mRNA Mixture Volume (µl)					Total formulation volume (µl) x **0.75**	
6)	Stock mRNA volume (µl)					6) Aqueous mRNA mixture volume (µl) x working mRNA (mg/ml) ÷ Stock mRNA(mg/ml)	
6)	Citrate buffer volume (µl)					6) Aqueous mRNA mixture (µl) - Stock mRNA (µl)	
	Synthesis of FLNP/mRNA						
	Aqueous mRNA mixture volume (µl)					Total formulation volume (µl) x 0.75	
	Organic Lipid Mixture Required volume (µl)					Total formulation volume (µl) ÷ 4	
	Total Formulation volume (µl)					Aqueous mRNA mixturevolume (µl) + Organic Lipid Mixture Required volume (µl)	

Fig. 2 Formulas and values for calculation of required quantities of each component for preparation of formulated lipidoid modRNA nanoparticles. *Grey box*: The input in these cells is determined by the investigator according to the planned experiments. *Blue box*: The output in this box is the required volume of organic lipid mixture in microliters. *Green box*: The output in this box is the aqueous mRNA mixture volume in microliters

3. Prepare the aqueous mRNA mixture. In a microcentrifuge tube, first add the citrate buffer, and then add the mRNA. Mix by pipetting several times.
4. Synthesize formulated lipid nanoparticles/mRNA (FLNP/mRNA). Add the "required volume" of the organic lipid mixture to the tube containing the aqueous mRNA mixture (1:3 volume/volume organic lipid–aqueous mRNA). Mix by repeated pipetting for 15 s.
5. Leave undisturbed for 10 min at room temperature to allow for self-assembly of FLNPs.

	Final mRNA concentration (mg/ml)					0.1	
	Working lipidoid concentration (mg/ml)					4	
1)	Total mRNA to be used (μg)					20	
1)	Total formulation volume (μl)					200	
2)	Required volume of Organic Lipid Mixture (μl)					50	
2)	Aqueous mRNA Mixture Volume (μl)					150	
3)	**Organic Lipid Mixture**					3)	5)
	Organic lipids	Stocks		Working mixture		Required Volume (μl)	Scale up Volume (μl)
		mg/mL	MW	Molar ratio	mg/mL		
	C14-113B	**10**	**541**	**50.0**	**4**	20	40
	DSPC	**10**	**790.15**	**10.0**	**1.168**	5.8	11.7
	Cholesterol	**10**	**386.66**	**38.5**	**2.201**	11.0	22.0
	PEG-DMG	**10**	**2555**	**1.5**	**0.567**	2.8	5.7
4)	Ethanol (μl) (Required and scale up, respectively)					10.3	20.6
	Total volume (μl) (Required and scale up, respectively)					50.0	100.0
5)	Lipid scale-up factor factor					2	
6)	Stock mRNA concentration (mg/ml)					1	
	Working mRNA concentration (mg/ml)					**0.13**	
	Aqueous mRNA Mixture						
	Aqueous mRNA Mixture Volume (μl)					150	
6)	Stock mRNA volume (μl)					20.0	
6)	Citrate buffer volume (μl)					130.0	
	Synthesis of FLNP/mRNA						
	Aqueous mRNA mixture volume (μl)					150	
	Organic Lipid Mixture Required volume (μl)					50.0	
	Total FLNPs volume (μl)					200.0	

Fig. 3 Example calculations of required quantities of each component for preparation of formulated lipidoid modRNA nanoparticles. Color coding same as Fig. 2

6. In a large glass beaker, add 3 l of 1× PBS and a magnetic stir bar. Pre-wet the dialysis cassette membrane by immersion of the dialysis cassette in the beaker for at least 2 min. Remove dialysis cassette from the beaker and gently tap to remove excess fluid (*see* **Note 5**). Cover the top of the beaker with aluminum foil. Bring the beaker with the PBS and stir bar to a cold room (4 °C) and place on a magnetic stir plate (*see* **Note 6**).
7. Dilute the FLNP/mRNA solution by adding 1× PBS as needed and pipet to mix.

8. Collect the sample from the microcentrifuge tube (FLNP/mRNA solution) into a 1-ml syringe with needle, leaving a small amount of air in the syringe.
9. Mark one corner port of the dialysis cassette with a permanent ink marker. Penetrate the gasket through a syringe guide port at the marked corner and inject the sample into the dialysis cassette while holding it upright. Use caution, as inserting the needle too far into the sample chamber may puncture the membrane (*see* **Note 7**).
10. Place the dialysis cassette in the beaker with PBS on a stir plate. Let it float vertically and set the stir plate to a low setting (gentle stirring). Dialyze overnight (or for at least 3 h) at 4 °C (*see* **Note 8**). For dialysis volumes lower than 750 μl there is no need to exchange the PBS dialysis buffer. For larger volumes, replace the dialysis buffer with fresh 1× PBS once after the first 2 h.
11. Remove the dialysis cassette from the beaker. Penetrate gasket with a new syringe and needle through the unused syringe guide port (the one that is not marked) and slowly inject air into the chamber to the maximum allowed volume to separate the membranes, which helps prevent needle puncture of the cassette membrane. With the needle in place, turn the cassette upside down so that the needle is at the bottom, and as sample collects near the port withdraw sample into the syringe.
12. Transfer the solution containing dialyzed FLNP/mRNA from the syringe into a microcentrifuge tube. Measure the new volume (post dialysis volume) (*see* **Note 9**) and adjust the concentration accordingly to calculate the volume/dose to be injected. Maintain the formulation at 4 °C until ready for use (*see* **Note 10**).

3.3 Intramyocardial Delivery of the Lipid mRNA Nanoparticles by Direct Injection in Rats

1. Calculate in advance of the procedure the total volume (μl) of FLNP/modRNA required for delivering the desired full dose of mRNA (μg). Bring the FLNP/modRNA to the procedure room and keep on ice until ready for use (**step 6**). As described below, the total volume will be delivered in multiple injections.
2. Anesthetize the rat by intraperitoneal injection of ketamine/zylaxine (60–80 mg/kg and 5 mg/kg, respectively). Shave the chest.
3. Once the animal is sedated, cannulate the trachea with a 16 G or 18 G (1¼″–2″ length) IV catheter (*see* **Note 11**), and connect to a mechanical ventilator providing room air (70–90 breaths/min) (*see* **Note 12**). Place animal in right lateral position, and secure the animal by the limbs to the operating board with adhesive tape. Use a heating pad to maintain a body temperature of 37 °C. Wipe the chest with povidone-iodine solution.

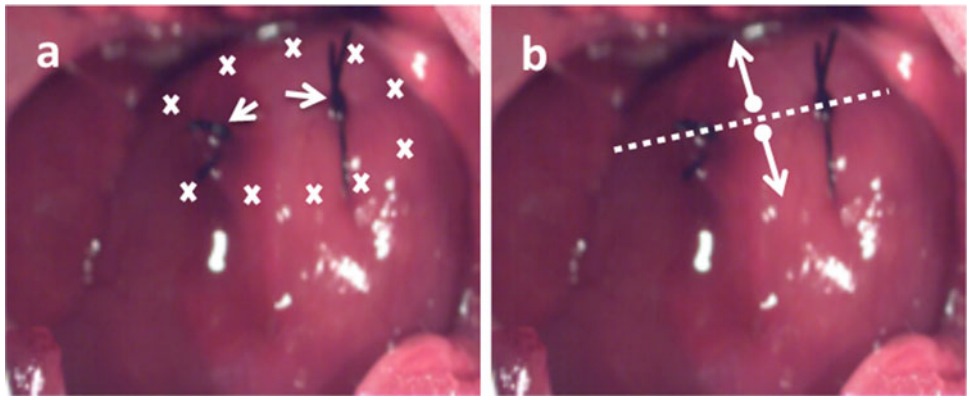

Fig. 4 Direct intramyocardial injection. (**a**) Image of rat heart with schematic of intramyocardial injection sites (*marks*) on either side of the silk sutures. *Arrows* point to loosely tied silk sutures used as landmarks; and (**b**) schematic illustrates the lengthwise cut (*dashed line*) through the 6-0 silk sutures placed as landmarks, splitting the heart in two to use one half for histology and the other for RNA extraction

4. Perform a left thoracotomy between the fourth and fifth ribs. Place a self-retaining retractor in the fourth intercostal space to stretch open the thorax. Incise the pericardium to expose the heart.
5. Thread two 6-0 silk sutures (with taper point needle) on the exposed left ventricular free wall of the heart and tie them loosely (Fig. 4a) (*see* **Note 13**). These sutures are to be placed superficially, avoiding the coronary vessels, and will serve as reference markers at the time of injection and also during subsequent tissue harvest (*see* **Note 14**).
6. Load the total amount of FLNP/modRNA to be delivered into a 1-ml syringe with a 25 G × ⅝″ needle. Change needle to a 30 G × ½″–1″ needle. Approach the heart surface with the needle at a shallow angle (30–45°) relative to the epicardial surface, advance the needle along the free wall of the heart (*see* **Note 15**). Inject the solution as you retract the needle. Distribute the injections on the anterior, apical and lateral regions of the heart to the left and right of the silk sutures placed as reference, administering 40–100 μl per injection site (*see* **Note 16**).
7. To close, first bring the rib cage together with interrupted sutures, and then the muscular and fascia layer with running sutures. Lastly suture close the skin (*see* **Note 17**).
8. Allow the animal to recover while being maintained connected to the ventilator until the animal is able to breath spontaneously, without signs of labored breathing.
9. For analgesia, administer buprenorphine 0.1–0.5 mg/kg subcutaneous, twice a day for 3 days.

3.4 Intracoronary Delivery of the Formulated Lipid mRNA Nanoparticles by Intraventricular Injection with Temporary Aortic Cross Clamping in Rats

1. Anesthetize and prep the animal as described in the previous section on intramyocardial injection (**steps 1–3**), with the exception that the rat is placed in a supine rather than lateral position, and the FLNP/modRNA are delivered in a single injection.
2. Make a skin incision (approximately 1.5-cm long) on the chest along the midline; continue the incision through the subcutaneous tissue. The heart can be approached through a mid sternotomy, or alternatively a left distal parasternal approach (*see* **Note 18**).
3. Incise the pericardium to expose the heart. Apply a cephalad retraction to the thymus and identify the aorta and pulmonary artery.
4. Partially exteriorize the heart by gently advancing a cotton tipped applicator from the apex towards the underside of the heart (*see* **Note 19**). Place a 6-0 silk suture (with taper point needle) at the apex as a purse string, without tying it. Introduce a 24 G × ¾″ catheter through the left ventricular apex at the center of the purse string. Immediately upon gaining access to the left ventricle, remove the needle, watch for blood return and connect the catheter to a 1-ml syringe containing the FLNP/modRNA solution to be injected.
5. Advance the catheter to the aortic root; it is likely that the catheter will be visualized below the surface of the aortic wall as it advances into the ascending aorta. Proceed cautiously to avoid puncturing the wall of the aorta. Then retract the catheter slightly to leave the tip of the catheter at the base of the aortic root.
6. Place an atraumatic clamp (a padded clamp can be used) across the ascending aorta just distal to the aortic root (Fig. 5) (*see* **Note 20**). Inject the FLNP/modRNA solution into the aortic root while the heart is pumping against a closed system, this allows the solution to circulate down the coronary arteries and perfuse the heart without direct manipulation of the coronaries (*see* **Note 21**). After 30 s of aortic cross clamping, release the clamp (*see* **Note 22**).
7. Withdraw the catheter and immediately tie the purse string suture on the apex of the heart to seal the catheter puncture site. Apply a cotton tipped applicator to the puncture site to aid stopping of bleeding.
8. Close the chest by first bringing together the edges of the sternum, and then the muscular and fascia layer in the midline. Lastly, suture close the skin layer (*see* **Note 17**).
9. Release the animal from the restrains while maintaining connected to the ventilator until the animal is able to breath spontaneously, without signs of labored breathing.
10. For analgesia administer buprenorphine 0.1–0.5 mg/kg subcutaneous, twice a day for 3 days (*see* **Note 23**).

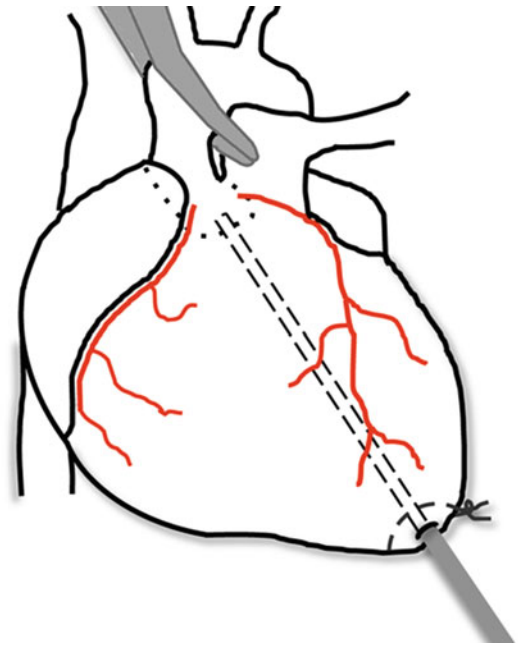

Fig. 5 Intracoronary injection. Schematic of rat heart depicting intracoronary injection with aortic cross clamping

4 Notes

1. Weigh the dried lipids into tared vials and add ethanol. Use a vortex-mixer to ensure complete dissolution. If the lipids do not dissolve, heat to 37 °C until the solution becomes transparent.
2. Aliquot and store mRNA at −70 °C. For use, thaw the required aliquot(s) of mRNA on ice. Always wear gloves and use RNAse-free tips and tubes when handling mRNA to avoid RNA degradation. We strongly recommend the use of modified mRNA, which incorporates both pseudouridine and 5-methylcytidine modified nucleotides, reducing the innate immune response to RNA [13–15].
3. When mixing the lipid components, make a volume larger than the minimum required; we recommend a 200% increase to ensure a sufficient amount of the required organic lipid mixture to add to the aqueous mRNA mixture (**step 4** of Subheading 3.2). It also helps to increase the working volumes when the required minimum of any of the components is impractically small (e.g., less than 1 μl).
4. When lipids are brought to room temperature, some particulates of the lipids will be visible. To dissolve the lipids properly for use, place in water bath at 37 °C while shaking for 1–2 min

and then vortex intermittently. Check that the lipids are well dissolved; no particles should be visible. Repeat as needed.

5. The beaker containing the PBS can be prepared beforehand, prior to bringing lipids to room temperature, but the best time to pre-wet the dialysis cassette membrane is during the 10-min incubation period for FLNP self-assembly.
6. To avoid malfunction of electronics due to condensation, it is recommended that the stir plate be brought to the cold room at least 24 h prior to its use.
7. Slowly advance the needle into the dialysis cassette cavity so that the sharp tip of the needle is barely visible. Inserting the needle too far into the cassette chamber may puncture the dialysis membrane and render it useless. Inject approximately half of the sample. Withdraw some air from the cassette by pulling back on the syringe piston and then inject remaining sample. With the needle in the cassette cavity, withdraw remaining air to compress the chamber windows to allow maximum contact of the sample with membrane surface area, maximizing the dialysis efficiency. Always use caution to prevent the needle from contacting the membrane.
8. Dialysis is required for 3 h but it can be extended to overnight, as convenient for the scheduled experiments, e.g., timing of the surgical procedures for injection of the FLNP/modRNA. The longer period of dialysis is not detrimental to the formulation.
9. To measure the post-dialysis volume, collect the entire volume of the sample into the 1-ml syringe and measure the volume according to the graduation marks on the syringe.
10. Successful transfection of myocardium in vivo was achieved when the FLNP/modRNA was injected within the first 24 h after preparation. However, in vitro tests showed that the formulation remains stable and retains activity for at least 14 days when stored at 4 °C [5].
11. Prepare the catheter prior to use by cutting off the sharp end of needle, a wire cutter is the ideal tool, and exercise caution to avoid personal injury when cutting the needle tip.
12. Ventilator settings must be adjusted per individual animal, with respiratory rate 70–90 breaths/min and inspiration time 0.4–0.6 s.
13. Reference markers are required when performing the procedure in a sham animal to later identify the sites of nanoparticle injection; such landmarks may not be required when injecting the nanoparticles into the heart of an animal with a clearly defined ischemic region, e.g., post LAD ligation.
14. The procedure of organ harvest must be carried out expeditiously to avoid tissue degradation. Label tubes and cryomolds

beforehand. To process heart tissue from animals that received direct intramyocardial injection, harvest the heart and rinse in PBS (avoid perfusion with fixative), cut the heart in half lengthwise through the 6-0 silk sutures placed as landmarks (Fig. 4b). One half, to be used for RNA extraction, should be cut into ≤1 mm^3 pieces, collect into a DNAse/RNAse free microcentrifuge tube and store at −80 °C until ready for processing. The other half is to be used for histology; place tissue sample in cryomold, embed in optimum cutting temperature (OCT) compound, place on dry ice, and once the OCT freezes store at −80 °C until ready for cryosectioning. Apply fixative to each cryosection prior to staining.

15. As you advance the needle the bevel should be parallel to the surface of the heart to avoid puncturing through the full thickness of the myocardium and into the ventricular chamber.
16. The recommended maximum volume at any single injection point is 100 μl.
17. The recommended suture sizes are 3-0 to 4-0, and 5-0 for skin layer. For the type of suture material, use slowly absorbed or nonabsorbable suture material, avoid using silk for skin closure.
18. For the parasternal approach, cut off the most distal two ribs from their attachment to the left side of the sternum, and then advancing proximally, cut off the next two ribs on the right side of the sternum, and lastly cut through the sternum diagonally. Place a self-retaining retractor to bring apart the distal part of the sectioned sternum towards the right and the proximal part of the sternum to the left.
19. Applying external pressure on the right side of the thorax can also help to exteriorize the heart.
20. The procedure that includes clamping of the pulmonary artery along with aorta is expected to result in a wider distribution of particles throughout the myocardium [6].
21. While one person alone without assistants performs most rodent surgeries, it is recommended that for this particular technique one person takes control of the clamp on the aorta while an assistant applies the injection. Once sufficient proficiency is acquired with the technique it may be performed by one person alone.
22. It is expected that during the cross clamping the heart rate will decrease, but it should recover to baseline within 30 s of clamp release. The general recommendation for a maximum volume to be delivered is to not exceed 500 μl due to risk of pulmonary edema [8]; we have delivered up to 600 μl in the rat without overt compromise of heart or lung function and with animal survival for at least 2 weeks, which was the longest time point at which the animals were euthanized [5].

23. It is expected that recovery from the sternotomy will induce greater discomfort for the animal than the left thoracotomy used for the direct injection, thus requiring adjustment of buprenorphine dosage for appropriate analgesia.

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Part IV

Gene Delivery Methods

Chapter 11

Gene Transfer in Isolated Adult Cardiomyocytes

Kjetil Hodne, David B. Lipsett, and William E. Louch

Abstract

During the past few decades, gene delivery using recombinant virus has made tremendous progress. With a higher than 80% transduction efficiency, even in non-dividing cells, viral transduction has become the method of choice for efficient gene transfer into cardiomyocytes. However, in vitro gene delivery is dependent on a robust cell isolation protocol, as prolonged cultivation is needed to initiate gene expression and target specific cellular processes. This chapter describes some of the important steps that need to be considered for successful in vitro gene transfer into adult cardiomyocytes. Included are detailed protocols for isolating cells, maintaining rod shaped cardiomyocytes in culture over several days, and employing adenovirus for gene transduction.

Key words Gene transfer, Virus, Transduction, Cardiomyocyte, Cell isolation, Cell culture

1 Introduction

Combined with cellular electrophysiology and imaging techniques on single isolated cardiomyocytes, gene transfer is an invaluable tool for investigating cellular physiology. In non-dividing cells like adult cardiomyocytes, efficient gene transfer using conventional transfection techniques like calcium phosphate [1–4], or liposome/Lipofectamine [5–8] has proved to be challenging (reviewed in [9]). Even in neonatal cardiomyocytes, transfection efficiency is usually low with these techniques, with values reported near 6% [6, 7]. The low transfection efficiency in non-dividing cells has made viral gene transduction the preferred method for delivering genetic material into these cells.

1.1 Selecting a Viral Vector

To date, several recombinant (non-virulent) viruses including adenovirus (AV), adeno-associated virus (AAV) and lentivirus (LV) have been shown to efficiently deliver exogenous genetic material into adult cardiomyocytes in vitro and in vivo [10–14]. However, the viruses differ in terms of their viral backbone, insert size, infection strategy and transcription efficiency (Table 1). These

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Table 1
Comparison of viral vectors

	Adenovirus (AV)	Adeno-associated virus (AAV)	Lentivirus (LV)
Cloning capacity for the transgene[a]	8 kb	4.7 kb	8.5 kb
Viral backbone	dsDNA	ssDNA	ssRNA
Immune response (target cells)	High	Low	Low
Integration (target genome)	No	No	Yes/No
Initiation of expression	Fast	Slow	Slow
Duration/characteristics of gene expression	Short, transient	Long, but transient	Long, often stabile

[a]Cloning capacity is dependent on the viral capsid and the amount of genomic material that needs to be carried for efficient transduction. Typically, virulent genes not necessary for transduction are removed and replaced by the gene of interest

variations provide several different options for the researcher and choosing the right virus depends on the experimental objective(s).

AV has several advantages when performing in vitro overexpression- or knockdown (short hairpin (sh)RNA/small interfering (si)RNA)—experiments. The double-stranded (ds)DNA backbone found in AV facilitates direct transcription and limits the time required for cardiomyocyte cultivation. In addition, AV allows for larger inserts up to 8 kb long (AV serotype 5 with the early transcribed region E1 and E3 deleted). However, AV does not result in incorporation of the transgene into the host genome, meaning that gene expression is limited to approximately 2 weeks in isolated cells [15–18]; a fact that limits the applicability of AV for in vivo use. Another potential drawback of using AV is the strong immune response induced by the virus, although parallel experiments with empty vector can help control for these effects.

AAV transduction induces little immune response and can provide stable gene expression in cardiac cells for up to 1 year [19]. These benefits make these viruses well suited for in vivo work, and studies wishing to combine in vitro and in vivo techniques. In addition, a variety of serotypes (surface markers) with different tropism are available (e.g., [20–22]). The DNA backbone of traditional AAV is single-stranded and to efficiently transcribe the DNA template the cell needs to perform a second-strand synthesis to make readable dsDNA [23]. This process creates a transcriptional delay and, as a result, in vitro cultivation needs to be prolonged. A novel method has been developed to overcome this transcriptional delay. The so-called self-complementary (sc)AAV has been shown to facilitate transcription without the need of second-strand synthesis, allowing for faster onset of transcription compared to

conventional AAV [24]. Despite improvements in its transcriptional activity, a major drawback of using AAV is the limited insert size of 3 kb (+1 kb promoter).

As with AAV, LV cannot be directly transcribed following transduction. Rather, the single-stranded RNA of these viruses depends on reverse transcriptase to generate a transcription-competent dsDNA template. Because LV induces less of an immune response than AV, and can carry larger inserts than AAV, LV may be particularly practical for in vivo research. Moreover, LV can incorporate into the host genome and the long-term effect of LV has made this virus attractive for potential therapy.

1.2 Overview of Viral Transduction Techniques

In vitro gene delivery is dependent on a healthy, quiescent population of isolated cardiomyocytes. Thus, successful cell isolation is crucial and, in our experience, this begins with careful animal handling. As with all techniques using animal models, it is essential to reduce the stress on the animal at all times, as it can affect downstream processes of interest. In particular, the heart is highly sensitive to neurohumoral transmission and elevated respiratory metabolism, two factors that are altered as a result of elevated stress.

The importance of a well-practiced and reproducible cell isolation protocol cannot be overstated. The most commonly used protocol for cell isolation is the Langendorff technique [25, 26]. Following surgical removal, the intact heart is cannulated through the aorta and perfused retrogradely using either constant pressure perfusion (gravity feed) or constant flow (pump feed) of fresh, oxygenated cell isolation solution (Fig. 1). The pressure created by the reverse flow causes the aortic valve to close and directs the isolation solution through the coronary arteries. If mounted rapidly and correctly, ischemic conditions in the heart are minimized, the myocardium remains viable, and digestive enzymes are uniformly distributed [9]. Full details of the cell isolation method are outlined in the following sections.

Following isolation, stable cell cultivation is required for an extended time period (>48 h) to enable successful gene delivery. In general, two strategies for cultivation exist; namely the redifferentiation and rapid attachment methods. With the redifferentiation method, cardiomyocytes lose their rod shaped structure within 24 h following isolation and produce pseudopodia-like structures when attached to the surface of the culture dish. These cells also start to contract spontaneously (e.g., [27–29]). Although, this is a useful method for investigating, for example, genetic reprogramming, most studies would benefit from cells with morphological [rod shaped with a regular transverse (t) tubule network] and electromechanical properties highly resembling those found in freshly isolated cells. When applied correctly, the rapid attachment cell culture method can produce such myocytes [30], although some degradation of cardiomyocyte morphology and function over time

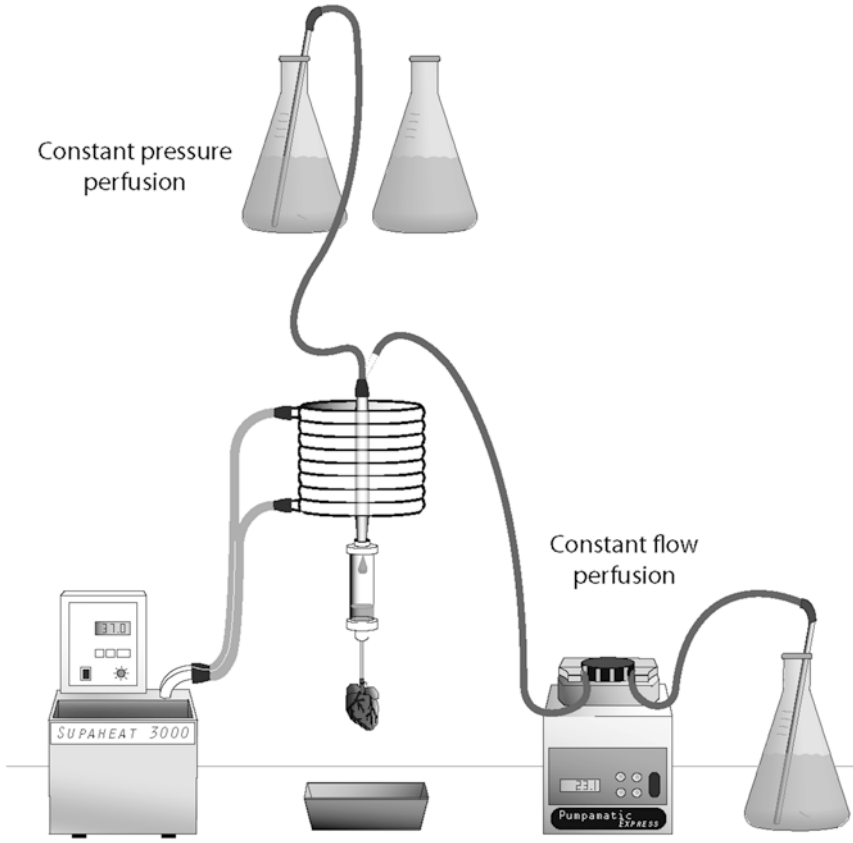

Fig. 1 Langendroff setup for ventricular cardiomyocyte isolation. With the Langendorff technique, the heart is mounted on a cannula via the aorta, and retrogradely perfused with solutions that are either gravity-fed (constant pressure perfusion) or pump-fed (constant flow perfusion). Cardiomyocyte isolation is accomplished by including enzymes in the perfusate, with careful regulation of temperature and extracellular [Ca^{2+}], as detailed in the methods protocols. Reproduced from ref. [9] with permission

seems to be inevitable ([9] and Fig. 2). Inclusion of growth medium is widely reported to minimize run-down of cell quality over time, possibly by strengthening plasma membranes [31], and to promote cell adherence to the bottom of the culture vessel [30]. Indeed, serum inclusion has become standard procedure in most protocols for mammalian cell culture. However, cardiomyocytes are not normally in direct contact with serum in vivo, and investigators should be aware that its precise effects on cultured cardiomyocytes are not completely understood. Addition of taurine, L-carnitine, and creatine to the culture medium have also been shown to improve cell maintenance, and several protocols have included the use of insulin and exclusion of L-glutamine (when not adding serum) [32, 33]. Lastly, in accordance with Tian and colleagues [34], we found that adding submicromolar cytochalasin D (cyto D) helped preserve the t-tubule network during extended cultivation periods (Fig. 2).

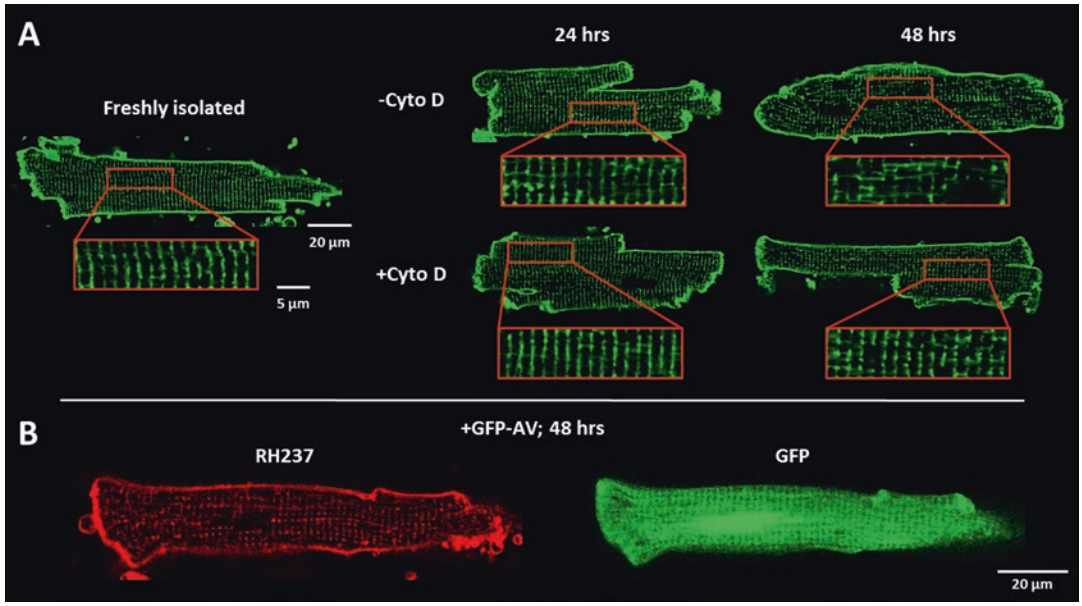

Fig. 2 Representative confocal micrographs of freshly isolated and cultured adult rat cardiomyocytes. (**a**) Progressive changes in Wistar rat cardiomyocyte morphology during cell culture. Cell membranes were stained for 20 min with 50 μM di-8-ANEPPS (Thermo Fisher) and imaged using a Zeiss LSM710 microscope (Carl-Zeiss AG, Oberkochen, Germany). Inclusion of 0.5 μM cytochalasin D (cyto D) in the culture medium minimized time-dependent disruption of t-tubule structure. (**b**) Representative rat cardiomyocyte transduced with an adenovirus construct that drives green fluorescent protein (GFP) expression. The t-tubule network was visualized by co-staining with 12.5 μM RH237 (Thermo Fisher) for 15 min

Detailed protocols for successful adenoviral gene transfer are outlined in the following sections, starting with advanced preparation of materials and equipment, and including required methods for cell isolation and culture. These protocols are based on both previous published work and our own developed methods.

2 Materials

Cultivation of isolated adult cardiomyocytes requires a sterile environment. Therefore, all materials should be treated accordingly (*see* **Note 1**).

2.1 Commercial Products

Langendorff apparatus (*see* Fig. 1 and **Note 2**).

Heparin LEO, 5000 IE/ml.

Coverslips (type varies depending on experimental requirements).

Multiwell plates (sterile).

Natural mouse laminin.

Poly-L-lysine solution.

Medium 199.

Fetal bovine serum.

Penicillin–streptomycin.

Sodium chloride.

Magnesium chloride hexahydrate.

Potassium chloride.

HEPES.

D-(+)-glucose monohydrate.

Cytochalasin D.

L-carnitine hydrochloride.

Taurine creatine.

Bovine serum albumin.

Calcium chloride solution.

Sodium dihydrogen phosphate monohydrate.

Collagenase, Type 2 (catalog number: LS004176; Worthington Biochemical Corporation, Lakewood, NJ, USA; *see* **Note 3**).

Deoxyribonuclease I (DNase I, catalog number: LS002006; Worthington).

Cell counting chamber (e.g., glass slide with a Bürker pattern).

Open Mesh Fabric with ≈250 μm openings (e.g., catalog number: 03-255/46; SEFAR, Heiden, Switzerland).

2.2 Custom Solutions

Cell Isolation Solution (CIS, Table 2).

Adult Cardiomyocyte Maintenance Medium (ACMM, Table 3).

Table 2
Components in CIS

Chemical	Molecular Weight (g/mol)	Concentration (mM)
NaCl	58.44	140
HEPES	238.30	5
$MgCl_2 \bullet 6\ H_2O$	203.30	0.5
KCl	74.55	5.4
$NaH_2PO_4 \bullet H_2O$	137.99	0.4
D-Glucose monohydrate	198.17	5.5

Table 3
Components in ACMM

Chemical	Molecular Weight (g/mol)	Concentration
Creatine	131.13	5 mM
L-carnitine hydrochloride	197.66	2 mM
Taurine	125.15	5 mM
Cytochalasin D	507.62	0.5 μM

3 Methods

3.1 Preparation of Custom Solutions (See Note 1)

3.1.1 CIS (Table 2)

1. Dissolve all components in Table 2 in a beaker containing roughly 900 ml MQ dH_2O.
2. Adjust pH with 1 M NaOH to 7.4.
3. Correct volume to 1 l using a tall-necked glass volumetric flask.
4. Sterile filter using 0.22 μm millipore filter into an autoclaved bottle.
5. Store at 4 °C for up to 1 week.

3.1.2 ACMM (Table 3)

1. Dissolve all components in Table 3 in a beaker containing roughly 900 ml of M199.
2. Sterile filter using 0.22 μm millipore filter.
3. Add 100 units/ml of penicillin and 100 μg/ml of streptomycin.
4. Adjust volume to 1 l using a tall-necked glass volumetric flask.
5. Add 1 % fetal calf serum (FCS) to 1/3 of the medium in a separate bottle (used for initial seeding; medium lacking serum used for all subsequent cultivation).
6. Store at 4 °C for up to 2 weeks.

3.2 Isolation of Adult Cardiomyocytes

Carry out all steps at room temperature unless otherwise specified.

3.2.1 Day Before Isolation

1. Autoclave coverslips if performing imaging or other types of experiments requiring a glass substrate.
2. Prepare laminin coating solution by diluting natural mouse laminin in M199 medium to a final concentration of 10 μg/ml.
3. Make appropriate volumes of both CIS and ACMM (*see* Subheading 3.1).
4. Dilute bovine serum albumin (BSA) in MQ dH_2O to a final concentration of 20 mg/ml (*see* **Note 4**) and aliquot into appropriate working volumes; store at −20 °C.

5. Coat coverslips with poly-L-lysine for 15 min, followed by laminin coating solution overnight at 4 °C.

3.2.2 Day 1: Isolation and Seeding

Isolation Protocol

1. Thaw BSA solution, and keep on ice along with CIS, collagenase, and DNase.
2. Aerate CIS solution with medical-grade O_2 (approximately 10 min for 1 l solution).
3. Prepare following solutions and maintain at 37 °C:
   - *CIS/BSA*: 28.5 ml CIS + 1.5 ml BSA solution.
   - *0.1 mM* Ca^{2+}: 14.25 ml CIS + 750 ml BSA solution + 1.5 µl 1 M $CaCl_2$.

   Additionally, aliquot enough CIS for **step 13** and warm to at least room temperature.
4. Prepare 2 mg/ml collagenase solution by mixing 50 mg collagenase in 25 ml CIS, and add 4 µl 0.1 M $CaCl_2$ to activate the enzyme.
5. Prime the Langendorff apparatus (*see* Fig. 1) with the collagenase and CIS solutions.
6. Adult rats are anesthetized in a chamber filled with 5% isoflurane (Abbott Scandinavia Ab, Solna, Sweden) and a 95% mixture of N_2O and O_2. Hearts are quickly excised by thoracotomy and placed in ice cold 0.15 M NaCl solution containing roughly 1 ml of heparin (*see* **Notes 5–7**).
7. Trim away excess tissue so that the aorta is easily accessible; hang heart on appropriately sized cannula and fasten around remaining aorta with sutures.
8. Start perfusion with Langendorff apparatus. If using a constant-flow set-up, adjust pump speed so that flow rate is 3 ml/min (1 drop per 1.8 s). Perfuse heart with CIS until clear of blood (*see* **Note 8**).
9. Switch to collagenase solution and digest for 10–12 min from the time the collagenase reaches the cannulated heart (this time can vary and it is advisable to optimize all newly ordered batches of collagenase); recycle the collagenase solution as needed.
10. Once digested, the myocardium becomes pale and soft. When approaching this point, let sufficient circulating collagenase solution accumulate in a disposable vessel. Cut down the heart and submerge in collagenase solution. Trim away the left and right atria and right ventricle; divide the remaining left ventricle into small pieces (3–4 mm^3).
11. Use thin-tipped forceps to tease the left ventricular tissue apart, thereby increasing the amount of surface area exposed for secondary digestion.

12. Transfer approximately 8 ml of tissue and collagenase solution to a 10 ml Falcon tube containing 0.2 mg DNase in 500 µl BSA. Pipette up and down **GENTLY** with a plastic pipette (*see* **Note 9**) to free additional cells from tissue.
13. Filter cell suspension through SEFAR Nitex® open mesh fabric with a pore size of 255 µm (*see* **Note 10**). Adjust filtered cell suspension to a volume of 10 ml with pre-warmed CIS.
14. Allow cells to pellet, then wash out the remaining collagenase solution by removing most of the supernatant and rinsing the cell pellet with the CIS/BSA solution followed by the 0.1 mM Ca^{2+} solution. Avoid disturbing the cell pellet as much as possible.
15. Allow cells to acclimatize for 10 min, then gradually increase [Ca^{2+}] by adding the following amounts of **1 M** $CaCl_2$ directly to the cell suspension (per 10 ml cell suspension; allow cells to acclimatize between each increase):
    - 1.0 µl = 0.2 mM Ca^{2+}.
    - 3.0 µl = 0.5 mM Ca^{2+}.
    - 5.0 µl = 1.0 mM Ca^{2+}.
16. Remove the supernatant and resuspend the cell pellet in ACMM containing 1 % fetal bovine serum (FBS) before plating.

Cell Seeding

17. Count the cell density with a hemocytometer (e.g., a Bürker chamber) (*see* **Note 11**).
18. Seed cells at a density between 5.0×10^3 and 1.0×10^4 cells/cm^2 (e.g., for a 6-well plate: 4.75×10^4–9.5×10^4 cells per well).
19. Use a microscope to check the confluency of the seeded cells, then allow to attach for 30 min–1 h.
20. Once cells are attached, replace growth medium with ACMM lacking serum. When exchanging, gently swirl medium around wells to remove as many dead/unattached cells as possible.
21. If virally transducing cells, change to serum-free medium and add vector(s) at desired multiplicity of infection (MOI, *see* **Note 12**).
22. Incubate cells at 37 °C and 5 % CO_2; exchange growth medium every 24 h.
23. If using fluorescently tagged constructs, check for expression of your fluorophores 24–48 h following transduction.

4 Notes

1. Make sure that the water used is pure. Normally, MilliQ water (MQ) with resistivity of 18.2 MΩ cm is adequate. Depending on the tubing mounted to the filtration system, fungus, bacteria,

molds, etc. may be present in the water. Thus, autoclaving should also be considered. All bottles and tubes used in the Langendorff apparatus should also be autoclaved. Between each use the Langendorff apparatus should be cleaned using 70% ethanol. If possible the cell isolation should be conducted inside a biosafety cabinet with HEPA filter.

2. In a typical Langendorff apparatus used for isolating cardiomyocytes [25], tubing leads perfusate (CIS) from a solution reservoir down to a cannula (Fig. 1). The temperature is regulated via a heating coil that surrounds the perfusion tubing. The heart is mounted via the aorta and depending on the size of the aorta the cannula must be carefully chosen (for mice between 22 and 16 gauge (0.6–1.3 mm) and for rats from 14 to 8 gauge (1.6–3.2 mm)). The cannula can be made from glass or metal. A groove near the bottom of the cannula may aid in securing the aorta if a ligature is to be used.
3. In order to have stable conditions when chemically dissociating the cells, different lots of collagenase should be tested before the experiment. Worthington provides an online system where several different lots can be ordered and tested before ordering larger stocks. Typically, three different lots are tested. In addition, if using modified (e.g., genetically, banded, infarcted) animals the collagenase treatment should be tested on these animals as well. Choose the collagenase that maximizes cell yield and quality.
4. Slowly dissolve the BSA in a glass beaker with a magnetic stir bar. Too much agitation will cause a large amount of foam to form, which makes subsequent volume measurements difficult.
5. The protocol described has been optimized for male and female Wistar rats (Møllergaard Breeding and Research Center, Skensved, Denmark) weighing between 150 and 230 g. It is important to minimize the stress experienced by the animals as it can affect the neurohumoral state of the cells. Animals can also be anesthetized using injectable (pentobarbital, ketamine/xylazine) anesthetics [35–37]. However, some reports have demonstrated that inhalable anesthetics may be preferable in small animals as they avoid potential myocardial ischemia [38].
6. When operating animals, make sure to use clean, well-maintained surgical equipment. Lay the anesthetized animal on its back, exposing its ventral side. Using surgical scissors, remove a small patch of skin/fur from just below the rib cage. Cut through the muscle so as to expose the top of the liver and the diaphragm. Make a small incision on one side of the diaphragm, and cut across the body, detaching the diaphragm from the rib cage (take care to avoid the heart). Cut through the ribs towards the anterior end of the animal, and lift the rib cage in order to expose the lungs and heart. Carefully pierce

the pericardium and remove from around the heart. Using curved forceps, clamp the ascending aorta just below the aortic arch while gently lifting the heart to better expose the aorta. Using sharp, small scissors, cut the aorta just above the forceps, then quickly clip all other circulatory vessels and transfer the heart to ice-cold CIS. Avoid accidentally puncturing or cutting the heart during all steps.

7. Animals can also be injected with heparin (400–5000 u/kg body weight) 10–20 min before the anesthetization to prevent blood clotting and possible myocardial infarction [35, 37, 39, 40]. We recommend making an injectable heparin solution by diluting stock heparin in 0.9% NaCl at a 1:5 ratio.
8. Sufficient perfusion of the coronary arteries is crucial during adult rodent cardiomyocyte isolation. The cannula should be carefully inserted into the ascending aorta, making sure not to puncture the aortic valve. If properly positioned, the incoming perfusate will cause the aortic valve to close, and thereby be circulated through the coronary arteries. This process is easily monitored, as the larger coronary arteries are clearly visible in rodent hearts. If the blood remaining in the arteries following excision does not clear during perfusion, then either try repositioning the cannula or take down the heart and reattempt cannulation.
9. When titrating tissue, it is recommended to use a graduated Pasteur pipette. In order to prevent additional shearing forces on the cells, use scissors to remove the lower portion of the pipette tip so that the digested tissue chunks fit through the enlarged opening.
10. Cut out a small (roughly 5–6 cm^2) piece of SEFAR Nitex® Open Mesh Fabric and fold into a funnel shape.
11. Adult cardiomyocytes are large cells and may get caught in the small opening between the base of the hemocytometer and the coverslip. It is therefore recommended to place a small volume of cell suspension (roughly 5 μl) directly over the grid etched in the base of the hemocytometer. Afterwards, gently sit the coverslip over each grid and secure as tightly as possible. Count the total number of cells according to the instructions provided with your hemocytometer of choice. We found that the cardiomyocytes adhere faster by adding 1% serum to the culture medium for 1 h prior to further cultivation without serum (similar to ref. [41]).
12. Care must be taken when optimizing your working virus concentration. The virus titer is provided with all commercially ordered viruses and is typically listed as either PFU (plaque forming unit) or IFU (infectious unit), which reflects the number of *live/infectious* virus particles in suspension. This measurement, along with the total number of cells seeded in culture, can be used to calculate the multiplicity of infection (MOI):

$$\text{MOI} = \frac{\text{Number of active virus particles}}{\text{Number of cells in culture}}$$

The MOI describes how many *live* virus particles are *available* to each cell in your culture. As an example, if your virus titer is 1.0×10^9 IFU/ml and you add 10 µl of stock virus suspension to 1.0×10^6 cells, the MOI is calculated as follows:

$$\text{Number of active virus particles} = \text{Titer} \times \text{Added volume of stock virus}$$

$$\text{Number of active virus particles} = 1.0 \times 10^9 \frac{\text{IFU}}{\text{ml}} \times 0.010\ \text{ml}$$

$$\text{Number of active virus particles} = 1.0 \times 10^7$$

Therefore:

$$\text{MOI} = \frac{\text{Number of active virus particles}}{\text{Number of cells in culture}} \quad \text{MOI} = \frac{1.0 \times 10^7}{1.0 \times 10^6} \quad \text{MOI} = 10$$

Assuming Poisson distribution (i.e., that all cells are equally susceptible to infection), an MOI of 10 would be sufficient to infect 99% of all cells in culture. This can be assumed for most clonal cell lines, however, may not necessarily be the case for a primary cell line like adult cardiomyocytes. Therefore, it is imperative that this value be optimized for each experiment as infectivity can also be influenced by factors like cell density and volume of culture medium. Keep in mind that in order to determine transfection efficiency you need a reporter like green fluorescent protein (GFP; ~715 bp, Fig. 2b) in addition to the promoter and poly(A) tail signal. These modifications minimize the cloning capacity further. However, to avoid using long bicistronic cloning vectors with Internal Ribosomal Entry Site (IRES) when expressing two genes, a novel strategy using a self-cleaving 2A peptide sequence may be used (for details *see* refs. [42, 43]).

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Chapter 12

Gene Transfer in Cardiomyocytes Derived from ES and iPS Cells

Francesca Stillitano, Ioannis Karakikes, and Roger J. Hajjar

Abstract

The advent of human induced pluripotent stem cell (hiPSC) technology has produced patient-specific hiPSC derived cardiomyocytes (hiPSC-CMs) that can be used as a platform to study cardiac diseases and to explore new therapies.

The ability to genetically manipulate hiPSC-CMs not only is essential for identifying the structural and/or functional role of a protein but can also provide valuable information regarding therapeutic applications. In this chapter, we describe protocols for culture, maintenance, and cardiac differentiation of hiPSCs. Then, we provide a basic procedure to transduce hiPSC-CMs.

Key words Human pluripotent stem cells (hPSCs), hPSC-derived cardiomyocytes, Transduction, Gene therapy, Cardiac differentiation

1 Introduction

Gene transfer is defined as a technique to efficiently and stably introduce foreign genes into the genome of target cells [1]. This technique is essential not only for studying the structural and/or functional role of a gene/protein but also as a novel approach for the investigation and potential treatment of a variety of diseases, including cardiac disease [2–7]. Studies in isolated cardiomyocytes have increasingly employed gene transfer technology to investigate the mechanisms regulating myocyte structure and function, providing significant insights into Ca^{2+} cycling and the role played by individual sarcomeric proteins in modulating contractile function [7–11]. Gene delivery to cardiomyocytes is currently of interest as a treatment approach in cardiovascular disease, and targeted genetic alteration strategies established in single myocytes are now being investigated for their efficacy in vivo [12–18]. Current preclinical platforms for evaluating gene therapy approach often use animal models, which can be

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inaccurate predictors of human cardiac pathophysiology due to interspecies differences in cardiac structure, electrophysiology, and gene expression [19–21].

Indeed, despite positive results from numerous preclinical studies in animal models of cardiovascular diseases [7, 16, 17, 22, 23], the translational progress into clinical practice has been remarkably slow.

Therefore, there is a critical need for using "human-based" cellular platforms to better investigate gene transfer/delivery processes in order to validate the potential of gene therapies, not only in animal models but also in "human" derived cardiomyocytes. Due to the fact that human cardiac tissues are difficult to obtain, cardiomyocytes derived from human pluripotent stem cells (hPSC-CMs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) [24, 25], may offer a valuable tool for gene therapy studies.

The ability to differentiate hPSCs in a directed manner has progressed considerably in the past 10 years and a substantial effort has been made to develop strategies to efficiently and reliably direct stem cell differentiation to the cardiovascular lineage [26, 27].

hPSC-CMs, in particular hiPSCs-CMs, can be used as a human model to study cardiac disease [28–30] which implies also the evaluation of the therapeutic potential of different processes, and importantly gene therapy. Recently, the effectiveness of gene delivery systems in hPSC-CMs has been revealed and viral vectors that can be used to genetically manipulate the hESC/hiPSC system have been identified [31]. Our laboratory routinely produces hPSC-CMs and utilizes viral-mediated gene transfer. Here, we describe in detail our current experimental protocols for culturing hESC/iPSCs, cardiac differentiation of hESC/iPSCs and gene transfer into hPSC-CMs.

2 Materials

Use sterile techniques to prepare all solutions.

2.1 Feeder-Free Cultivation of Human Pluripotent Stem Cells

1. mTeSR®1 media (Basal Media + 5× Supplement).
2. 0.2 μm low-protein binding filter.
3. DMEM/F12 Media.
4. BD Matrigel™ hESC-qualified Matrix (*see* **Note 1**).
5. ROCK inhibitor, Y-27632 (*see* **Note 2**).
6. Dulbecco's phosphate-buffered saline (DPBS) 1×, without Ca^{2+}/Mg^{2+}.
7. Tissue culture plates, 6-well.
8. Cell Lifter.

9. Conical 50 ml tube.
10. Sterilized tissue culture hood.
11. Ultrapure water.
12. 5 ml serological pipette.
13. CO_2, O_2 adjustable incubator.

2.2 Direct Cardiac Differentiation

1. mTeSR®1 media.
2. StemPro®-34 complete media (StemPro®-34 Basal Media, StemPro®-34 Nutrient Supplement, l-ascorbic acid, 1× GlutaMAX) (*see* **Note 2**).
3. 0.2 µm low-protein binding filter.
4. Conical 15 ml tube.
5. Ultra-Low attachment surface 6-well plates.
6. Human Recombinant BMP4 (*see* **Note 2**).
7. Human Recombinant Activin-A (*see* **Note 2**).
8. Blebbistatin (*see* **Note 2**).
9. IWR-1 (*see* **Note 2**).
10. D0 media: mTeSR®1, BMP4, Blebbistatin, details in Table 1.
11. D1 media: StemPro®-34 complete, BMP4, Activin A, details in Table 2.
12. D3 media: StemPro®-34 complete.
13. D4 media: StemPro®-34 complete, IWR-1, detail in Table 3.

2.3 Gene Transfer

1. Dulbecco's phosphate-buffered saline (DPBS) 1×, without Ca^{2+}/Mg^{2+}.
2. DetachKit 2: Trypsin/EDTA Solution (0.025%/0.01%) plus Trypsin Neutralizing Solution (TNS) (*see* **Note 3**).
3. StemPro®-34 complete media.
4. ThermoMixer F1.5.
5. Centrifuge machine.
6. Incubator.
7. Vector for gene transfer.

Table 1
D0 media composition

Reagent	Stock solution	Working solution	Concentration (v/v)	Volume for 6 wells
mTeSR®1				12 ml
BMP4	10 µg/ml	10 ng/ml	1 µl/ml	12 µl
Blebbistatin	50 mM in DMSO	5 µM	0.1 µl/ml	1.2 µl

Table 2
D1 media composition

Reagent	Stock solution	Working solution	Concentration (v/v)	Volume for 6 well = 12 ml
StemPro®-34 complete				12 ml
BMP4	10 μg/ml	10 ng/ml	1 μl/ml	12 μl
Activin A	25 μg/ml	25 ng/ml	1 μl/ml	12 μl

Table 3
D4 media composition

Reagent	Stock solution	Working solution	Concentration (v/v)	Volume for 6 wells
StemPro®-34 complete				6 ml
IWR-1	10 mM in DMSO	2.5 μM	0.75 μl/ml	4.5 μl

3 Methods

Equilibrate all media reagents to room temperature before starting.

Perform all steps under a sterile tissue culture hood.

3.1 Feeder-Free Cultivation of Human Pluripotent Stem Cells

In order to propagate hPSCs successfully, cultures must be serially passaged when colonies become large and near-confluent by dissociation into small cell aggregates and seeding onto new plates (*see* **Note 4**). The following protocol is a new, enzymatic-free alternative for passaging hPSCs, using DPBS 1×, without Ca^{2+}/Mg^{2+}.

3.1.1 Preparation of Matrigel-Coated 6-Well Plates

1. Thaw one aliquot of the hESC qualified Matrigel slowly at 4 °C in the fridge (~1–2 h), and dilute it in a conical 50 ml tube that contains 24 ml of DMEM/F12. Mix well in sterilized tissue culture hood immediately before coating 6-well plates.
2. Add 1 ml per well of the diluted Matrigel ensuring that the whole surface is covered with a liquid film (1 aliquot of the hESC qualified matrix is sufficient to coat four 6-well plates).
3. Allow hESC qualified Matrigel to set for 30 min at room temperature under the hood. Use plates immediately, or if desired, store at 4 °C immediately after plating for up to 1 week and equilibrate to room temperature for 30 min prior to use.

3.1.2 Passaging and Maintenance of Human Pluripotent Stem Cells (hPSCs)

1. Thaw mTeSR™1 5× Supplement (*see* **Note 5**) and add to 400 ml of mTeSR™1 Basal Media. Mix thoroughly. Filter using a 0.2 μm low-protein binding filter and use immediately or store at 4 °C for up to 2 weeks.
2. Maintain hPSCs in culture in 6-well plates and use 2 ml/well of mTeSR®1 media. Prepare the desired volume of mTeSR®1 with ROCK inhibitor.
3. If stored at 4 °C, warm the prepared Matrigel coated plates to room temperature by placing them in sterilized tissue culture hood for at least 30 min prior to plating cells.
4. Use a microscope to visually identify regions of differentiation and sterilely remove them by scraping with a pipette tip.
5. Aspirate the media, add 1 ml of DPBS 1×, without Ca^{2+}/Mg^{2+} and incubate for 7–10 min (clone-dependent) at room temperature.
6. While cells are incubating, prepare the Matrigel plate. Aspirate the Matrigel; label the new plate with the cell line name, the new passage number and the date. Add 1 ml of mTeSR®1 with ROCK inhibitor to each well.
7. Aspirate the DPBS 1× and add 1 ml of mTeSR®1 media with ROCK inhibitor.
8. Scrape the cells using a cell lifter. Use a 5 ml serological pipette to break the clumps by pipetting. Do not break the colonies into single cells.
9. Add more mTeSR®1 media to achieve the desired split ratio for the new plate. For example: 1:6 split of 1 well into 6 wells- scrape with 1 ml total, add 5 ml more and plate 1 ml into each well.
10. Add 1 ml of the cell suspension into each well of the new Matrigel plate.
11. Gently move in a front-to-back and side-to-side motion to uniformly disperse cells across the well, and return to 5 % CO_2, 6 % O_2 incubator.
12. Beginning 24 h after passaging, replace media daily using 2 ml/well mTeSR®1 without ROCK inhibitor.

3.2 Direct Cardiac Differentiation

Directed cardiac differentiation protocol consists of two stages: in stage 1 (days 0–4.5), the hPSCs grown in feeder independent conditions are differentiated toward a multipotent cardiovascular progenitor population by the combinatorial activation of the BMP and nodal/activin signaling pathways. In stage 2 (days 4.5–8), the uncommitted progenitors are terminally differentiated toward ventricular-like cardiomyocytes by the inhibition of the WNT signaling pathway with the small molecule IWR-1 [26] (Fig. 1).

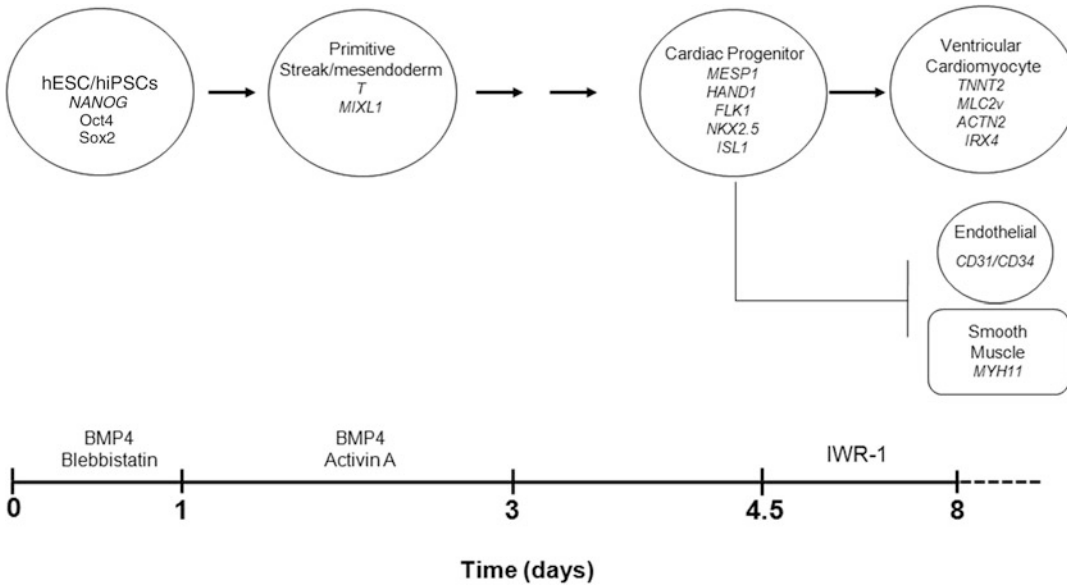

Fig. 1 Summary of the hPSC-CM differentiation protocol

3.2.1 Day-0: Embryoid Bodies (EBs) Formation (See Note 6)

1. Aspirate mTeSR®1 media.
2. Add 2 ml of DPBS 1×, without Ca^{2+}/Mg^{2+} to each well and incubate for 7–10 min at room temperature.
3. Aspirate DPBS 1×, without Ca^{2+}/Mg^{2+} and add 1 ml of D0 media.
4. Scrape the cells very gently using a cell lifter; use a 5 ml serological pipette to break the clumps by gently pipetting. Do not break the colonies into single cells.
5. Distribute the cells into a low cluster plate (1:1 split) and add 1 ml more of D0 media.
6. Incubate for 24 h in 6% O_2/5% CO_2/37 °C.

3.2.2 Day-1 Mesoderm Induction

1. Prepare StemPro®-34 complete media: thaw StemPro®-34 Nutrient Supplement (*see* **Note 5**). Aseptically add the entire contents (13 ml) of StemPro®-34 Nutrient Supplement directly to a 500 ml bottle of StemPro®-34 Basal Media before use. Add 2.5 ml Ascorbic acid and 1× GlutaMAX. Swirl to mix. Filter using a 0.2 μm low-protein binding filter and use immediately or store at 4 °C for up to 2 weeks.
2. Transfer EBs in suspension to a 15 ml tube; allow ~10–20 min for settling by gravity at room temperature or 37 °C for longer incubation.
3. Remove supernatant, resuspend the EBs in D1 media, and transfer back into the original low cluster plate.
4. Incubate for 48 h at 20% O_2/5% CO_2/37 °C.

3.2.3 Day-3 Cardiac Induction

1. Transfer EBs in suspension to a 15 ml tube; allow ~10–20 min for settling by gravity at room temperature or 37 °C for longer incubation.
2. Remove supernatant, resuspend the EBs in D3 media, and transfer back into the original low cluster plate.
3. Incubate for 24 h at 20 % O_2/5 % CO_2/37 °C.

3.2.4 Day-4.5 Cardiac Induction

1. Prepare D4 media and add 1 ml/well (total volume: 3 ml/well).
2. Incubate at 20 % O_2/5 % CO_2/37 °C.

3.2.5 Day-5–7

1. Early spontaneous beating activity should be observed. If the media turns yellow add 1 ml of StemPro®-34 complete media in each well. Return the plate to the incubator.

3.2.6 Day-8

1. Replace the media with fresh StemPro®-34 complete media. Continue to replace with StemPro®-34 complete media every 3–4 days for desired time of culture.

3.3 Gene Transfer into Human Pluripotent Stem Cells

1. Set the thermo-mixer at 37 °C before starting.
2. Transfer EBs in suspension to a 1.5 ml tube; allow ~10–20 min for settling by gravity (*see* **Note 7**).

3.3.1 EBs Dissociation

3. Remove supernatant, add 750 µl of 0.025 % trypsin and place the tube on a thermo-mixer; set the thermo-mixer at 1400 rpm, 37 °C, and incubate for 10 min.
4. Pipet up and down with a P1000 pipet until complete dissociation is obtained (*see* **Note 8**).
5. Add 750 µl of trypsin inhibitor (TNS) and centrifuge at 300 × *g* for 5 min.
6. Aspirate the supernatant; resuspend the cells in 500 µl of StemPro®-34 Complete media.
7. Count the cells.
8. Seed the cells into a 6-well plate freshly coated with Matrigel in StemPro Complete media to allow attachment of dissociated PSC-CMs (*see* **Note 9**).
9. Wait until the cells start beating again (1–2 days) before gene transfer.

3.3.2 Gene Transfer

1. Change the old media with 2 ml of fresh StemPro Complete media per well and add the desired volume of the concentrated vector solution (containing the desired vector genome per cell) into the well. Distribute the vector solution equally by moving the plate.
2. Place the cells into the incubator (37 °C, 5 % CO_2) for 48–72 h and monitor for gene expression.

4 Notes

1. Slowly thaw hESC qualified matrix (5 ml) on ice at 4 °C overnight. Dispense aliquots into prechilled, 1.5 ml sterile microcentrifuge tubes and immediately store at –20 °C. The volume of the aliquot will vary based on lot and typically ranges 270–350 µl. Manufacturer provides details regarding volume of aliquot required to achieve a 1× concentration upon dilution into 24 ml of DMEM F-12.
2. Prepare Solutions using ultrapure water as a diluent unless otherwise indicated. Store each as aliquots at –20 °C: Rho kinase (ROCK) inhibitor Y-27632 (10 mM in DPBS, 100 µl aliquot); l-ascorbic acid (10 mg/ml, 2.5 ml aliquot); Human Recombinant BMP4 (10 µg/ml, 100 µl aliquot); Human Recombinant Activin A (100 µg/ml, 50 µl aliquot); IWR1 (10 mM in DMSO, 10 µl aliquot); Blebbistatin (50 mM in DMSO, 10 µl aliquot).
3. DetachKit 2 is a kit which contains Trypsin/EDTA Solution (0.025%/0.01%) plus Trypsin Neutralizing Solution (TNS). Make aliquots of 10 ml and store at –20 °C in the dark immediately after arrival. Thawed solutions can be stored at 4–8 °C and used for up to 6 weeks.
4. This protocol is for passaging of cells as small aggregates of approximately 50–200 µm in diameter. hPSCs are ready to passage when the majority of colonies are large, compact, and have centers that are dense compared to their edges. The passaging time usually occurs on day 4 or 5 after plating.
5. Thaw supplements at room temperature (15–25 °C) or overnight at 4 °C. Do not thaw in a 37 °C water bath.
6. Start cardiac differentiation when hPSCs are 80–90% confluent. hPSCs spontaneously form clusters which have compact appearances after 24 h in suspension culture, suggesting the development of a normal organized and complex EB structure. The formed EBs are highly uniform with high viability (Fig. 2).
7. Transfer EBs from one well to one 1.5 ml tube (1:1 split). Transfer 1.5 ml of EBs in suspension into 1.5 ml tube. Allow ~10 min for settling by gravity. Aspirate the supernatant and repeat this step until all EBs from one well are transferred.
8. Transfer one drop of cells on the top of a cover-slip and check at the microscope that single or very small groups of cells are obtained after EBs dissociation.
9. Use a split ratio 1:1- Dissociate EBs from 1 well, count the cells and plate to 1 well of a tissue culture 6-well plate freshly coated with Matrigel. About 10^6 cells are usually obtained using this protocol.

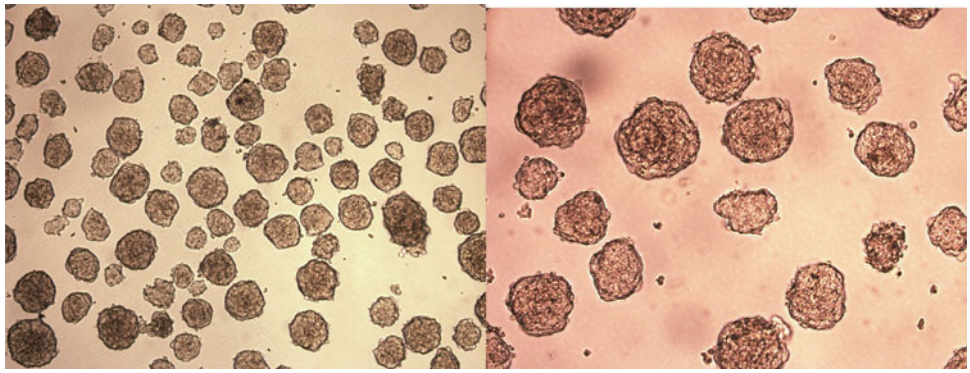

Fig. 2 EBs at day 4 of differentiation

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Chapter 13

Gene Transfer to Rodent Hearts In Vivo

Federica del Monte, Kiyotake Ishikawa, and Roger J. Hajjar

Abstract

Altering expression of specific genes in rodent models allows the dissection of various pathways involved in various disease states. Advances in gene transfer technologies produced vectors with tissue specificity, various time courses, increasing or knocking down genes in an externally controlled fashion. To achieve this goal, appropriate vector delivery method is essential for successfully conducting experiments. In this chapter, we focus on cardiac gene transfer in rodents that can be employed for delivering both viral and nonviral vectors.

Key words Rat, Mouse, Small animals, Adeno-associated virus, Adeno virus, Plasmid, Modified RNA, Gene delivery

1 Introduction

1.1 General Introduction

Gene transfer technologies have advanced tremendously over the last 10 years [1]. Even though the ultimate goal of gene transfer is to correct monogenic diseases or increase/decrease expression of genes clinically, in rodent models, however, altering expression of specific genes is very useful [2]. It allows the dissection of various pathways involved in various disease states. To allow for optimal pathway's targeting for therapy or for advancing the knowledge of diseases, molecular mechanisms, it is critical to carefully mind the design and delivery of vectors. Vector development allows for in vivo gene expression with tissue specificity, various time courses, increasing or knocking down genes in an externally controlled fashion [3]. In addition to the design of the vectors, their delivery method is important to achieve tissue expression without off site vector sequestration and viral inactivation as well as modifying the desired molecular aspect and not otherwise artificially manipulating the disease state (e.g., induction of ischemia or traumatic injury to the organ). These improvements in the vectors and their delivery are quite important in manipulating disease states. In this chapter we focus on cardiac gene transfer in rats. These methods can be,

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however, applied to mice models with slight modifications, although some procedures may be technically more demanding due to the size differences.

1.2 Cardiac Gene Transfer

Delivering genes through vectors to the myocardium has been attempted for over 20 years [4]. Initial attempts focused on using dsDNA injected directly into the ventricles but this initial approach had limited success. This was followed by the use of viral vectors to deliver the gene product. These provided higher efficiency of gene expression and allowed for easier delivery modalities as well as tissue specificity and persistence of expression when injected directly [5], throughout the coronary circulation by transiently blocking the cardiac outflow using cross-clamping techniques [6], or throughout the systemic circulation. However, the significant inflammation limited the usefulness of recombinant adenoviruses. The advent of recombinant adeno-associated vectors (AAV) and especially cardiotropic AAV serotypes (AAV9 and AAV10) has simplified gene transfer to the myocardium and intravenous injections of AAV9 results in significant myocardial uptake that is persistent [7]. Modified RNA used as a gene therapy vector has been very potent in inducing gene expression for a short period of time [8]. We discuss the various techniques of gene transfer regardless of the types of vector (viral or nonviral) used.

1.3 Summary

Genetic manipulation of specific genes in rodent models allowed a better understanding of pathways involved in various disease states. Short- and long-term gene expression can be achieved using various vectors to manipulate disease pathways acutely or testing the sustained effect of genetic loss or gain of function. For short-term expression, modified RNA has become the vector of choice, but it requires more invasive procedures. The development of cardiotropic AAV vectors instead has allowed noninvasive, simple injection of these vectors in the tail vein to obtain long-term gene expression or suppression. Selection of vectors is available not only for different time of expression but also to target specific tissues or cell types. AAV isotypes allows to target specific organs whereas the use of cell specific promoters in adeno or AAV vectors target all cells but express only in individual cell types. Cell types targeting can be achieved also with the use of different vectors. Lentiviral gene transfer for example can also be used in cases where fibroblasts need to be transduced for differentiation purposes (direct reprogramming and iPS cells). As vectors become more sophisticated at targeting specific cell types the delivery methods should become easier in animal models and ultimately in human.

2 Materials

1. *Gene Therapy Product*: The gene therapy product can be a viral or nonviral product. For cardiovascular application the most common viral-based vectors include: recombinant adenoviruses [9, 10], AAV [11], and lentiviruses [12] while the most common nonviral vectors include: double stranded DNA [13] and modified RNA [14]. Materials and methods for both viral and nonviral preparations are discussed elsewhere in this series (*see* **Note 1**).
2. *Solution/nanoparticles used with vectors*: *Depending on the vectors used*, *different solutions are used during targeted cardiac delivery*.
   (a) Vector suspension solutions for the adenovirus: 10 mM Tris–HCl pH 7.4, 1 mM $MgCl_2$, 10% (volume/volume) glycerol stock solution (*see* **Note 2**).
   (b) Vector suspension solutions for lentivirus, AAV vectors, and double stranded DNA: Phosphate Buffered Solution (PBS) or normal saline.
   (c) Vector suspension solutions for modified RNA, cationic lipid based nanoparticles: Active epoxide-derived lipidoid reagent, stabilizing lipid excipients including distearoyl phosphatidylcholine (DSPC), cholesterol, a polyethylene glycol (PEG)–lipid conjugate.
3. *Rats*. 150–350 g male or female rats can be obtained from many vendors. The strains of animals can be Fisher 344, Brown Norway, Sprague Dawley, or Wistar. It is important that the animals are acclimated for 2–3 days prior to surgery.
4. *Ventilator*.
5. *Medications for anesthesia*, *analgesia*, *and prophylactic antibiotics*.
6. *Standard surgical instruments including*:
   2 Dumont tweezers.
   2 Adson forceps.
   2 Kelly hemostatic forceps.
   1 surgical scissor, 14 cm.
   1 self retaining retractor.
   1 μ bulldog clamp 3 cm long.
   16–18 G soft catheters for tracheal intubation.
   22 G soft catheter.
   1 High temperature cautery.
   4.0 nylon sutures.
   7.0 nylon sutures.

3 Methods

The feasibility of in vivo cardiac gene transfer by viral vectors has been consistently demonstrated both in large and smaller animals. A number of mechanical approaches have been used to achieve cardiac gene transfer as shown in Table 1. These methods have been refined for rodent hearts and specifically for rat models. Below we cover the methodologies for the different delivery approaches in the rat.

3.1 Preparation of the Rat

1. Rats of different types of colonies (Fisher 344, Sprague Dawley, Brown-Norway, or Wistar) are obtained. Experimental groups should be appropriately designed and randomized [15]. The rats should be free of antibody titers to a number of routinely tested rat viruses. In addition, the rats should be free of all endoparasites and ectoparasites and mycoplasma species.
2. The rats are maintained in a barrier room at 72 ± 2 °F with a relative humidity at 50 ± 10 %, and fed a commercial laboratory diet and water ad libitum. Ventilation in the room should be between 12 and 15 air changes per hour of 100 % pre-filtered outside air. The light cycle period should be controlled at 12 h of light and 12 h of dark with no twilight transition. The Institutional Animal Care and Use Committee will need to approve the study.

3.2 Anesthesia

1. Rats are anesthetized with intraperitoneal pentobarbital (40–60 mg/kg) or other appropriate drugs approved in the institutional protocols.
2. A rectal temperature probe is placed in the animal and a heating lamp is used to maintain body temperature while the animal lies dorsally.
3. The animals are intubated via the larynx by using a 16 or 18 G soft catheter. A small light source is placed on the neck over the

Table 1
Methods of gene transfer in rats

• Intra-myocardial Injection
• Intra-pericardial Injection
• Clamping of the aorta
• Cross-clamping of the aorta and pulmonary artery
• Ultrasound destruction of micro-bubbles combined with vectors
• Tail vein injection (mainly with AAV9)

larynx and the tongue is lifted. This allows the larynx to be clearly visualized permitting the angiocath to be accurately introduced.

4. The rats are ventilated with tidal volumes of 2 cm^3 at 50 cycles/min with an FiO_2 of 0.21. The ventilation should be operated in volume-cycled, positive-pressure mode. To produce tidal volumes, a constant airflow is gated into the animal during inspiration (*see* **Note 3**).

3.3 Viral/Nonviral Vector Preparation

(a) For recombinant adenoviruses, the glycerol solution is diluted at least 1:1 with normal saline.

(b) For lentivirus, AAV vectors, and dsDNA, PBS or normal saline can be used, and these solutions can be directly injected into the animals or appropriately diluted.

(c) For modified RNA, use cationic lipid based nanoparticles. Prior to injection the epoxide-derived lipidoid reagent is made by nanoprecipitation of lipidoid with modRNA at high concentration followed by dialysis against phosphate buffered saline (PBS) for a minimum of 3 h at 4 °C.

1. Dilute the vector in appropriate solution to the desired concentration, and prepare in a syringe (*see* **Note 1**). For adenovirus and modified RNA, the syringe should be kept on ice until just prior to injection.

3.4 Direct Injection into the Ventricular Wall

Direct injection of gene vector solution into the ventricular wall using an epicardial approach has also been shown to induce significant expression of reporter constructs (Fig. 1). There are different approaches for this intervention. In an open-chested, ventilated animal, direct inspection of the heart is possible and injection directly into the area of interest is possible.

1. Remove the hair on the chest and thoroughly disinfect the surgical area.
2. Gently cut the skin and open the chest by dissecting the sternum.
3. Open the pericardium and expose the heart.
4. Using a 30 G needle, the anterior, lateral, and infero-posterior walls can be readily penetrated (*see* **Note 4**). For each injection, it is important to introduce the needle only a few millimeters (since the walls are 1–3 mm in thickness). Angling the needle at 30–60° in relation to the ventricular wall yields the best results. Investigators have used volumes of 10–50 μl per injection. In this case, it is important to choose the solution in which the vector is placed judiciously (*see* **Notes 5**–**7**).
5. Close the chest and recover the animal.
6. Administer appropriate analgesic drugs and antibiotics.

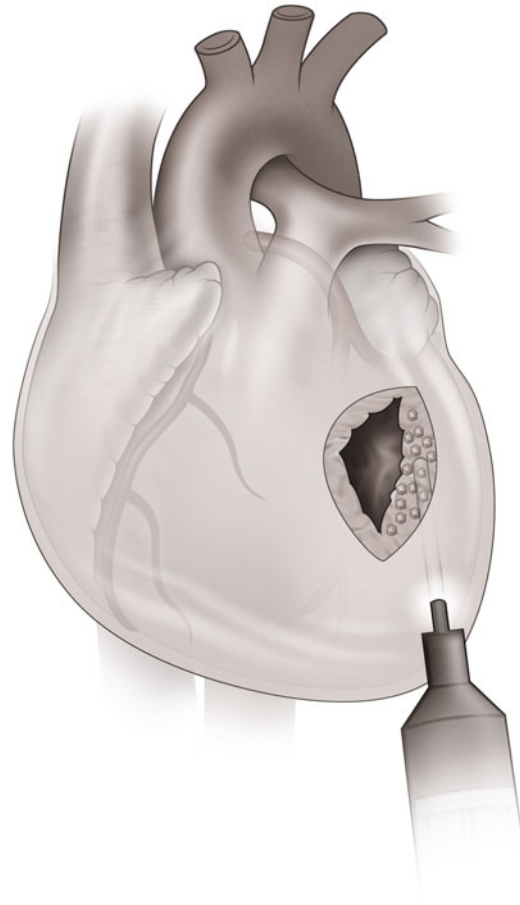

Fig. 1 Direct injection of the vector solution into the myocardium

3.5 Pericardial Gene Transfer

This method yields mainly epicardial staining.

1. Remove the hair on the chest and thoroughly disinfect the surgical area.
2. A small incision is made below the xiphoid in a transverse fashion.
3. Identify the pericardial sac and obtain a good view.
4. Using a 30 G needle, deliver the vector (100 μl) inside the pericardium (*see* **Note 8**).
5. Close the chest and recover the animal.
6. Administer appropriate analgesic drugs and antibiotics.

3.6 Cross-Clamping of the Aorta and Pulmonary Artery

This method achieves grossly homogeneous transduction of cardiac myocytes throughout the left and right ventricles of the heart. More importantly, this technique can produce dramatic, transgene-specific physiological effects on ventricular function in vivo. The success of this approach likely reflects in vivo optimization of the

parameters previously shown to be important for ex vivo gene transfer which: (1) the use of crystalloid solution as opposed to whole blood, (2) high coronary flow rate, (3) exposure time, (4) virus concentration, and (5) temperature (*see* **Note 9**).

1. Remove the hair on the chest and thoroughly disinfect the surgical area.
2. Gently cut the skin and open the chest by dissecting the sternum (*see* **Note 10**).
3. Open the pericardium and expose the heart.
4. Place a 7-0 suture at the apex of the left ventricle.
5. Identify the aorta and pulmonary artery, and gently isolate these vessels for clamping.
6. Advance a 22 or 24 G catheter (depending on the size of the animal) containing 200 μl of vector solution from the apex of the left ventricle to the aortic root (*see* **Note 11**).
7. Clamp the aorta and pulmonary arteries distal to the site of the catheter and inject the solution as shown in Fig. 2. Aortic

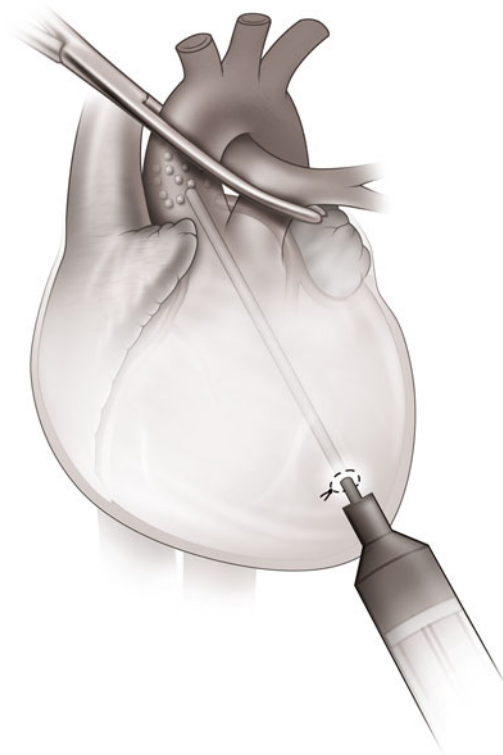

Fig. 2 Cross-clamping of the aorta and pulmonary artery while injecting the vector solution within the aortic root

clamping can be performed, but the expression with aortic clamping alone is low. Clamping both the aorta and pulmonary arteries yields better results.

8. After 10–60 s, release the clamp on the aorta and pulmonary. The clamp can be maintained for 10 up to 60 s while the heart is pumping against a closed system (isovolumically). This allows the solution that contains the vectors to circulate down the coronary arteries and perfuse the heart without direct manipulation of the coronaries (*see* **Notes 12** and **13**).
9. Close the chest and recover the animal.
10. Administer appropriate analgesic drugs and antibiotics.

3.7 Tail Vein Injection

Injection of vectors in the tail vein usually ends up in the liver to a large degree. Cardiotropic AAV vectors (AAV9 or AAV10), however, have high tropism to the heart.

1. Prepare the vector solution. The amount of vector injected is usually about 100–200 μl. The concentration of the vector is critical with 10^{11}–10^{12} vg being capable of infecting 30–80 % of cardiac cells in the rats 1 month post gene transfer using AAV.
2. Immobilize or lightly anesthetize the animal and insert a 28 or 30 G catheter in the posterior tail vein of the animal.
3. Inject the vector solution (*see* **Note 14**).

4 Notes

1. It is critically important to have the correct concentration for each of these vectors.
2. The viral solutions should be in aliquots of 50–200 μl and stored at −80 °C. During the preparation for gene delivery, the vector solution should be placed at appropriate temperature.
3. The FLOW RATE multiplied by the INSPIRATORY TIME gives the tidal volume. RESPIRATORY RATE is set independently. Typical settings for 150–250 g rats are: respiratory rate: 50 breaths/min, tidal volume of 2 cm^3, and inspiratory time of 0.5 s.
4. The interventricular septum can also be targeted by identifying the interventricular branch of the coronary artery and directing the needle parallel to that branch.
5. Solutions containing high concentrations can cause increased edema in the area of injection.
6. Using the left anterior descending artery as a guide, investigators have injected directly around the vessel for optimal left ventricular gene transfer. This has yielded excellent results in terms of functional effects. Coupled with needle injury, this method of injection induces focal expression within the myocardium. For global myocardial expression, this method has obvious shortcomings, but for local expression such as the

biological pacemaker or various growth factors used for blood vessel growth, this is an ideal approach.

7. Echo-guidance injection can be also employed to determine the location.
8. Collagenase and hyaluronic acid have been added to loosen the collagen fibers and allow further entry of the vectors and greater transduction of the cardiomyocytes (up to 40% when using adenovirus). However, a great deal of inflammation is present with this procedure raising the concern that pericarditis and its sequelae may limit the usefulness of this approach.
9. The high perfusion pressure presumably allows the opening of capillaries and optimizes the myocardial area of vector exposure. By cross clamping both the pulmonary artery and the aorta, the left ventricular end-diastolic pressure does not increase since blood return to the left ventricle is minimal. This allows perfusion of the virus at relatively low downstream pressure and the endocardium can be efficiently infected.
10. An incision from the left third intercostal space can be also used.
11. It is important to either feel or visualize the catheter as it is advanced from the ventricular apex all the way to the aortic root.
12. During the period of cross-clamping, the right and left ventricles become visibly pale as clear viral solution perfuses the myocardium through the coronary arteries. During the procedure, heart rate decreases from ~300 bpm to ~50 bpm, but recovers to baseline within 30 s of clamp release. Ventricular pressure returns to baseline within 60 s.
13. Pretreatment of the animals with either adenosine or serotonin by injecting a solution containing 1–10 μM into the inferior vena cava can also be used. This induces increased permeability of the capillaries, resulting in enhanced attachment of the viruses to myocardial cells.
14. Although it is relatively more invasive, AAV injection through the jugular vein by cut down achieves higher infection efficiency. This approach is also mainly used to achieve a global gene expression in rat models of heart failure and in transgenic mice models of cardiomyopathies.

Acknowledgment

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Chapter 14

Ultrasound-Targeted Microbubble Destruction for Cardiac Gene Delivery

Shuyuan Chen and Paul A. Grayburn

Abstract

Ultrasound targeted microbubble destruction (UTMD) is a novel technique that is used to deliver a gene or other bioactive substance to organs of living animals in a noninvasive manner. Plasmid DNA binding with cationic liposome into nanoparticles are assembled into the shell of microbubbles, which are circulated by intravenous injection. Intermittent bursts of ultrasound with low frequency and high mechanical index destroys the microbubbles and releases the nanoparticles into targeted organ to transfect local organ cells. Cell-specific promoters can be used to further enhance cell specificity. Here we describe UTMD applied to cardiac gene delivery.

Key words Gene, Gene delivery, Microbubble, Ultrasound, Ultrasound targeted microbubble destruction

1 Introduction

A number of techniques have been proposed for gene delivery to the heart, including intracoronary, intrapericardial, and direct myocardial injection. We have previously demonstrated successful transfection of rat myocardium in vivo by ultrasound-targeted microbubble destruction (UTMD) of microbubbles containing an adenovirus encoding reporter gene, plasmids, and/or proteins [1–11]. Other groups also successfully applied this technique to cardiac gene delivery [12–35].

The delivery of bioactive substances by ultrasound targeted microbubble destruction is based upon assembling perfluorcarbon-filled microbubbles together with a bioactive substance, thus incorporating it into the shell of the bubbles (Fig. 1). During intravenous infusion of these bubbles, they can be destroyed with high mechanical index ultrasound in the target organ as they distribute through the vasculature, thus releasing the transported substance into the surrounding tissue (Fig. 2). To show that DNA is indeed incorporated into the shell, confocal microscopy was utilized.

Kiyotake Ishikawa (ed.), *Cardiac Gene Therapy: Methods and Protocols*, Methods in Molecular Biology, vol. 1521,
DOI 10.1007/978-1-4939-6588-5_14,

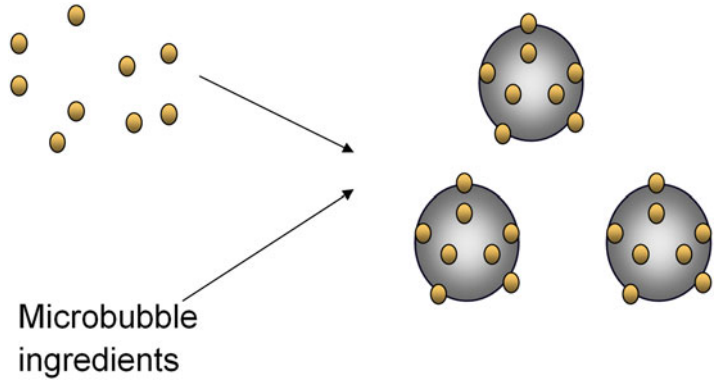

Fig. 1 Schematic representation of microbubble production with a bioactive substance (*yellow*) that is incorporated into the shell of the microbubbles

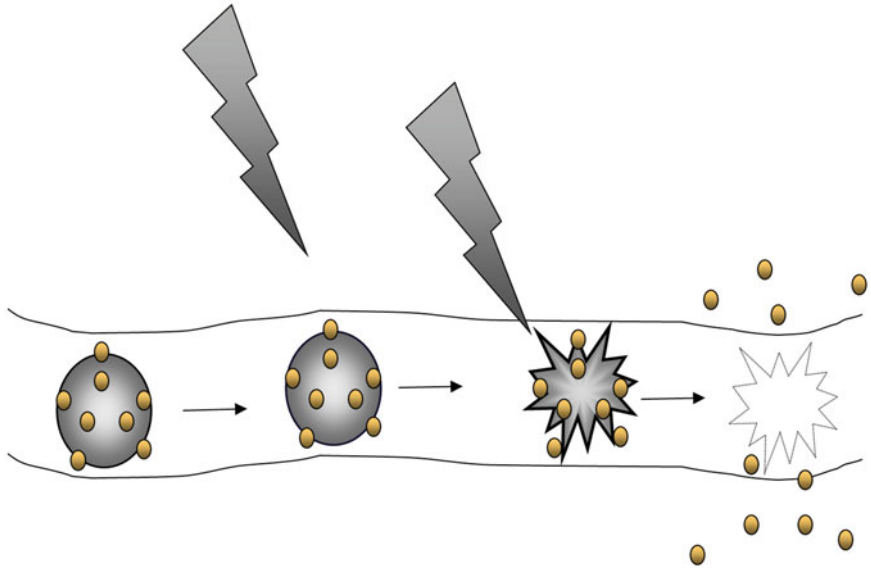

Fig. 2 Schematic representation of microbubbles in a capillary being destroyed by high mechanical index ultrasound and releasing the transported bioactive substance into the surrounding tissue

Washed microbubbles were stained with propidium iodide (stains DNA) and fluorescein (to stain the lipid shell) (Fig. 3).

It is important to measure microbubble size and concentration when trying new bubble protocols. This can be easily done in a Beckman coulter counter (Multisizer3). Typical concentrations are in $1–3 \times 10^9$ bubbles per ml. Mean size should be around 2.0–2.3 μm (Fig. 4).

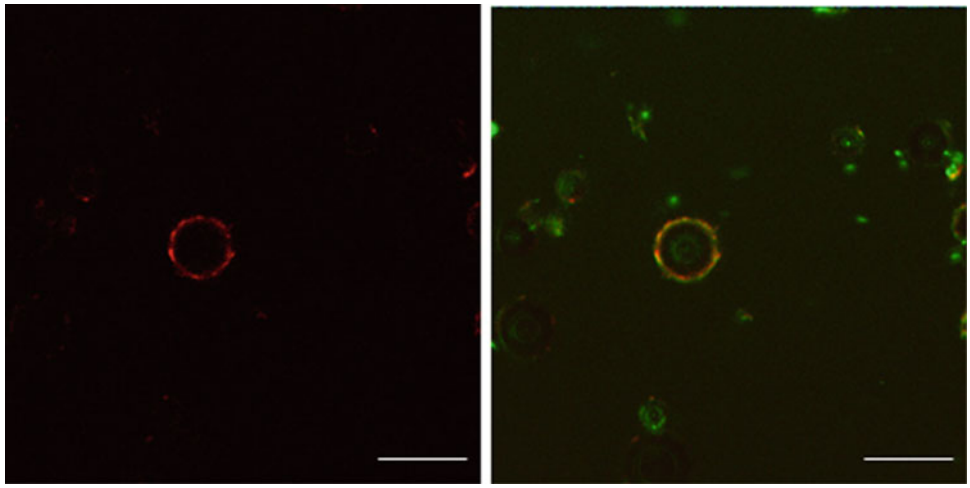

Fig. 3 *Left*: lipid microbubble with incorporated DNA stained by propidium iodide. *Right*: confocal microscopy of propidium iodide and fluorescein labeled microbubble

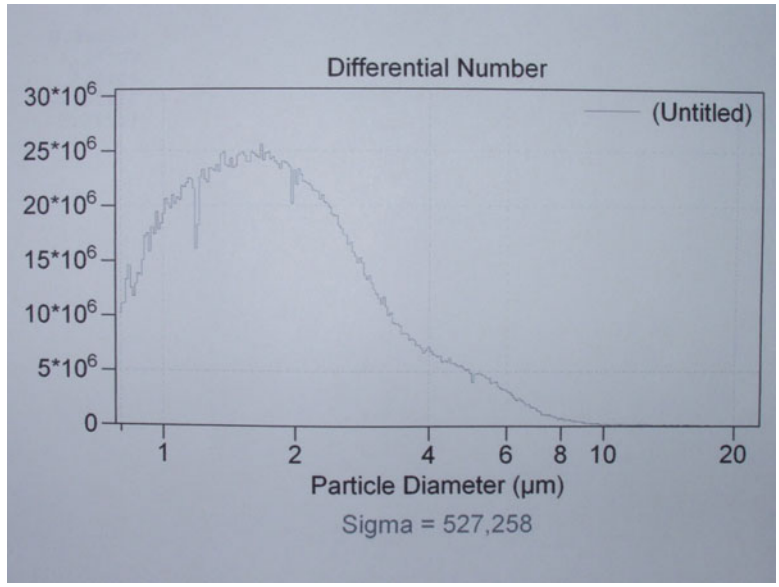

Fig. 4 Typical distribution curve of microbubble size measure in a Beckman Coulter Multisizer 3

2 Materials

2.1 Preparation of Microbubbles

1. Lipid stock solution: 270 mg DL-α-phosphatidylcholine, dipalmitolyl, 30 mg DL-α-phosphatidylethanolamine, dipalmitolyl, 1 g Glucose, 10 ml PBS.
2. 100% glycerol.

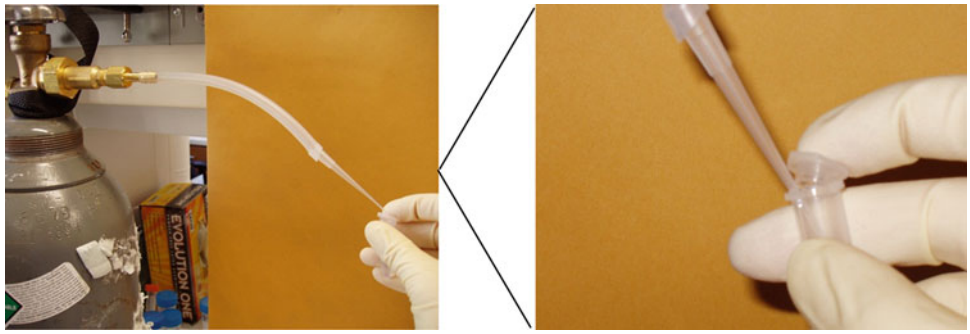

Fig. 5 An Eppendorf tube with lipid solution is filled with octafluoropropane gas

3. 100% alcohol.
4. Plasmid DNA of interest.
5. Ice.
6. Water bath 37 °C.
7. Vialmix.
8. Octafluoropropane gas (Fig. 5).
9. Gauze.
10. 10% albumin solution.
11. PBS-solution.
12. Lipofectamine 2000.

2.2 Preparation of the Rat Model

1. Gauze.
2. Luer Stub Adapter 23 G (alternatively 23 G needle).
3. 6-0 silk sutures.
4. 4-0 silk sutures with needle.
5. Polyethylene tubing PE50 (external diameter 1 mm) [for mice PE 10–0.6 mm].
6. Nair.
7. Ultrasound gel.
8. EKG electrodes.
9. Avertin (40 ml): 2 g 2,2,2-tribromethanol, 1.24 ml 2-methyl,2-butanol (tert-Amyl alcohol), 38.76 ml H_2O.
10. PBS-solution.
11. Precision infusions pump.
12. Surgical instruments (Fig. 6).
13. Ultrasound machine (Fig. 14).

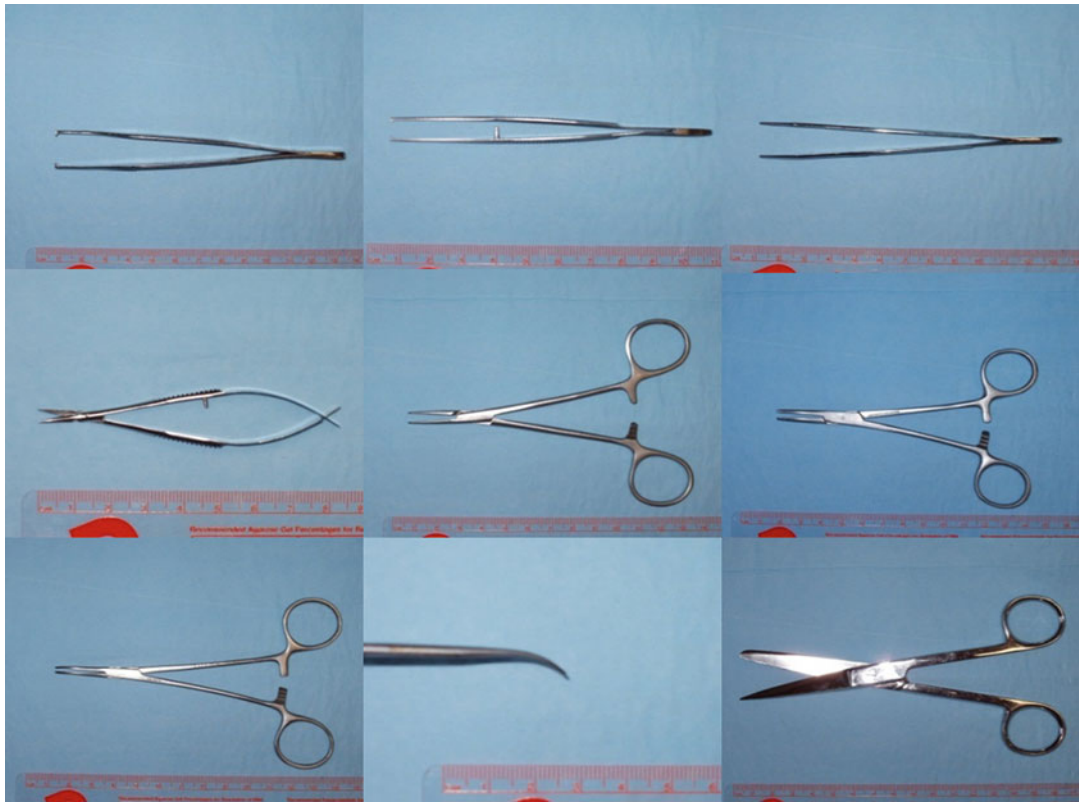

Fig. 6 Surgical instruments: (**a**) large forceps with sharp teeth; (**b**) small forceps with sharp teeth; (**c**) blunt forceps; (**d**) Vannas Scissors Curved 80 mm; (**e**) needle holder; (**f**) curved clamp; (**g**) curved clamp (fine); (**h**) tip of curved clamp (fine); (**i**) scissors

3 Methods

3.1 Lipid Microbubble Loading Plasmids DNA

1. Prepare lipid stock solution. To dissolve ingredients, heat in boiling water-bath (approximately 20–30 min) and pipette up and down every 5 min until no particles are visible. Filtering through gauze may be helpful. This stock solution can be stored at 4 °C.

To make microbubbles:

2. Add 50 μl of glycerol in a 1.5 ml tube.
3. 1 ml of alcohol added to a tube contains 2 mg of plasmid DNA, spin down and throwaway supernatant, incubating DNA pellets to dry in 37 °C for 30–60 min.
4. Add 250 μl of lipid stock solution to the tube contains 2 mg of dried DNA.
5. Merge 1 and 3. And keep them on ice.

6. Replace air with octafluoropropane gas (Fig. 5).
7. Shake in a Vialmix for 30 s in 4 °C.
8. Before infusion add 0.5 ml PBS and mix carefully.

3.2 Lipid Microbubble Loading Cationic Liposome/ Plasmids DNA

1. Add 250 μl of lipid stock solution and 50 μl of pure glycerol to 1.5 ml tube mixed and kept on ice.
2. Add 50 μl of Lipofectamine 2000 to a 1.5 ml tube that contains 2 mg of dried plasmid DNA pellets and incubate for 15 min at room temperature.
3. Add the lipid stock solution and glycerol from **step 1** to cationic liposome/DNA.
4. Add 5 μl of 10 % albumin solution, and mix well with a pipette. Do not use a vortex. And then keep on ice.
5. Replace air with Octafluoropropane gas (Fig. 5).
6. Shake in a Vialmix for 30 s in 0–4 °C.
7. Keep this preparation on ice for injection.

3.3 Procedure

1. Choose rats between 200 and 300 g for experiments. Inject 2–3 ml of 4× Avertin i.p. After rat is anesthetized shave off the hair on the chest and neck. Then add Nair™ on the chest and let sit for 2–4 min. After removing the Nair, no hair should be visible on the rat chest (Fig. 7).

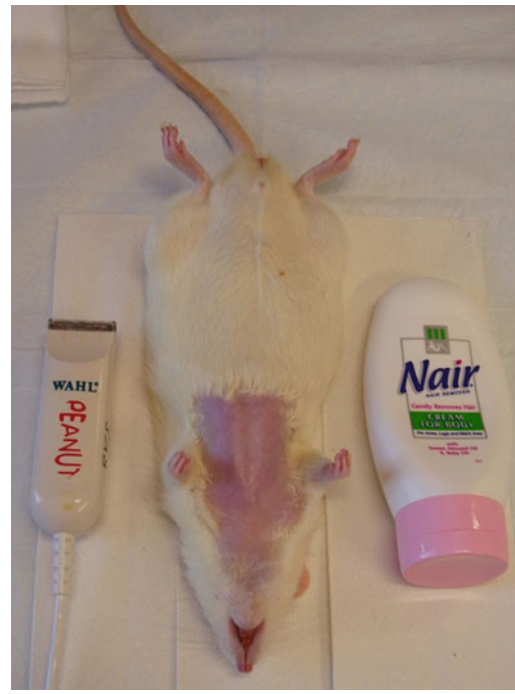

Fig. 7 Rat after shaving and application of Nair

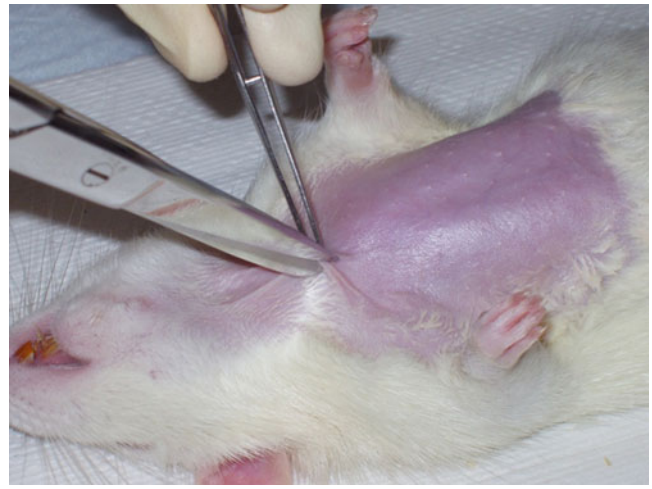

Fig. 8 5 mm skin incision above the jugular vein mediolateral of the neck

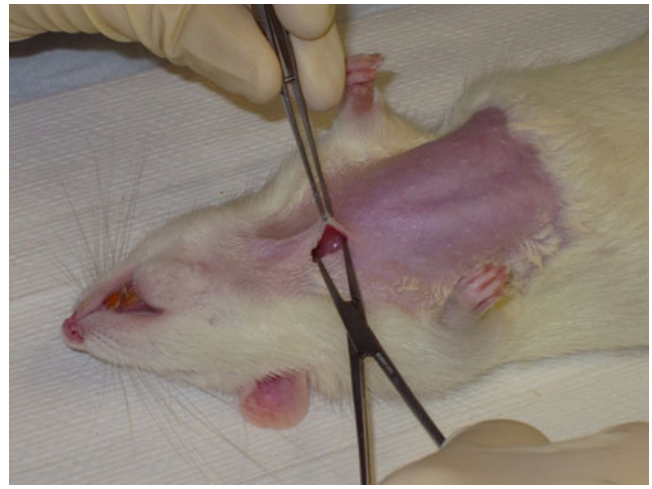

Fig. 9 Use the curved clamp to bluntly enlarge the incision and then prepare the jugular vein

2. An incision will be made (about 5 mm) mediolateral of the neck where a pulsation is visible (Fig. 8). With the curved clamp enlarge the incision. Then use the fine curved clamp to bluntly prepare the jugular vein. When the vein is exposed carefully tunnel it with the fine curved clamp. Then use the clamp to pull a 6-0 silk suture underneath the vein. Be careful not to twist the vein when pulling the suture through. Adding a drop of water can help. Ligate the vein with a cranial suture and prepare a caudal suture to bind over the catheter later (Figs. 9 and 10).
3. To prepare the catheter cut about 30 cm of the polyethylene tubing. By using an oblique position of the scissors the tip of the

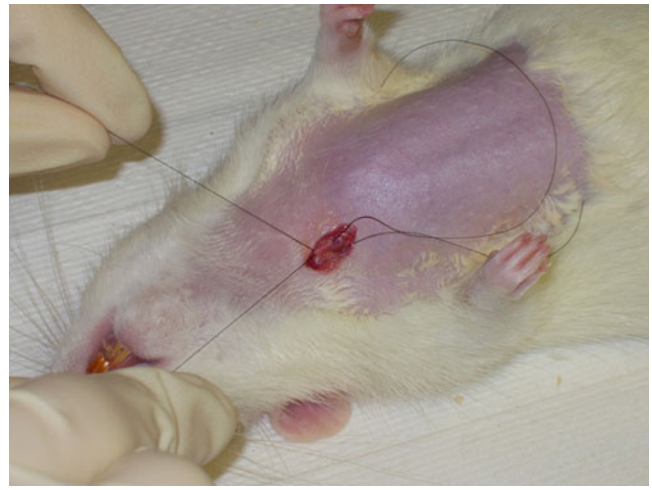

Fig. 10 Ligate the vein cranially and prepare a suture caudally to bind later over the catheter

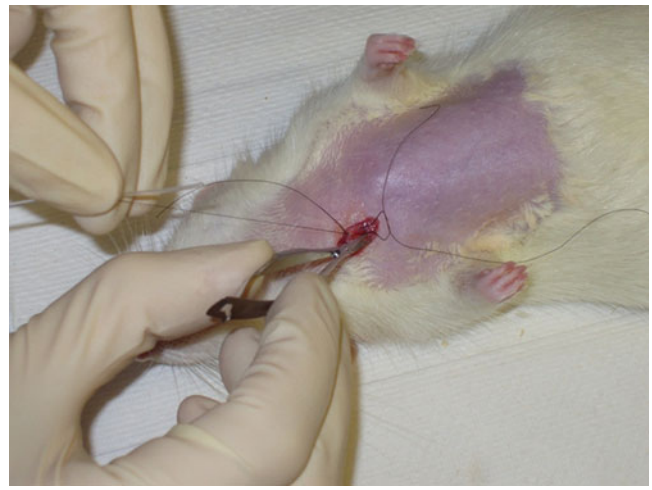

Fig. 11 The left hand holds the vein by its cranial suture as well as the catheter. With the right hand the incision is made

catheter becomes pointed. A 45° angle is favorable. Then insert the Luer Stub Adapter 23 G (or alternatively 23 G needle) in one end and flush the catheter with saline or PBS (*see* **Note 1**).

4. To insert the catheter hold the vein by the cranial suture and make a careful superficial cut with the Vannas scissors. Alternatively, one can use a fine injection needle (especially in mice). Have the catheter in hand while incising the vein (Fig. 11).
5. After the incision is made, let go of the scissors and take a blunt forceps to grab the catheter and insert it into the vein (Fig. 12). After insertion, proper catheter position can be verified by aspirating the syringe. Then the caudal suture can be tied tightly around the catheter. Now, the catheter can be connected with the infusion pump (Fig. 13) (*see* **Note 2**).

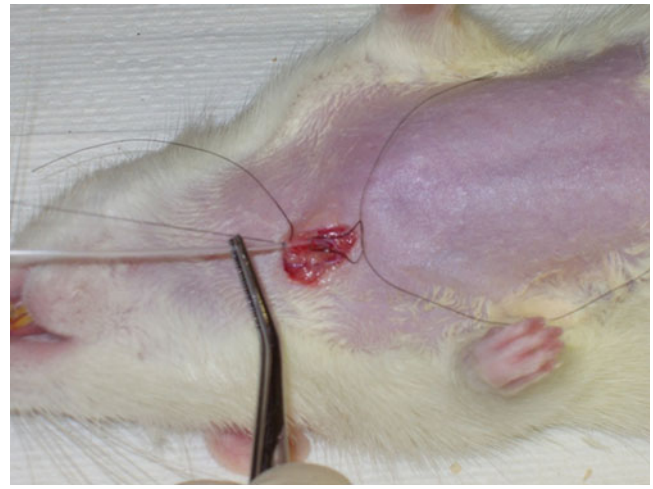

Fig. 12 The catheter is inserted into the jugular vein while holding the vein tight with the cranial suture

Fig. 13 Infusion pump connected with the catheter

3.4 Ultrasonic Microbubble Destruction

1. After the catheter is inserted, three EKG electrodes will be attached to three paws. Then a small amount of ultrasound gel is distributed on the chest. This helps to avoid small air bubbles trapped under few hairs (*see* **Note 3**).
2. After localizing the heart (feeling the pulse with the finger), a large amount of gel is added on the chest to produce a standoff for the probe (*see* **Note 4**).
3. Then the S3 ultrasound probe will be clamped on the chest leaving at least 1 cm of gel between the probe and the skin (Figs. 14 and 15).
4. Ultrasound machine settings (for example, Sonos 5500) (*see* **Notes 5–12**).

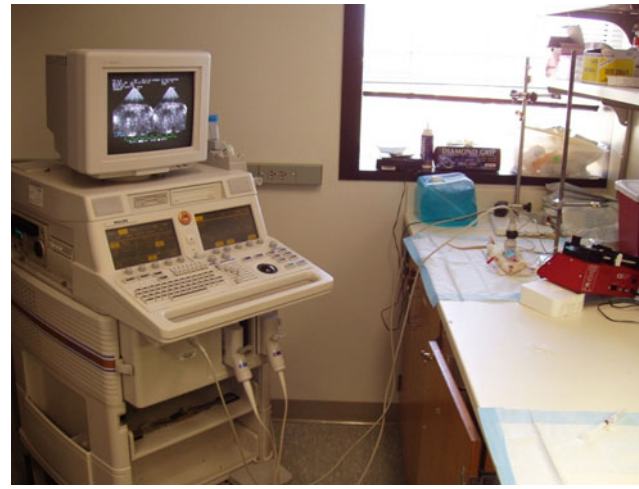

Fig. 14 Setup of ultrasound probe and machine

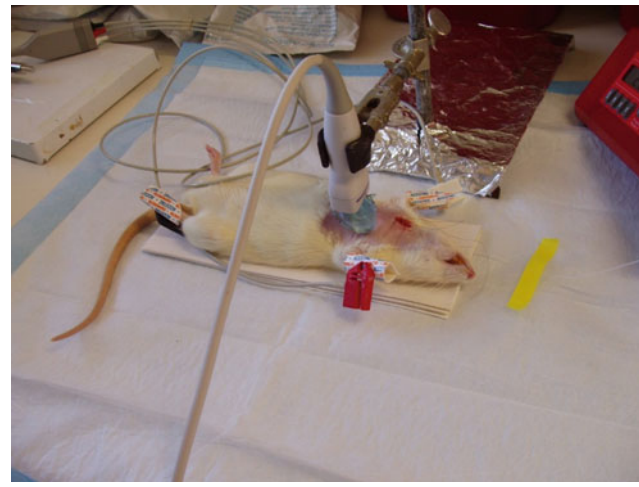

Fig. 15 Setup of ultrasound probe

5. A mid-ventricular short axis view is to be acquired. This is mostly possible after the contrast agent has been injected. A good quality image is crucial for efficient transfer of bioactive substance. If the image is good, myocardium and cavity are clearly distinguishable. After high MI ultrasound the myocardial opacification should be significantly lower than before destroying the microbubbles (Fig. 16).
6. Microbubbles suspension should be infused over a 5–10 min period (Fig. 17). It is important to agitate the infusion pump regularly in order to maintain a homogeneous bubble suspension.
7. After finishing the experiment, the catheter is removed and the skin is closed with a 4-0 silk suture. The animal should awake within 30–60 min.
8. Potential complications (*see* **Notes 13–15**).

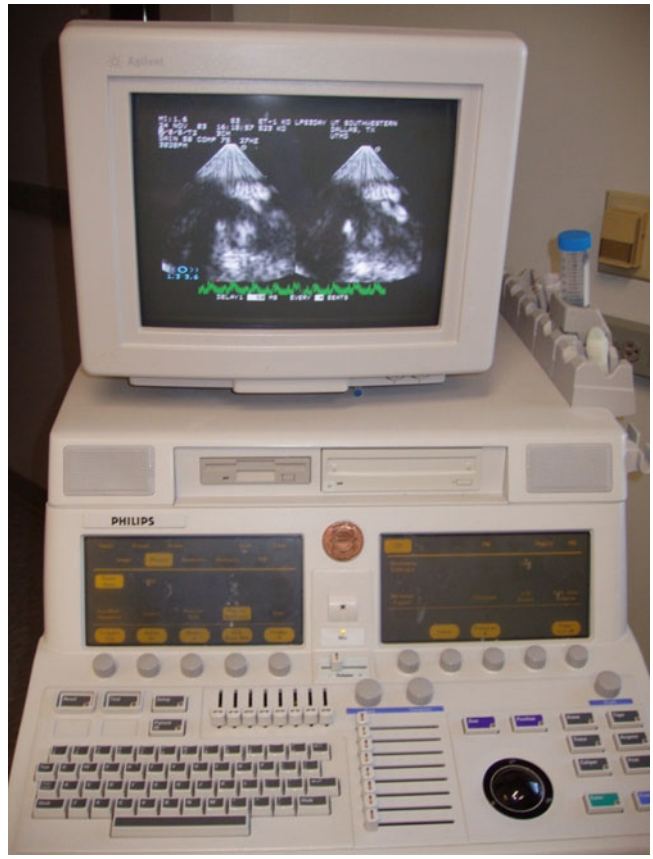

Fig. 16 Ultrasound machine settings

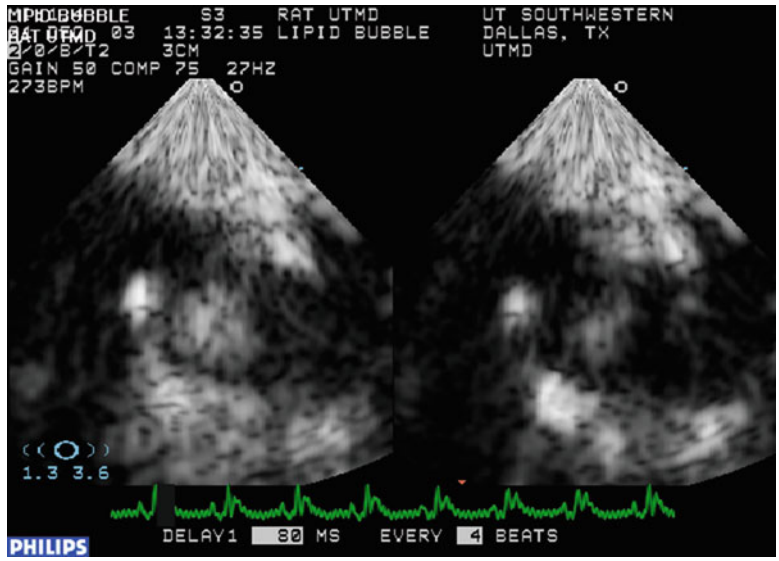

Fig. 17 Rat left ventricle in triggered ultraharmonic mode during microbubble infusion in a mid-short axis view. *Left* image shows left ventricle before high mechanical index ultrasound, *right* image after. The lower opacification of the myocardium indicates that microbubbles have been destroyed

4 Notes

1. Be careful not to leave any air in the catheter, since low volumes are sufficient to kill the rat by embolization.
2. When changing the PBS-syringe with the one containing the microbubbles, it is important that no air is introduced into the system.
3. A drop of ultrasound gel on each electrode helps to improve EKG signal.
4. Be careful to avoid air bubbles. Optionally, the gel can be centrifuged before applying it.
5. Ultraharmonic mode (transmit 1.3 MHz).
6. Mechanical Index 1.4.
7. Triggered imaging—every fourth beat—four frames of ultrasound.
8. Delay 80 ms.
9. All segmental gains to 0.
10. Gain 50.
11. Compress 75.
12. Postprocess B.
13. Arrhythmia: The high MI ultrasound may cause premature ventricular beats. As long as these occur rarely they can be tolerated. If however they occur with each high MI US and come in doublets and triplets, the mechanical index should be reduced, since the animal will be at risk for ventricular tachycardia or even ventricular fibrillation. Usually, reducing the MI to 1.0 will reduce the arrhythmia and after a few minutes the MI can be gradually increased again. If an animal develops ventricular tachycardia, the probe should be immediately removed. Pumping of the chest with a thumb may help revive the animal.
14. Pulmonary embolism: Pulmonary embolism occurs if air was injected by mistake or if microbubbles are too large (foamy microbubbles). This event is normally characterized by missing contrast in the left ventricle and a highly opacified large right ventricle. Usually, the rat will stop breathing within a minute and rarely survives.
15. Insufficient contrast effect of microbubbles: If microbubbles do not develop a sufficient contrast effect after injection or if contrast effect is too short, then the tube most likely had too much air when bubbles were made (either empty gas cylinder or leaking tubes). Always use high quality tubes that will close tightly and make sure enough gas is available.

Acknowledgment

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Chapter 15

A Needleless Liquid Jet Injection Delivery Approach for Cardiac Gene Therapy

Anthony S. Fargnoli, Michael G. Katz, and Charles R. Bridges

Abstract

Fundamentally, cardiac gene therapy clinical trials have demonstrated that route efficiency is paramount in achieving maximum myocardial expression within safety limits. Gene transfer phenomena are largely influenced by physical transport principles (i.e., pressure, residence time, dispersion trafficking, mechanical resistance) that are independent of therapeutic characteristics. An alternative to intracoronary infusion methods, in an effort to improve efficiency in terms of cardiac specificity, is direct myocardial delivery via surgical injection. Direct injection methods circumvent the blood's immunological components and the cardiac system's native anatomical barriers by directly administering product into the myocardium. In addition, this approach offers the advantage of precise site selection. Two unresolved problems with direct delivery wherein the novel needleless liquid jet approach may resolve are: (1) initial therapeutic retention and (2) subsequent host responses associated with highly focal expression.

In this protocol, we present a novel approach to improve direct cardiac gene delivery using a needleless liquid jet methodology. The liquid jet application is essentially a device concept that accelerates and disperses the therapeutic at a targeted myocardial site. The core hypothesis offered is that this approach, with optimized settings, could result in increased therapeutic retention in the initial delivery phase. This would theoretically result in more total myocardial expression per dose while at the same time providing a more homogenous profile around the injection site. Therefore, this would increase efficiency in terms of transduced muscle per delivery site and offer a significant improvement to standard intramuscular injection.

Key words Cardiac injection, Needleless, Delivery, Liquid jet, Intramuscular, Gene transfer

1 Introduction

The majority of cardiac gene therapy clinical trials have reported largely negative results in terms of efficacy. It is believed that the disparity between the preclinical and clinical results is directly attributable to the very low to undetectable levels of therapeutic expression in human myocytes. Therefore, achieving sufficient gene transfer in cardiovascular diseases with a reliable and safe delivery method has been a major impediment that must be addressed. Certainly for cardiac gene therapy, the predominant mechanism is therapeutic myocyte gene expression which can only result from

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vector transduction. Unfortunately, the inability to transfer the genome copies into a substantial percentage of cells diminishes any chance of achieving documented therapeutic efficacy.

Needleless liquid jet injection is characterized as a ballistic delivery based approach. The technology was originally developed in the early 1960s and applied for these simple medical applications: mass routine vaccinations, administration of local anesthetics, tuberculin testing, and dermatology treatments of minor skin lesions. Another similar and more widely known in vitro application of particle bombardment is the "gene gun" approach. This method was originally reported in the same period which utilizes heavy metal particles associated with DNA to be introduced with a high velocity pressurized inert gas into the target cell. Naked DNA is complexed with these particles prior to loading, and after injection they enter the cell and gradually released. Acceleration can be achieved by a high-voltage electric spark or a helium pressure gun. For optimal gene expression the following parameters have been taken into account: (a) the properties, density and sizes of gold particles for bombardment; (b) the DNA doses, and (c) the discharge voltage for optimal gold particles penetration. Using a submicrogram amount of DNA per bombardment, 1000–10,000 copies of DNA can be delivered to each target cell [1, 2]. The fundamental principal of the gene gun is based on the bombardment of micrometer-sized heavy metal particles coated with therapeutic DNA. The transfer to the target cells is achieved by means of direct penetration through the many holes in the plasma membrane. However, a problem with penetration throughout the thickness of the myocardium was noted, as only the surface layer of cardiomyocytes was transfected [3]. Uncoated metal particles could also be delivered through a solution containing DNA surrounding the cell thus picking up the genetic material and proceeding into the living cell. Despite some interest for clinical applications these problems have prevented use: technical maintenance cost, complexity of parameters and complexity issues with combining vectors to relatively large metallic particles. Given the stability and formulation advantages of modern viral vectors, ballistic delivery of active therapeutic is more cost effective through liquid jet delivery.

1.1 Liquid Jet Delivery Principles

The ballistic method of jet injection is performed using a high-speed pressurized gas, typically air or carbon dioxide however the driving gas can be designated per application. The injection consists of piercing center jet stream and surrounding micro jets. The resultant impact force of the jet expands the distance between the cell pores, increases pores, and generates new pores in the cellular and endothelial membranes, and promotes a better distribution of the proteins and genes in the interstitial compartment (Fig. 1). The penetration power depends on three factors: (1) the applied

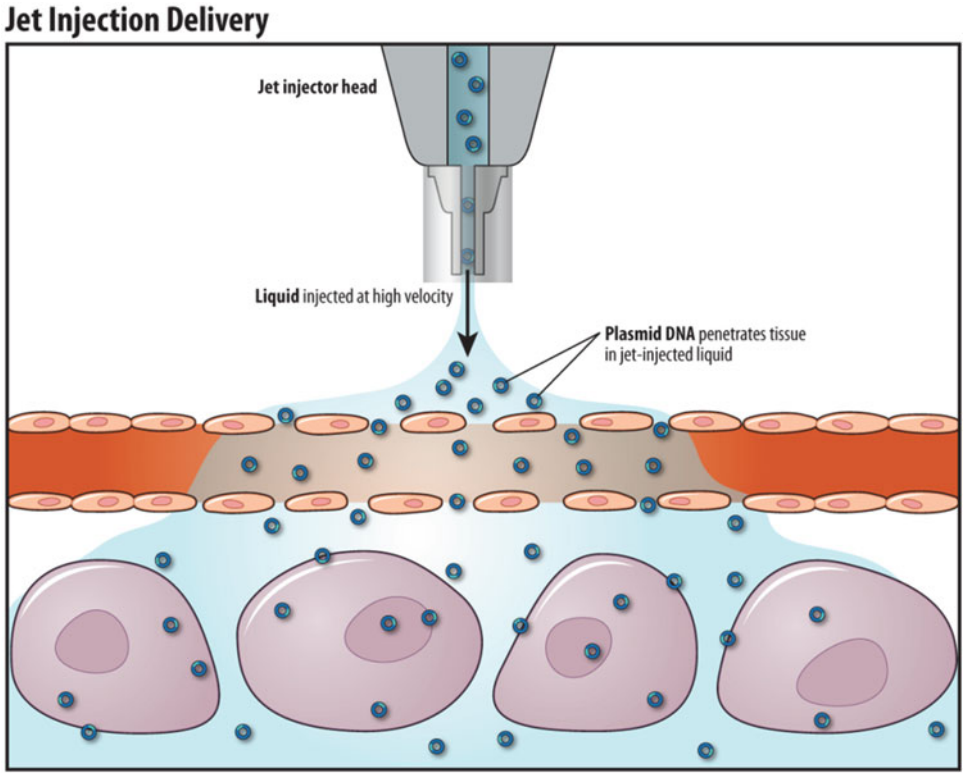

Fig. 1 Liquid jet delivery of plasmid DNA. Liquid jet delivery is demonstrated featuring DNA vector suspended in a liquid formulation being accelerated and dispersed at the target myocytes

pressure of the gas, (2) the dispersion factor of the jet as a function of the nozzle bore, and (3) the target tissue's mechanical and host response properties [4]. Levels of gene expression by jet injection were found to be 50-fold higher than by conventional needle injection in one preliminary study [5]. The jet injection gene transfer is usually well tolerated without side effects provided that the driving pressure settings are optimized.

These previous applications are only described for nonviral based techniques of gene transfer using relatively high pressure. Macromolecules of varying chemistries and properties can be delivered such as stable active compounds, DNA and to a lesser degree proteins. Pressure used is typically less than 3–4 bars with resultant velocity profile of the droplets ranging from 100 to 300 m/s. The velocity of the fluid in the jet injection contributes to the transmural distribution in the tissues, whereas the diameter of the jet and the injected volume limit the penetration depth [6]. The efficiency of this method depends on nozzle diameter (150–300 μm), velocity of the liquid jet and distance between the nozzle and surface of the tissue [7, 8]. The penetration of the injected molecules inside the cells subsequently results in direct uptake, maximizes bioavailability

in the tissue microenvironment for additional molecules, and in worst case inadvertently reaches off target organs via local systemic circulatory penetration. In another study, the intramuscular jet injection of DNA combined with electroporation was demonstrated to be feasible in a mouse model [9]. We and others could not find publications on the applications of this method for cardiac gene therapy, thus indicating its novelty for viral vector based therapy [10–12].

1.2 Device Description and Specifications

The stainless steel needleless liquid jet (DERMOJet™) was purchased from Robbins Instruments (Chatham, NJ). The device itself was invented by Dr. A. Krantz in France with the medical device manufacturer Akra DermoJet (Pau, France). The device consists of a stainless steel design with polycarbonate reservoir with dimensions: 8 oz (weight), 6.5 in. (length), and 11/16th in. (Diameter) injector head. The exit orifice is 0.0079″ (32 gauge; 235 μm) diameter in a jewel shape design. The size transmission limit is crystalline particles up to 200 μm. The device is actuated by a very high constant spring mechanism which drives a piston that subsequently accelerates a fixed volume fluid load. Injection is executed by a loading lever mechanism which sets the spring lock element, while the push button trigger releases for the acceleration of drug through the orifice. The ejection orifice features an internal jewel shaped configuration for a dispersive effect. The ejection volume is fixed at 100 μl, which is a typical ideal retention limit for most tissues. Various mechanical components in the device are tunable, most notably the spring and orifice characteristics.

2 Materials

2.1 Rats

All animal studies must follow National Institute of Health Guidelines (*Guide for the Care and Use of Laboratory Animals*, NIH publication No. 85–23, revised 1996) and be approved by the appropriate Institutional Animal Care and Use Committee.

2.2 Strain, Age, Weight, Gender

Male Sprague–Dawley, 250–400 g.

1. Preoperative analgesia: SQ Buprenorphine (0.03 mg/kg), SQ Enrofloxacin (5.0 mg/kg), SQ Ketoprofen (5.0 mg/kg).
2. Induction anesthesia: SQ ketamine (100 mg/kg), xylazine (10 mg/kg).
3. Maintenance anesthesia: intratracheal isoflurane. 1.5–3%.
4. Postsurgical medication: SQ Bupivacaine 0.5%(5 mg/ml), lactated ringer (2–3 ml), buprenorphine (0.03 mg/kg), cefazolin (100 mg/kg).
5. *Additional devices*: anesthesia mask, anesthesia induction camera, endotracheal tubes, pulse oximeter, eye lubricant, thermometer

probe, heating pad, ECG pads, ventilator, oxygen source, operative table with appropriate work area, lights, hair trimmers, scale and weight dish.

2.3 A Needless Liquid Jet Injection Vector Delivery

1. Needless liquid jet injector.
2. An optical system consisting of a field laser.
3. Microsurgical set including scissors, forceps, rib spreader, scalpel and blades, mosquito-tip hemostats.
4. Sutures: 4-0, 7-0 Prolene, 5-0 Vicryl, 3-0 Silk.
5. Solutions: iodine solution 5 %, ethyl alcohol 70 %, saline solution 0.9 %.
6. Gauze, 2 × 2 and 4 × 4.
7. Insulin syringe (for viral vector), 0.5 ml, U-100 29 G 1/2 needle.

3 Methods

3.1 Preoperative Care and Preparation

1. At least 30 min prior to surgery rats will receive analgesic as well as antibiotic and nonspecific anti-inflammatory as listed above preoperative analgesia. Shave the surgical site.
2. Rats will be anesthetized using induction and maintenance anesthesia, then intubated (ventilator setting: ventilation ~120–150 ml/min; a tidal volume of 2–2.5 ml and respiratory rate of 50–70) and transferred to a warming pad.
3. An eye lubricant is applied to prevent the animal's eyes from drying. The next step is to connect the rat to an EKG machine and a SpO_2 monitor.
4. Animal is monitored for any movement or tail pinch response to assure proper depth of anesthesia. Secure endotracheal tube in position. Connect the rat's endotracheal tube to the ventilator with the rat in left lateral decubitus position.
5. On day of injection, prepare sufficient sterile diluted vector to allow for a 350-μl injection per pup as well as an additional 150 μl to account for losses during preparation and injection. Keep diluted vector on ice until injection.

3.2 A Needless Liquid Jet Injection Vector Delivery

1. Scrub the rat's chest two times with chlorhexidine solution, and drape the rat in a sterile fashion.
2. Make a 2-cm skin incision at the left chest, divide the subcutaneous tissue and underlying thoracic muscles, and enter the thorax through the fourth intercostal space. Once the chest is entered the lung is reflected posteriorly and the pericardium is incised without disrupting the phrenic nerve to expose the left ventricle.

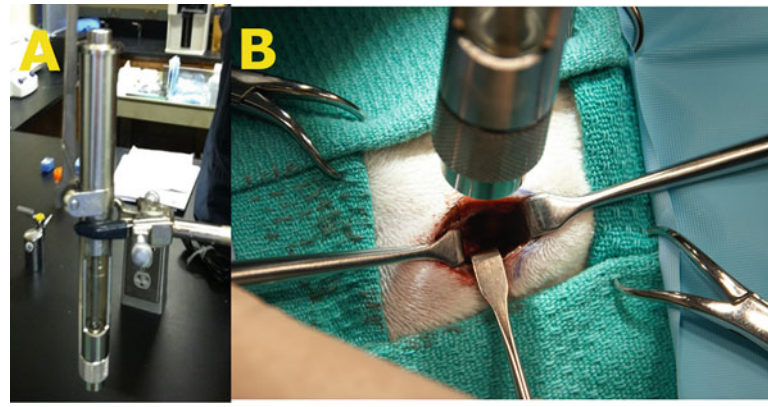

Fig. 2 Needleless injection experimental setup in the rodent model. (**a**). The DermoJet™ needleless jet injector mounted to an adjustable control arm. (**b**). The surgical field application demonstrating the injector aligned optimally for repeat safe injections toward the left ventricular surface area via thoracotomy access

Fig. 3 Transmural AAV.GFP mediated transduction (*purple*) at 4 weeks following delivery. Equal distribution in the basal and mid left ventricular cross sections. Resultant 4 week post-harvest expression results from needleless left ventricular delivery of AAV9 (10^{11}gc) encoding GFP. The expression profile indicated via IHC stain (i.e., GFP purple) revealed transmural expression profiles presented in the middle and basal LV sections. The composite left ventricular coverage scoring as a percentage of 2D transmural area was in the range of 50 to as high as 70 % for the majority of sections

3. Load liquid jet injector with a vector (*see* **Note 1**). The key parameter is the injector head distance normal to the target cardiac plane. To execute, the pen configuration injector is mounted to a stainless steel arm apparatus secured to a solid base (Fig. 2) (*see* **Note 2**). The optimal distance range is determined between 8 and 10 in. (i.e., nozzle tip to heart surface) (*see* **Note 3**). Sufficient delivery volume is loaded into the chamber to ensure consistent kinetics as per DermoJet™ manufacturer instructions (*see* **Note 4**). Three separate shots, each containing 100 μl, are fired perpendicular to the LV surface. Hits are confirmed by detection of pinhole piercing on the epicardial surface with surrounding coloration changes. Left ventricular cross section GFP distribution is presented in Fig. 3.
4. Once needless injections are complete, place an 18-G angiocath through the skin into the thorax via the fifth or sixth inter-

costal space. This will serve as a chest tube to restore negative intrathoracic pressure. Give a manual sigh breath from the ventilator to reinflate the lungs completely.

5. Once hemostasis is achieved, closure of the ribs is performed with a 4-0 Prolene suture followed by closure of the chest muscles using a 5-0 Vicryl suture. After tying these sutures, a 3 ml syringe is used to evacuate the air/fluid in the thoracic cavity in order to regain the normal negative pressure. The skin incision is closed using a running 5-0 Vicryl absorbable suture. After this suture is finished, chest tube removed, isoflurane flow is halted and the animal is weaned off the ventilator and oxygen.
6. If multiple procedures are being performed the same day, the rat can be moved to the optional recovery ventilator until spontaneous respirations are observed.
7. Standard rodent pain treatment courses with this particular model are typically between 24 and 72 h post-operative time. Animals are provided a local anesthetic to the wound area prior to emergence from anesthesia as well as fluids for support and kept on a warming pad with supplemental oxygen for 24 h. Animals also receive analgesic and antibiotics as directed above (Post-surgical medication).
8. The animals will be observed for signs of pain, anxiety, and or respiratory distress. Criteria for this level of distress will be poor general appearance of coat, lethargy, eye color, breathing rate >130 beats/min or labored breathing.

4 Notes

1. A liquid jet device is acquired and modified for cardiac delivery applications. The spring actuator length is reduced by 80–1.9 cm, which resulted in a significant driving power reduction. A super speed camera (Zeiss International, Thornwood NY) at the University of Pennsylvania Complex Fluids Laboratory is used in a custom analysis suite to quantify jet fluid exit velocity and to compute estimated driving pressure. The nozzle jet velocity is measured at 110 m/s based on a 30,000 images per second capture rate. The driving pressure ranged from 150 to 250 kilopascals (kPa). Prior to the modification, the factory setting results were 330 m/s velocity at greater than 550 kPa.
2. The next key parameter to optimize for a surgical application is the injector head distance normal to the target cardiac plane. To execute, the pen configuration injector is mounted to a stainless steel arm apparatus secured to a solid base. For alignment

with the cardiac targeted surface plane, an optical system consisting of a field laser and digital level are utilized in conjunction to ensure accuracy between injections.

3. The safe liquid jet delivery distance range is determined by performing a limited number of acute studies with methylene blue dye injection. Safety criteria are defined as tolerance of six consecutive 100 μl needleless injections without visible bleeding and ECG changes. Confirmation of hits is validated via marks by surface retention of dye. The optimal distance range is determined between 8 and 10 in. (i.e., nozzle tip to heart surface). This range is found effective where sufficient dye is retained transmurally, but no incidence of major cardiac damage was observed.
4. The experiments indicated that the DermoJet™ device as is would not be suitable for any cardiac application since the potent jet is piercing with high force and simultaneously providing limited distribution. Advancing to distances greater than 24 in. from the target organ are required to remotely obtain anything of value in terms of dye transfer, thus it is imperative to modify the settings. Since the device is actuated by a spring, we worked with the manufacturer to cut the constant down by 80% of the original length. The original jet velocity is measured and confirmed by the factory at just over 330 m/s out of the 100–200 kPa driving pressure range.

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Chapter 16

Cardiac Gene Delivery in Large Animal Models: Antegrade Techniques

Shin Watanabe, Lauren Leonardson, Roger J. Hajjar, and Kiyotake Ishikawa

Abstract

Percutaneous antegrade coronary injection is among the least invasive cardiac selective gene delivery methods. However, transduction efficiency is quite low with a simple bolus antegrade injection. In order to improve the transduction efficiency using antegrade delivery, several additional approaches have been proposed.

In this chapter, we briefly discuss important elements associated with intracoronary delivery methods and present protocols for three different catheter-based antegrade delivery techniques in a preclinical large animal model. Despite the lower transduction efficacy relative to more invasive delivery techniques, antegrade techniques have the advantage of being clinically well established and having safer profiles which is important when treating patients with cardiac disease.

Key words Cardiovascular diseases, Gene therapy, Minimally invasive, Homogenous distribution, Intracoronary, Vectors, Gene delivery, Balloon occlusion

1 Introduction

Percutaneous antegrade coronary injection is among the least invasive cardiac selective delivery methods with relatively safe and simple profiles. However, transduction efficiency has been reported to be compromised with a simple bolus of antegrade injection [1]. To increase the transduction efficiency, additional approaches (slow intracoronary perfusion, intracoronary perfusion + coronary artery occlusion, intracoronary perfusion + coronary artery occlusion + coronary sinus occlusion) have been proposed and tested in preclinical animal models [2]. In this chapter, we briefly describe features of each method and present practical protocols to perform preclinical gene transfer experiments in large animals.

In ex vivo gene transfer experiments using adenovirus, it has been shown that higher coronary flow, vector dwelling time,

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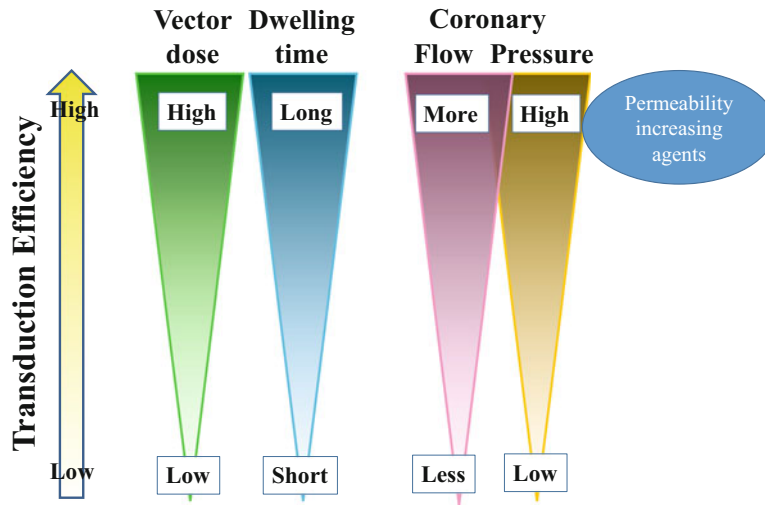

Fig. 1 The important factors to increase transgene efficacy. Coronary flow and the perfusion pressure are somewhat related to each other. Vector dwelling time, vector dose, and use of permeability enhancing agents affect transgene efficacy independently

vector dose, and use of permeability enhancing agents are the most important factors that lead to increased transduction efficiency [3].

The importance of these factors was validated in vivo with perfusion pressure as an additional factor [1, 4, 5]. Figure 1 shows the important factors for increasing gene transduction efficiency through vascular administration of vectors.

1.1 Slow Intracoronary Perfusion

This method is the simplest procedure in terms of antegrade approaches, and the vectors are administered slowly into the coronary arteries without interruption of the coronary flow [6–11]. This procedure is similar to coronary angiograms that are performed routinely in patients and the method is well established. It is especially attractive for patients with end-stage heart failure, as the procedure takes less time and is relatively less invasive compared to other cardiac targeting delivery methods. However, it suffers from lower transduction efficiency. Visual guidance of this procedure is available elsewhere [12].

1.2 Intracoronary Perfusion + Coronary Artery Occlusion

This method delivers vectors through the lumen of an inflated balloon catheter with a temporary occlusion of the coronary artery. Temporarily blocking the coronary artery flow with a balloon catheter and injecting the vectors distal to the balloon results in higher delivered vector concentrations as well as longer dwell time [4, 13]. Depending on the injection rate, higher coronary flow and perfusion pressure can also be achieved. However, the efficacy of coronary arterial blockade remains controversial. Boekstegers et al. showed ischemia during coronary artery infusion did not significantly

increase myocardial transduction [14]. In contrast, Shah et al. reported infusion with temporary coronary occlusion with a considerably higher flow rate and consequently elevated coronary pressures resulted in higher gene expression, but this was also associated with more myocardial injury [15]. A brief coronary occlusion may induce favorable effects on gene uptake due to myocardial ischemic conditioning, however it comes with the risk of ischemia induced myocardial stunning as well as coronary vessel injuries.

1.3 Intracoronary Perfusion + Coronary Artery Occlusion + Coronary Sinus Occlusion

Since the cardiac circulation is predominately supplied by the coronary arteries and drained through the coronary sinus, both of these vessels can be the target of gene transfer. A combination of coronary artery and coronary sinus blockade increases the pressure in the closed circuit between the balloons inflated in the coronary artery and the coronary sinus [1, 13]. It can also increase the vector dwelling time and this method results in elevated transgene expression. As with intracoronary perfusion with coronary arterial balloon occlusion, high coronary pressure can cause myocardial injury. Since the outflow of coronary flow is also blocked in this method, the injury can be more severe.

2 Materials

2.1 Slow Intracoronary Slow Perfusion

1. A heating pad (a heat therapy pump) for all procedures.
2. A mechanical ventilator for large animals.
3. A standard catheter pack.
4. Vital monitors including pressure sensors.
5. Y-connector with hemostatic valve.
6. Contrast agent for angiogram.
7. An introducer sheath for vascular access.
8. A guiding catheter for coronary artery access (*see* **Note 1**).
9. Two 0.014 in. coronary guide wires with soft tip for coronary artery access.
10. Disinfectants: 70% isopropyl alcohol and povidone–iodine.
11. Analgesics and anesthetics: Telazol (tiletamine/zolazepam), buprenorphine, propofol.
12. Heparin sodium.
13. Vector solution.
14. Saline or phosphate buffered saline (PBS).
15. Two syringes for vector infusion.
16. Infusion pump tubes.
17. Infusion pump.
18. Nitroglycerine.

2.2 Intracoronary Perfusion + Coronary Artery Occlusion

1. The materials for the coronary artery access are same as Subheading 2.1.
2. An inflation device for a balloon catheter.
3. An over-the-wire (OTW) balloon dilation catheter for the occlusion and of the coronary artery and injection of the vectors. The balloon size should be selected depending on the size of the coronary artery based on baseline coronary angiogram (*see* **Note 2**).
4. A manual external defibrillator.

2.3 Intracoronary Perfusion + Coronary Artery Occlusion + Coronary Sinus Occlusion

1. The materials for the coronary artery access and vessel occlusion are same as Subheadings 2.1 and 2.2.
2. A guiding catheter for coronary sinus access (*see* **Note 3**).
3. A 0.014–0.035 in. hydrophilic wire for the coronary venous access.
4. A balloon catheter for the concomitant occlusion of the coronary vein. This is usually larger than the balloon catheter used to occlude the coronary artery. In addition, the lumen size should be large enough to allow the passage of the wire selected to engage the coronary vein.

3 Methods

3.1 Slow Intracoronary Perfusion

1. The animal (a pig or other large animal) is premedicated using Telazol (tiletamine/zolazepam) (8.0 mg/kg) and buprenorphine (0.6 mg) (*see* **Note 4**).
2. The animal is intubated and then ventilated with 100% oxygen. Obtain a venous access on the ear vein. General anesthesia is maintained with Propofol (8–10 mg/kg/h) throughout the procedure (*see* **Note 4**).
3. The animal is placed in dorsal position on a heating mat during the procedure, with its legs held tight by ropes. Connect the monitors on the animal. The puncture site is prepared with 70% isopropyl alcohol followed by povidone–iodine. A percutaneous puncture provides access to the artery for sheath placement (*see* **Note 5**).
4. Puncture the peripheral artery (femoral or carotid artery) to obtain an arterial using the Seldinger method (*see* **Note 6**).
5. Administer heparin sodium at the dose of 200–300 U/kg IV to achieve an activated coagulation time of 250–300 s.
6. Advance the guide wire with the guiding catheter to the ascending aorta. Special care should be taken when the guiding catheter passes the aortic arch.

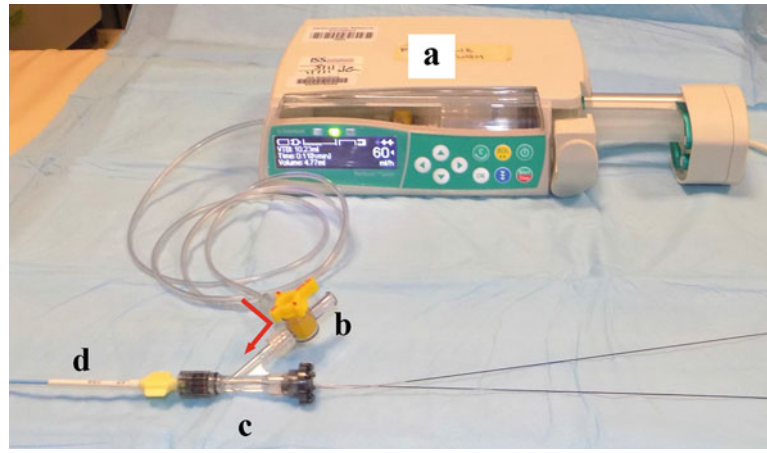

Fig. 2 Slow intracoronary perfusion system. During the infusion, the Y-connector valve should be tightly locked to prevent the vector solution from loss. Pressure monitor line can be connected to femoral arterial sheath during injection. (*a*) Infusion pump. (*b*) Three-way stopcock for exchanging the infusion line and pressure monitor line (also to the manifold for angiography). (*c*) Y-connector with hemostatic valve and coronary wires. (*d*) Guiding catheter

7. Intravenous nitroglycerin (1 μg/Kg/min) infusion is started through the ear vein.
8. Set up the injection lines as presented in Fig. 2.
9. Engage the left coronary artery (LCA) and after the angiogram, advance two 0.014-in. guide wires, one into the left anterior descending artery (LAD) and one into the left circumflex coronary artery (LCX) to increase the stability of the catheter position (*see* **Note 7**).
10. Prepare two syringes. In the first syringe, dilute the vector solution to 20 ml with saline or PBS. Prepare another syringe filled with saline or PBS 10 ml (flush). This will be used to deliver the residual virus within the catheter lumen.
11. Confirm that the catheter tip is stable at just proximal to the bifurcation of LAD and LCX. The pressure through the catheter is monitored to ensure that the catheter is not wedging.
12. The vector solution (15 ml) is injected through the catheter over 15 min (1 ml/min) using an infusion pump into the LCA. After 15 min, the syringe is exchanged to the flush, and infusion is continued for 5 min (1 ml/min) (*see* **Note 8** and Fig. 2).
13. Remove the catheter from the LCA. Engage the catheter to the right coronary artery (RCA) (*see* **Note 9**).
14. A 0.014 in. wire and a catheter are fixed to the RCA the same way as the LCA.

15. The remaining 5 ml of saline or PBS mixed with virus is injected into the RCA for 5 min (1 ml/min) followed by 5 min (1 ml/min) of flush.
16. Withdraw the guiding catheter and the wire.
17. Withdraw the sheath from the artery and achieve hemostasis by applying direct pressure to the site for several minutes (*see* **Note 10**).

3.2 Intracoronary Perfusion + Coronary Artery Occlusion

1. The methods for the coronary artery access are same as Subheading 3.1.
2. Prepare a manual external defibrillator for ventricular fibrillation that can be induced by the coronary artery occlusion (*see* **Notes 11**).
3. For the coronary artery occlusion, advance an angioplasty balloon along with the coronary guide wire into the target artery.
4. Perform an angiogram and place the balloon in a proximal part of the coronary artery. Inflate the coronary balloon (total 3 min) and remove the wire inside the OTW balloon. Inject the vector solution slowly through the wire lumen of the angioplasty balloon (*see* **Notes 12–14**).
5. Deflate the balloon and wait for 5–10 min to allow the heart to recover from short ischemia.
6. Repeat the injection as needed depending on the volume of the vector solution. Upon finishing the injection, remove the balloon and the wire from the target coronary artery.
7. Advance a balloon along with the coronary wire into another target artery. Similarly occlude and inject the vectors.
8. Withdraw the guiding catheter, the balloon, and the wire.
9. Withdraw the sheath from the artery and achieve hemostasis by applying direct pressure to the site for several minutes (*see* **Note 10**).

3.3 Intracoronary Perfusion + Coronary Artery Occlusion + Coronary Sinus Occlusion

1. The method for the coronary artery access is same as Subheading 3.1. The method for the coronary artery ballooning is same as Subheading 3.2.
2. Advance the guide wire with the guiding catheter to venous system (*see* **Note 15**).
3. Select the great cardiac vein with a 0.025 (0.014–0.035) inch wire and advance an occlusion balloon to the great cardiac vein (*see* **Note 16**).
4. Occlude the artery and the vein simultaneously. Remove the coronary wire in the occluded arterial balloon and administer vectors slowly through the lumen. Wait for 3 min after occlusion of the coronary artery and sinus (*see* **Notes 12–14**) [3, 8].

5. Withdraw the guiding catheters, the balloons, and the wires.
6. Withdraw the sheaths from the artery and the vein and achieve hemostasis by applying direct pressure to the site for several minutes (*see* **Note 10**).

4 Notes

1. A Hockey Stick catheter is suitable for the femoral approach and an Amplatz Right catheter is suitable for the carotid approach in pigs. Usually a 5Fr Hockey Stick catheter can cannulate both the LCA and RCA.
2. The shorter balloon is preferred to minimize the damage of the endothelium from the expansion of the balloon. Usually 1–2 atm is sufficient to completely occlude the coronary artery using an appropriate size of the balloon.
3. An Amplatz Left catheter is suitable for the femoral approach to engage the coronary sinus and Judkins Right is suitable for the jugular vein approach in pigs.
4. Analgesia, anesthesia, and antibacterial drugs are approved by the animal committee at the facility for all procedures. Inhalational anesthetics can be used instead of propofol.
5. Pulling the leg of the puncture side will facilitate the vessel puncture. In young healthy animals, vessels can flexibly move and avoid needles when the legs are loosely pulled.
6. The femoral and the carotid arteries can also be accessed by a cut down method. Echo guidance will increase the accuracy of the puncture.
7. The administration of a vector solution into the coronary artery seems simple, but it can be tricky especially in some species. An appropriate injection catheter should be chosen for each species. This can vary depending on the size of the animals. Inserting the coronary wires into the branches will assure the catheter stability at the coronary ostium. The catheter tip should remain inside the left main tract; however, deep engagement of the catheter into the branches can cause an obstruction of the coronary artery and the formation of a thrombus. A large amount of heparin administration, the continuous monitoring of the catheter position under fluoroscopy, and checking the blood pressure and ECG for signs of ischemic changes are important.
8. Make sure the Y connector valve is tightly locked. A leak from loosely closed Y connecter results in loss of vectors.
9. An appropriate catheter for the RCA should be determined before the starting of LCA vector delivery. Catheters that fit both the LCA and RCA are preferred; however, stable RCA cannulation and easy manipulation should be prioritized.

10. Protamine sulfate can be administered slowly over 5 min to accelerate hemostasis if the vector does not interact with the drug. Although the frequency is low, protamine sulfate can cause hypotension. Continuous monitoring with ECG and pulse oximeter is necessary. If the signal of the pulse oximeter is lost, check the noninvasive blood pressure. If there is hypotension, inject 1–2 ml of atropine sulfate IV or, in a severe case, inject low dose of epinephrine or phenylephrine IV. With timely administration of these drugs, the pig will recover without any adverse effects.
11. In case of ventricular fibrillation, apply 200 J shock as soon as possible. If the rhythm does not recover, deflate the balloon and apply another shock. Chest compression should be maintained during the arrhythmia. Check the pressure monitor to evaluate the hemodynamics.
12. Continuously monitor the blood pressure and deflate the balloon if the pressure becomes too low. Preconditioning the heart by applying 10–15 s of occlusions may enable a more stable hemodynamics during 3 min injection.
13. Intracoronary adenosine (25 μg) can be injected to increase cellular permeability before injection of the vector solution [16].
14. It has been shown that higher injection rate is associated with myocardial injury [4].
15. The coronary sinus ostium is located between the atrial septum and the right ventricle. An injection of contrast from the coronary artery can facilitate identifying the location of the coronary sinus.
16. To prevent the perforation of the coronary vein, forming a loop with the tip of the wire is recommended.

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Chapter 17

Direct Myocardial Injection of Vectors

Guillaume Bonnet, Kiyotake Ishikawa, Roger J. Hajjar, and Yoshiaki Kawase

Abstract

Gene therapy holds great promise as a targeted treatment of cardiovascular diseases, which remain a major cause of morbidity and mortality in contemporary societies. Selection of the appropriate vector delivery method is critical for efficient transduction in the myocardium. Direct myocardial delivery is a feasible and effective method that has been shown to exhibit enhanced gene expression compared to coronary infusion and pericardial delivery. It is one of the most widely used gene transfer methods in both animal studies and clinical trials. The advantages, which result from a delivery that avoids exposure to the blood and bypasses the endothelial barrier, are a high local concentration at the injection site and a decreased leakage to off-target organs. The vectors are injected either with an endomyocardial or an epicardial approach, either surgically or percutaneously. In this chapter, we present the different approaches of direct myocardial injection, their advantages and their realization method in preclinical large animal models of cardiovascular diseases.

Key words Cardiovascular diseases, Gene therapy, Clinical application, Direct myocardial injection, Viral vectors, Endomyocardial approach, Epicardial approach, Surgery, Catheter-based method

1 Introduction

Selection of the appropriate vector delivery method is critical for efficient transgene expression in the myocardium [1, 2]. Although attractive, given its well-established technique, the coronary artery infusion method faces obstacles that limit an effective gene transduction in the myocardium: circulating DNases, neutralizing antibodies, cell-mediated immunity and endothelial barrier. Moreover, as a simple intra-coronary injection achieved a very low transduction with in vivo models in several studies, an occlusion of the coronary artery in order to extend the viral adhesion time has been suggested, which can cause acute ischemia. Even for a short duration, this may not be tolerated by patients with advanced heart failure or multi-vessel coronary artery disease. Finally, in a clinical setting, this administration route may be inaccessible in the cases of severe atherosclerosis or chronic total occlusions. The coronary venous system constitutes an alternate route whereby access to the

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myocardium can be achieved regardless of atherosclerosis severity or coronary obstruction. However, as balloon blockade of the coronary artery and sinus is necessary to prevent rapid vector washout to the periphery, it has the same risk related to acute ischemia for patients.

All these hurdles can be circumvented if the vector is injected directly into the targeted myocardium. Direct myocardial injection thus provides an attractive alternative insofar as it bypasses the blood and anatomical barriers, resulting in a more efficient and cardiospecific delivery. It results in localized high transgene expression compared to other methods [3, 4] and is therefore one of the most widely used gene transfer and stem cell therapy methods in clinical trials focused on myocardial repair.

Direct injection of the vectors into the myocardium can be achieved either surgically or percutaneously.

In clinical trials, surgical gene transfer is widely applied to patients whose myocardium is easily accessible such as during cardiothoracic surgeries. It can also be done during a dedicated procedure without cardiac arrest via lateral minithoracotomy [5]. Surgical delivery offers direct visual confirmation, which allows precise control of the injection. Limited exposure of the vectors to the blood reduces vector degradation. Together with bypassing of transendothelial crossing and local delivery, this method provides a high concentration at the injection site. It also enables gene transfer to the avascular and inaccessible areas. Moreover, training requirements are not too demanding [6] and animal as well as clinical studies have demonstrated its feasibility for gene delivery and cell administration [7]. This procedure carries, however, several risks mostly related to the invasive nature of the injection procedure itself. Perforation at the injection sites inside the left ventricle (LV) could be harmful in patients with thinned and scarred myocardia, causing cardiac tamponade. Cardiac arrhythmias may also arise secondary to tissue inflammation [6]. In addition, open thoracotomy and the general anesthesia, even with a minimally invasive surgical approach, constitutes a significant risk of morbi-mortality. Finally, besides the risks, this procedure presents some drawbacks, namely a limited access to certain areas of the left ventricle such as the septum. It can also cause leakage of the vectors from the injection sites, which can result in decreased vector retention and minimized but still significant exposure of the vectors to off-target organs.

The percutaneous approach offers an alternative that preserves the characteristics of intramyocardial delivery, but without the risks specific to the open-chest surgery. Both animal and clinical studies report its feasibility and safe use [8]. There are few data comparing both direct methods, but in a swine cell injection study, percutaneous endomyocardial injection was associated with a better total cell retention than surgical epicardial injection [9]. Similarly, several studies have shown a better cell retention with

direct intramyocardial injection compared to intracoronary infusion [3]. LV septum can be also targeted by this method. Considering the extensive use of coronary catheterization procedures, this approach thus appears to be preferable to surgery for the delivery of vectors, as it is less invasive and easier to implement in the clinical setting.

Catheter-based needle myocardial injection is associated with equivalent or even superior transduction efficiency compared to surgical injection [9, 10].

There are currently five catheter-based devices used in clinical trials for gene and cell therapies: four intended for a transendocardial approach (Helix™, MyoCath™, Myostar™, Stiletto™) and one designed for an epicardial approach via the coronary sinus (TransAccess Delivery System™). All of these devices have been shown to be effective and safe in both animals and clinical studies. Their composition comprises a core element, terminated distally in an injection needle with a variable shape, dedicated to the delivery of the vectors. The core is protected by a support catheter which helps to direct it toward the myocardial area of interest. MyoCath™ and Myostar™ catheters utilize an integrated system, meaning that the core and support catheters are combined into a single unit, whereas Helix™, Stiletto™ and TransAccess™ have separate core and support units [11]. Endomyocardial methods may have limited access to the myocardium in the area of the submitral valve apparatus while the right coronary territory is difficult to access with the transcoronary sinus approach. The interpersonal variability of coronary sinus anatomy may render the procedure more complex for the latter, whereas ventricular wall mobility may cause instability of the catheter in the case of endomyocardial approach. The transcoronary sinus approach allows parallel vector injection, which may result in greater vector retention compared to the injection perpendicular to the LV wall. Vector leakage may also occur into the LV cavity with the endocardial approach [9], and from the coronary sinus mostly to the lungs.

Procedures are performed under different imaging guidance modalities for determining the injection site including fluoroscopy [12], echocardiography [13], intravascular ultrasound [14], electric mapping systems [15], and magnetic resonance imaging [16]. Several authors believe that the electromechanical mapping-guided approach allows for better deployment of the tip of the catheter around areas of ischemia [10, 17]. It also allows to assess electromechanical function that help distinguish between normal, ischemic and infarcted myocardium, and is therefore useful to identify the areas of interest and guide the catheter towards them more accurately. These characteristics explain why this approach, in particular the NOGA mapping system, is the most frequently used in clinical trials [18–22]. However, the system is quite complex and expensive, and no imaging guidance is currently more recommended than another.

2 Materials

2.1 Surgical Procedure

1. Analgesia, anesthesia and antibacterial drugs that are appropriate for the animals and approved by the animal committee at the facility.
2. Monitors (pressure, ECG, SpO2).
3. A mechanical ventilator for large animals.
4. A standard surgical kit for a thoracotomy.
5. Towels.
6. Cautery pen.
7. Sterile direct DC pads.
8. Local anesthesia drug.
9. A 500 μL syringe and a 27 G needle with modified L-type shape to control the amount and place of injection (30 G needle can be used alternatively) (*see* Fig. 1).
10. Vector of interest.
11. Bioabsorbable sutures.
12. A nylon suture.
13. A 24F silicone thoracic drain tube.
14. A 60 mL syringe adapted to the drain.
15. Triple antibiotic ointment. Waterproof transparent dressings.

2.2 Percutaneous Procedure

1. Cine angiogram machine (biplane recommended).
2. Monitors (pressure, ECG, SpO2).
3. Analgesia, anesthesia and antibacterial drugs that are appropriate for the animals and approved by the animal committee at the facility.
4. Heparin.
5. A mechanical ventilator for large animals.
6. An introducer sheath for vascular access.
7. A 6-F pig-tail catheter.
8. A 8-F injection catheter.

Fig. 1 27 G needle with modified L-type shape

9. A 0.035-in. J-tip guidewire.
10. Iodinated contrast agent.
11. Standard cath pack.
12. Vector of interest.

3 Methods

3.1 Surgical Procedure

1. The procedure should be performed after an overnight fast. Prophylactic antibiotic is given an hour before the beginning of the procedure and analgesia.
2. The animal is anesthetized, intubated, and placed on the ventilator in right lateral recumbency (*see* **Note 1** and Fig. 2). Connect the monitors to the animal. The thoracic area is then clipped and scrubbed in preparation for surgery.
3. Locate the target intercostal space by palpation of the left chest (*see* **Note 2**). Perform intercostal nerve block with an approved local anesthesia drug on the left side of the chest.
4. Create a 8–10 cm incision on the skin on top of the fourth intercostal space, approximately centered on both sides of the horizontal level which passes through the left shoulder joint (beginning ~5 cm lateral to the sternum in a pig weighing ~10 kg, which is outside of the operating field and therefore hidden). Dissect the muscular layers to enter the thoracic cavity. Achieve hemostasis using a cautery pen (*see* **Note 3**).
5. Expand the surgical field by using two small rib spreaders perpendicular to one another. The larger the surgical window is, the easier it is to perform injection. However, a larger scar will increase the risk of infection and invasiveness. Pay special attention not to damage the lung and the pericardial sac (*see* **Note 4**).

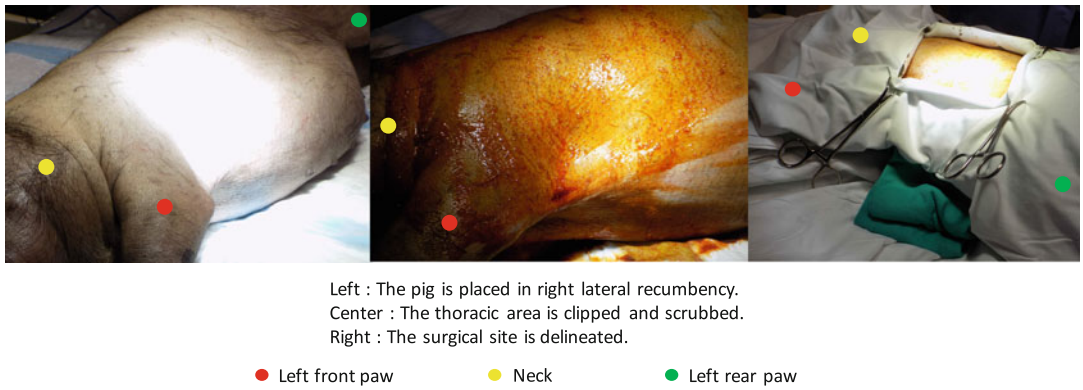

Fig. 2 Position of the pig for surgery. *Left*: The pig is placed in right lateral recumbency. *Center*: The thoracic area is clipped and scrubbed. *Right*: The surgical site is delineated. *Red*: Left front paw, *Yellow*: Neck, *Green*: Left rear paw

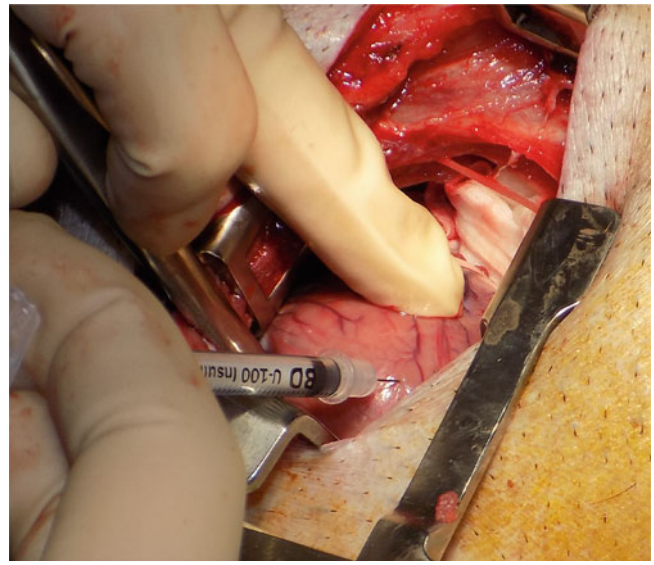

Fig. 3 Direct myocardial injection with a surgical epicardial approach. The bent part of the 27 G L-shaped needle is inserted into the target myocardium while applying pressure on a draining coronary vein

6. Delicately push back the left lung. Open up the pericardial sac after retracting it away from the myocardium and expose the heart (*see* **Note 5**).
7. Optimize the surgical window for good manipulation of the heart with a good view (*see* **Note 6**). By direct visualization of the heart, identify the area of interest.
8. Prepare (second operator) the vectors in injection syringes (*see* **Notes 6** and **7**).
9. Insert the bent part of the 27 G L-shaped needle into the target myocardium, taking care not to injure the epicardial coronary vessels (*see* **Note 8**). Before injection, check the absence of blood return after aspiration with the syringe. Inject vectors slowly (*see* **Notes 9–13** and Fig. 3). Continuously monitor ECG throughout the procedure since multiple direct injections sometimes cause ventricular fibrillation (*see* **Note 14**).
10. Create ~1 cm skin incision caudal at the second intercostal space from the injection space and place a silicon thoracic drain tube through this incision into the thoracic space.
11. After verifying the absence of bleeding inside the thoracic cavity, close layer by layer, using bioabsorbable sutures. Buried suturing will circumvent a later knock down of the animal to remove sutures.
12. Aspirate air from the chest cavity through the silicon thoracic drain tube.

13. Withdraw the silicon thoracic drain tube while keeping a negative pressure and close the incision using a nylon suture. This will create an airtight chest cavity.
14. Clean the surgical sites in sterile manner. Apply triple antibiotic ointment to prevent infection of the scar before covering it with waterproof transparent dressings.

3.2 Percutaneous Procedure Using Fluoroscopy

1. The procedure should be performed after an overnight fasting. Premedication comprises intravenous prophylactic antibiotic an hour before the beginning of the procedure and analgesia.
2. The animal is anesthetized, intubated, and placed on the ventilator in supine recumbent position.
3. Scrub the groin (and/or) the neck with Betadine and alcohol solution for disinfecting.
4. Obtain an access to the femoral or carotid artery with the Seldinger technique and insert an 8-F arterial sheath (*see* **Note 15**).
5. Administer heparin at the dose of 100 U/kg IV. Supplements are added as needed to maintain an activated clotting time of 200–250 s throughout the procedure.
6. Advance the guide wire with the guiding catheter to the arterial system. This will prevent accidental damage to the aorta. Special care should be taken when the guiding catheter passes the aortic arch.
7. A 6-F pig-tail catheter is advanced to the aortic valve in a retrograde fashion. After crossing the aortic valve, its tip is directed toward the apex within the LV cavity. Left ventriculography is then performed in left anterior oblique (LAO) 30° and right anterior oblique (RAO) 90° views (*see* **Note 16**).
8. After completion of LV angiography, the 6-F catheter is replaced by the 8-F injection catheter, the distal tip of which incorporates a 27 G needle that can be advanced or retracted (*see* **Note 17**). Before insertion, the catheter is flushed with a sterile heparinised saline.
9. The injection catheter is oriented to the target myocardial area using fluoroscopic guidance. Once a stable position is attained, the injection needle is extended 4–6 mm into the target myocardium. Inject vector slowly. Wait for a short period before removing the needle in order to minimize washout (*see* **Note 18**).
10. The volume of solution injected as well as the time of infusion is dependent upon the device used (*see* **Note 19**). However, exceeding 100 μL/single injection site may result in lower vector retention.
11. After completion of each injection, the needle is retracted and the catheter is moved to another endocardial site. Injections should be at least 5 mm apart.

12. Withdraw the sheath from the artery and achieve hemostasis by applying direct pressure to the arterial access site for several minutes (*see* **Note 20**).
13. Clean the puncture site in sterile manner and apply triple antibiotic ointment to prevent infection.

4 Notes

1. Inserting a thick surgical towel under the chest will raise the heart in the surgical area and facilitate injection procedures.
2. A left thoractomy provides a good surgical view of the anterior to lateral wall of the LV. The fourth intercostal space is suited for a naive heart without dilation, whereas the fifth intercostal space may be more suitable for a dilated heart after MI. If fluoroscopy is available in the operation room, visualization of the silhouette of the heart helps to determine an appropriate access site.
3. When entering the chest cavity, be careful not to injure the lung. Cutting the pleural membrane during the exhalation will reduce the risk of lung injury.
4. When dissecting the intercostal tissue, be careful not to damage the internal thoracic artery.
5. To facilitate heart exposure and minimize the risk of lung injury, the inspiratory volume on mechanical ventilator may be reduced during this operating time.
6. Pre-shaping the needle to curved or L shape will facilitate a more stable injection on the beating heart and prevent puncturing through the myocardium.
7. Pre-operative echocardiograms can determine the thickness of the LV wall. Left ventriculograms and coronary angiograms may help locating the infarct zone.
8. Inserting a wet gauze under the heart may sometimes facilitate the injection procedures. Do not forget to remove the gauze after the procedure.
9. The total volume for each injection site should not exceed 100 μL.
10. Applying pressure on a draining coronary vein will increase the vector dwelling time. However, it will increase the intramyocardial pressure and can cause ventricular tachycardia/fibrillation. The coronary sinus can be compressed for each injection, but should be released before the next injection.
11. Placing a suture for reference may help identify the injection site at the chronic stage. However, detailed notes together with epicardial vessel pictures can substitute this without causing extra injury on the heart.

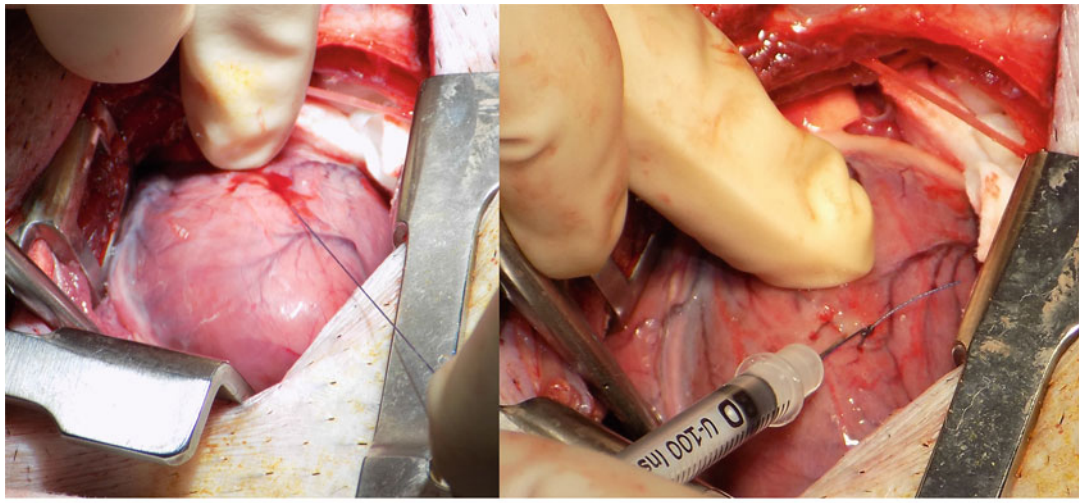

Fig. 4 Placing a mattress suture and injection inside the area surrounded by the suture. *Left*: Placing a mattress suture on an area without epicardial vessels. *Right*: Injection inside the area delimited by the mattress suture

12. Placing a mattress suture may increase the vector retention [23], however this is technically difficult and can be applied on a very limited area where no epicardial vessels are present (*see* Fig. 4).
13. Potential utility of sealing of the epicardial injection site with fibrin glue and reduction of ventricular rate by IV adenosine for the purpose of increasing cell retention have been suggested [7].
14. When ventricular fibrillation occurs, manually pump the heart while the assistant prepares for direct current shock. Apply a 10–20 J shock directly on the heart to restore sinus rhythm.
15. The femoral and the carotid arteries can also be accessed by a cut down method.
16. The LAO projection identifies the basal lateral, mid-lateral, apical lateral, apical septal, mid-septal and basal septal segments. The RAO projection identifies the anterior basal, mid-anterior, apical anterior, inferior, mid-inferior, and inferior basal segments.
17. Using a steerable guide sheath can increase stability of catheter position and facilitate injection procedures.
18. Note that in a clinical study, direct intramyocardial injections of genes and stem cells were followed by a minor release of cardiac biomarkers of myocardial damage, whereas intraventricular catheter movements in relation to a diagnostic electromechanical mapping procedure do not seem to lead to enzyme release exceeding the normal upper limits [24].

19. The electrocardiographic monitoring detects premature ventricular beats as evidence of needle penetration into the myocardium. Note that puncture of a nonviable myocardium may not generate premature ventricular beats. Under electromechanical mapping guidance, injections are made into ischemic areas, suggested by the combination of preserved voltage and abnormal wall motion.
20. Protamine may be used to reverse heparin unless there are known counteractions with the vectors. Protamine should be injected after the removal of the sheath to prevent acute thrombus formation inside the vessels. Inject protamine slowly as it can cause hypotension.

Acknowledgments

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Chapter 18

Selective Pressure-Regulated Retroinfusion for Gene Therapy Application in Ischemic Heart Disease

Rabea Hinkel and Christian Kupatt

Abstract

Coronary heart disease is still the leading cause of death in industrialized nations. Even though revascularization strategies such as coronary artery bypass graft surgery, percutaneous coronary intervention and enhanced drug therapy significantly improved the outcome, about 30% of patients develop chronic heart failure. Ischemic heart disease and heart failure are characterized by an adverse remodeling of the heart, featuring cardiomyocyte hypertrophy, increased fibrosis and capillary rarefaction. Therefore, gene therapeutic approaches for the treatment of heart failure, such as the modulating contractile function or therapeutic neovascularization, seem to be promising. To achieve an efficient transduction of the gene therapeutic agent, the time point and the application route seem to be important for the therapeutic success. In contrast to the classical systemic application regional intra-coronary application offers the possibility of higher transduction efficacy in the target area accompanied by a reduced off-target contamination. Antegrade delivery however, may be impaired by coronary heart disease, such as stenosis or occlusion of a coronary artery. Coronary veins appear not to be affected and might therefore be the preferable application route for gene therapy. For an effective and safe retrograde application in gene therapy, selective catheterization of the coronary vein draining the target area is necessary. In addition, to avoid coronary vein injury, a pressure regulated infusion enhances safety. Therefore, a selective pressure regulation of retroinfusion (SSR) seems to be a favorable approach for gene therapy transduction in combination with reduced systemic contamination.

Key words Gene therapy, Retrograde delivery, Ischemic heart disease, Coronary artery disease, SSR

1 Introduction

Coronary artery disease (CAD) is still the leading cause of death in western world countries. It is caused by atherosclerotic plaque formation in a coronary artery and treated with interventional or surgical revascularization therapy, thereby improving cardiac-specific mortality [1–3]. As of today, despite current strategies of rapid percutaneous revascularization, still 30% of patients with acute myocardial infarction will develop chronic heart failure over time [4]. Although drug therapy with antiplatelet agents, angiotensin-converting enzyme inhibitors and lipid lowering agents has improved the outcome following revascularization within the last

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years, the demand for primary and secondary prevention is large [5, 6]. For the USA, total costs (direct and indirect) for heart failure treatment will increase from $30.7 billion in 2012 to an estimated number of $69.8 billion in 2030 [7]. Moreover, a growing number of patients have exhausted interventional and surgical procedures of revascularization, but still suffer from angina. Hence, a molecular revascularization approach, such as gene-therapeutic neovascularization appears warranted.

Gene therapy for induction of neovascularization is not established in clinical settings up to date, mostly because of a lack of evidence of its efficacy. First generation attempts have included naked DNA and adenoviral formulations of vascular growth factor [8]. However, significant improvements have been made during the last decade, redefining the essentials of vascular gene therapy. Besides utilization of long-acting, low-immunogenic adeno-associated viruses (AAVs), balanced vascular growth has been developed, which provides vessel maturation in addition to capillary growth (e.g., [9, 10]).

In addition to the choice of the therapeutic agent, the application route seems of utmost importance. There are basically two different types of application: systemic and regional application. The regional catheter based administration can be divided into antegrade and a retrograde application [11, 12]. The antegrade application, however, has the disadvantage of utilizing the diseased vessel with a limited access to the target area and an altered transduction/uptake of the therapeutic agent. Therefore the retrograde approach seems to be favorable for patients with CAD, since in this case the venous tree that is not altered by the disease is used for assessing the ischemic/target region of the left ventricle [11, 12] (Fig. 1a). For an effective and safe retrograde application in gene therapy various application approaches are used: (1) The selective pressure-regulated retroinfusion system (SSR) [9, 13–16]; (2) The modified selective retroinfusion [17–19] and (3) The Retrograde coronary venous infusion [20]. In contrast to the SSR application the modified selective retroinfusion utilizes a 7-F balloon wedge-pressure catheter for the venous side. Application is performed in three periods each 3 min with a blocked AIV while the left anterior descending coronary artery (LAD) is occluded distal of the first diagonal branch. The retrograde coronary venous infusion blocks in addition the distal site of the AIV via an 1.5 mm over the wire balloon. SSR uses a pressure regulated infusion to avoid coronary vein injury, enhancing the safety. The SSR-system for retrograde delivery of therapeutics, such as medication or peptides [21, 22], cDNA [16, 23, 24], miRNA-inhibitor [25] and gene therapeutic agents [9, 15, 26, 27] has already been shown to be efficient and safe in preclinical pig models. Boekstegers and coworkers could show that the SSR system can be used safely in the clinical situation [13, 28]. In addition, for gene therapy, SSR offers a more

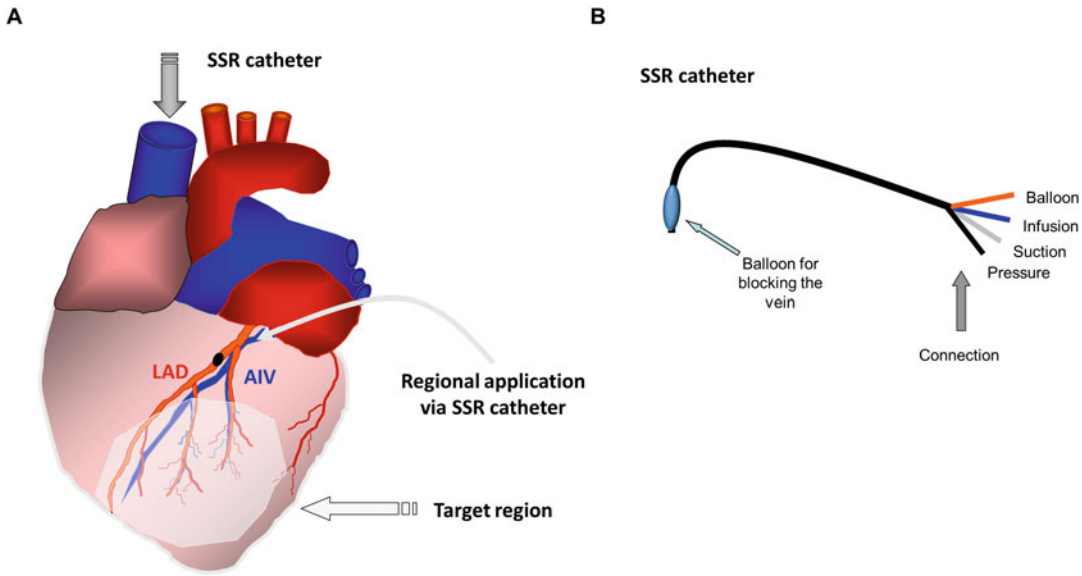

Fig. 1 Schematic illustration of the selective pressure regulated retroinfusion. (**a**) The retrograde approach via the cardiac vein (here AIV, *blue*) in the heart allows for a target area (*white area*) gene therapy, even if the arterial branch (LAD, *red*) is occluded (*black*). (**b**) Schematic illustration of the specific SSR catheter. This catheter contains four different lines, one connected to the balloon (*red line*) on the tip of the balloon for blocking the venous flow, one for the infusion of the gene therapeutic agent (*blue line*), one for the suction if applicable (*grey line*) and one for the continuous pressure measurement in the cardiac vein (black line) to allow a pressure regulated infusion

homogenous transduction than the direct intra-myocardial injection [29]. This procedure of SSR for gene therapeutic application in the pig model is described in detail in this chapter.

2 Materials

2.1 Arterial and Venous Peripheral Access

1. 8 French (F) sheath (including dilatator and wire) for the arteria carotis.
2. 11F sheath (including dilatator and wire) for the external jugular vein.
3. Saline solution.
4. Magnesium 1000 mg and amiodarone hydrochloride 150 mg added to the saline solution.
5. Heparin.

2.2 Angiography Equipment

1. X-ray C-arm with angiography tools.
2. Contrast agent.
3. Saline solution.
4. 3-way stopcock.

5. Pressure line for injection.
6. Y-adapter.
7. Torque handle.
8. Guidewire insertion tool.
9. Nitroglycerin (dilution 1:10 of 1 mg/ml glycerol trinitrate).

2.3 Catheterization of the Coronary Artery (LAD or Ramus Circumflex (RCx))

1. Judkins right (JR) catheter 7F with side wholes.
2. 0.014 in. guide wire.
3. Percutaneous transluminal coronary angioplasty (PTCA) balloon ($2.5–3.5 \times 10^{-15}$ mm).
4. Balloon inflation device (Fig. 2a).

2.4 Catheterization of the Coronary Vein (Anterior Interventricular Vein (AIV) or Middle Cardiac Vein)

1. Cournand catheter (6F).
2. 0.018 in. guide wire.
3. SSR catheter (Fig. 2b).

For the *retroinfusion of the middle cardiac vein* the following materials are required

4. 0.014 in. guide wire.
5. PTCA balloon ($3.5–4.0 \times 10^{-15}$ mm).
6. Balloon inflation device (Fig. 2a).

2.5 Infusion of Gene Therapeutic Agent

1. Infusomat (B. Braun Melsungen AG)
2. 50 ml perfusor syringe.

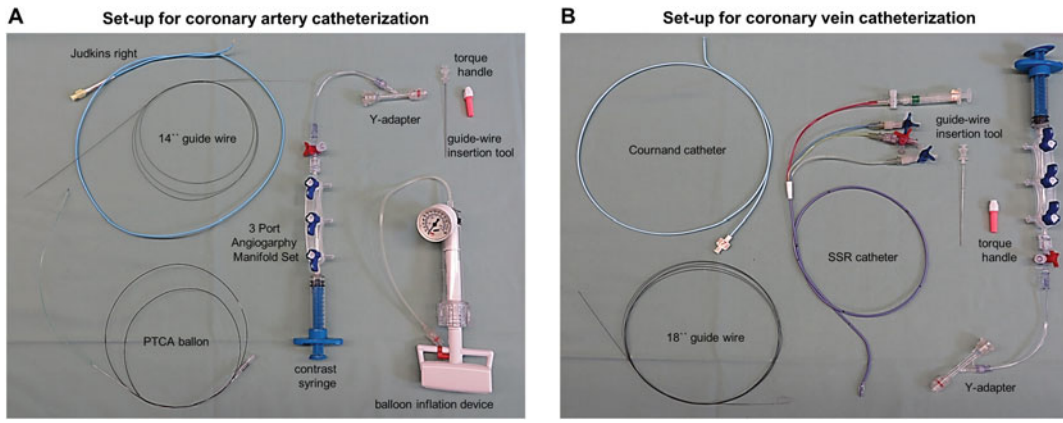

Fig. 2 Set-up for coronary artery and coronary vein catheterization. (**a**) Besides the Y-adapter, torque handle, guide-wire insertion tool, the 3 Port Angiography Manifold Set and the contrast syringe, which are used for both the arterial and the venous side, the specific requirements for the coronary artery (LAD or RCx) are: Judkins right (JR) catheter 7 Fr, 0.014 in. guide wire, PTCA balloon ($2.5–3.5 \times 10^{-15}$ mm), and balloon inflation device. (**b**) For the insertion of a specific selective pressure-regulated retroinfusion catheter (SSR) a Cournand catheter (6 Fr), 0.018 in. guide wire and a SSR catheter are needed

3. Perfusor line.
4. Stop cock.
5. Saline solution.

3 Methods

3.1 Venous and Arterial Assess

1. For the venous and arterial assess, the animals are placed in supine position and the legs are tied down, the neck region is shaved, scrubbed and draped in preparation for surgery (*see* **Note 1**).
2. Perform a 3 cm neck skin incision; here (1) the center of the incision is the middle of the triangle defined by the mandibular angle, (2) the shoulder joint and (3) the tip of the sternum is used as guidance. After incision of skin, subcutaneous fat and the subcutaneous muscle layer, the Musculus sternocleidomastoideus is used as guidance. On the medial part of the muscle the carotid artery is prepared and an 8F sheath is introduced into the cranially ligated common carotid artery via an introducer.
3. After successful introduction of the sheath, connect it to a monitor system allowing for pressure measurement. On the lateral side of the muscle, the external jugular vein is prepared and introduce an 11F sheath into the vein.
4. Then 10,000 IE heparin is applied systemically (acute clotting time (ACT) > 250 s) (*see* **Note 2**).
5. Before catheterization of the coronary artery a systemic infusion of saline solution including magnesium 1000 mg and amiodarone hydrochloride 150 mg is applied.

3.2 Blocking of the Antegrade Flow in Coronary Arteries

In order to achieve an optimal transfection efficacy up to the distal parts of the vein including the side branches, the antegrade flow needs to be blocked for the retroinfusion time. This step is not applicable in protocols where the coronary artery is already occluded (*see* Subheading 3.4).

1. For blocking either the LAD or the RCx, a Judkins right 7F with side wholes is needed. Connect the catheter to the Y-adapter and the 3 port manifold set/contrast syringe (Fig. 2a). After carefully flushing the catheter system with saline it is introduced into the 8F sheath in the A. carotis communis.
2. Adjust the X-ray to the heart and valve area and proceed the catheter forward till the height of the aortic arch. Here the tip of the catheter is torqued to the right side and further moved towards the left ventricle.
3. After passing the aortic arch branching, the catheter tip is turned to the left side and carefully advanced into the left main coronary artery (Fig. 3a).

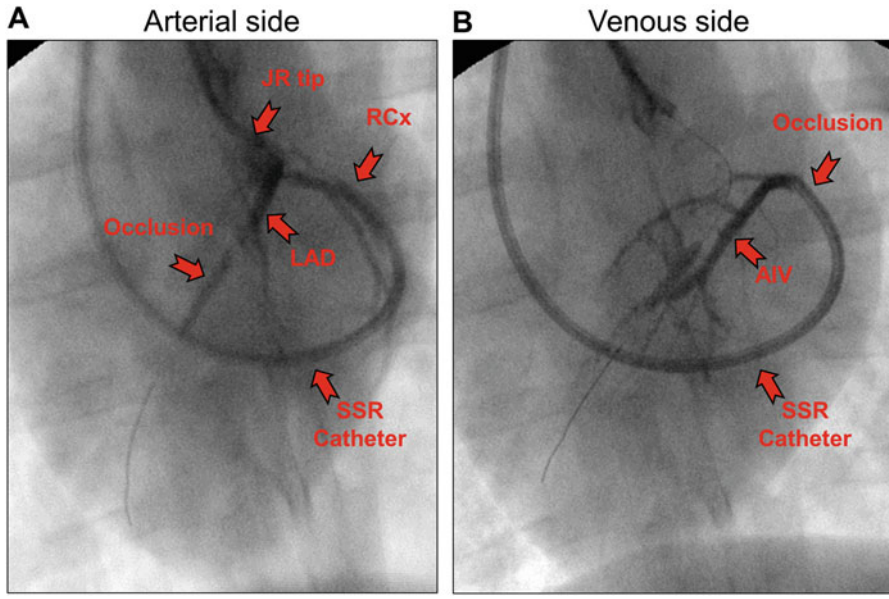

Fig. 3 Example of a selective pressure-regulated retroinfusion (SSR) into the anterior intraventricular vein (AIV). (**a**) Angiography of the left ventricular descending artery (LAD) and the ramus circumflexus (RCx) as indicated by the *red arrows* (LAO 30°). The antegrade flow is blocked by a balloon in the LAD distal to the first diagonal branch and the SSR catheter is in position for the retrograde application. (**b**) Angiography of the coronary vein through the SSR catheter (LAO 30°). This catheter is placed into the proximal AIV and blocked at the tip. Contrast agent is applied in the same manner as the gene therapy and shows a good distal filling of the AIV and its branches, allowing for effective transduction in the target area (the LAD perfused cardiac tissue)

4. Take an angiography of the coronary arteries via the 3-port manifold set/contrast syringe system to visualize the branching of the left coronary arteries (Fig. 3a).
5. Insert the 0.014″ guide wire into the JR catheter via the insertion tool and push forward to the tip of the catheter. After reaching of the catheter tip with the wire, rotate the catheter tip in the direction of the coronary artery (LAD) and the wire is forwarded into the LAD (Fig. 3a). If needed attach the torque handle to the distal end of the 0.014″ guide wire and rotate to allow for placement in the LAD.
6. Connect the balloon to the balloon inflation device (filled with 50% saline: 50% contrast agent, Fig. 2a).
7. Proceed the balloon along the 0.014″ guide wire to the tip of the catheter. For positioning the balloon in the LAD (distal to the first diagonal branch, in order to avoid ventricular fibrillation) the balloon is pushed forward while holding the wire in the same position.
8. Assure the correct positioning of the balloon by X-ray (Fig. 3a).

3.3 Placing of the Retroinfusion Catheter for the Selective Pressure-Regulated Retroinfusion in the AIV

1. First of all flush a 6F Cournand catheter with saline solution and introduce into the venous sheath.
2. Position the C-arm X-ray, such that the heart including the superior vena cava and the aorta are visible upon X-ray. The catheter is pushed forward until passing the right atrium and rotated, such that the tip of the catheter points to the left ventricle.
3. The catheter is moved carefully forward until the tip flips into the vena azygos (V. azygos). The great cardiac vein, which is needed to enter in order to move the catheter into the direction of the AIV, exits from the V. azygos where this turns in a 90° angle cranially. After inserting the Cournand catheter into the great cardiac vein, inject the contrast agent to depict the venous system (*see* **Note 3**).
4. Introduce the 0.018″ guide into the Cournand catheter via the guide wire insertion tool (avoiding damage in the sensitive tip of the wire) and push it forward into the great cardiac vein. Attach the torque handle to the wire directly before the Y-adapter and while softly twisting the wire, further push forward to the distal part of the AIV.
5. After ensuring a secure position of the guide wire, withdraw the Cournand catheter, while keeping the guide wire in its position. After removal of the Cournand catheter, the specific four-lumen SSR-catheter (Fig. 2b) is introduced into the vein via the guide wire (*see* **Note 4**). For this purpose the guide wire is inserted in the infusion or suction line at the tip of the SSR-catheter.
6. Then use the guide wire as guidance for the SSR-catheter and push forward till the proximal AIV. After correct positioning of the SSR-catheter in the AIV, remove the guide wire carefully.
7. After inflating the balloon (at the tip of the SSR-catheter, 1 ml air) the correct localization and the anatomic structure of the AIV is visualized via a single shot contrast agent (Fig. 3b).

3.4 Selective Pressure-Regulated Retroinfusion of Gene Therapy

1. Dilute the gene therapeutic agent in 15 ml saline solution in a perfusor syringe. Place the syringe into a perfusor and connect to the infusion line of the SSR-catheter via a perfusor line. Set the infusion velocity of the perfusor to 99 ml/h allowing for a continuous infusion over 10 min. Over an additional line connected to the SSR-catheter infusion lumen via 3-way-stop-cock (Figs. 1b and 2b) the infusomate is connected. This has an infusion solution reservoir with an infusion pump and a valve system for pressure regulated infusion.
2. Connect the pressure line of the SSR-catheter to a pressure measurement device.
3. Then first occlude the LAD (6–8 atm) followed by the SSR-catheter balloon (1.5 ml air) to determine the occlusion pressure in the AIV (*see* **Notes 5** and **6**). The infusion pressure reached

in the blocked AIV should be not more than 20 mmHg above the measured occlusion pressure to prevent hemorrhagic damage of the vein and the surrounding tissue (*see* **Note** 7).

4. Start the infusion via inflow from the high-pressure reservoir. It will be immediately interrupted by a valve upon reaching the predefined infusion pressure. Administration is carried out in a pressure controlled manner and can be varied from interval, and infusion time/heart beat allowing for an optimized contact time. Altogether, occlusion time of the LAD should not exceed 10 min, to prevent ischemic injury of the myocardial tissue.

3.5 Selective Pressure-Regulated Retroinfusion of the Middle Cardiac Vein

In case the RCx perfused area is the target area, the middle cardiac vein is utilized for the retroinfusion, e.g., in the chronic ischemic protocol displayed in Fig. 4a [9, 15, 26, 27].

1. Introduce the Cournand catheter and the 0.018″ guide wire as described in Subheading 3.3. In addition place a 0.014″ guide wire in the AIV before the Cournand catheter is removed.
2. Place the balloon (3.5–4 mm) in the proximal part of the AIV using the 0.014″ guide catheter as guidance (Fig. 4b).

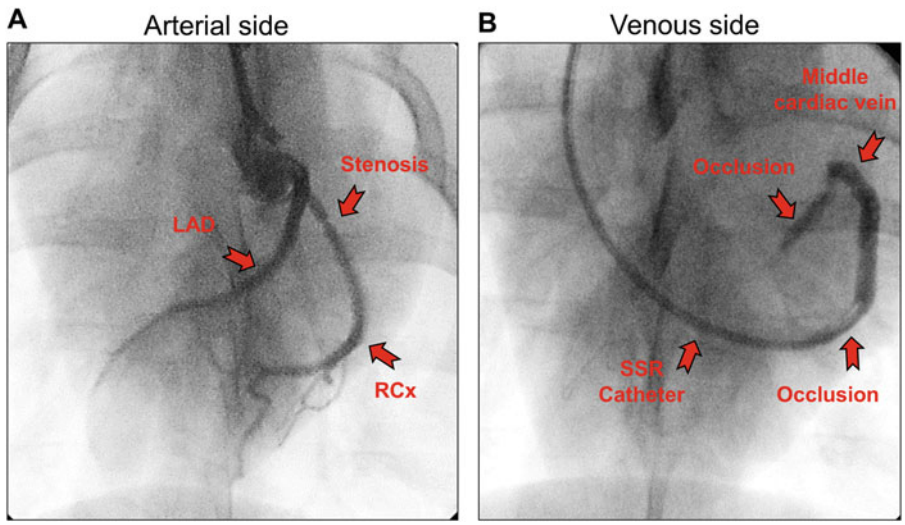

Fig. 4 Example of a selective pressure-regulated retroinfusion (SSR) into the middle cardiac vein. (**a**) Angiography of the left ventricular descending artery (LAD) and the ramus circumflexus (RCx) as indicated by the *red arrows* (LAO 30°). In the ramus circumflexus (RCx) a reduction stent is implanted at day 0 as displayed here, leading to a complete occlusion at time of retroinfusion (day 28 after implantation, not shown). The antegrade flow is blocked by the occluded stent in the distal RCx. (**b**) Angiography of the coronary vein through the SSR catheter. This catheter is placed into the great cardiac vein and blocked at the tip. In addition, to allow only for middle cardiac vein transduction, a PTCA balloon is placed in the proximal AIV, blocking flow from the distal AIV. Contrast agent is applied in the same manner as the gene therapy and shows a good retention in the middle cardiac vein, allowing for effective transduction in the target area (the RCx perfused cardiac tissue)

3. Introduce the SSR-catheter into the great cardiac vein as described in Subheading 3.3. Correct placement of the SSR-catheter and the balloon in the proximal AIV is monitored via X-ray and the occlusion pressure is measured while both, the SSR- and the AIV balloons are inflated (Fig. 4b). SSR is performed as described above.

4 Notes

1. *Animal size*
   The animals used for the SSR application of gene therapy should have a size of 20–25 kg body weight, to assure that the coronary vessels have a sufficient size. If the animals are lighter than 20 kg body weight, there is a high risk that the AIV diameter is too small to introduce the SSR catheter into the proximal AIV. When the catheter is not introduced far enough into the AIV, the gene therapy is not applied selectively to the LAD perfused area but also to the RCX perfused area.
2. *Activated clotting time (ACT) and embolic events*
   Before starting the catheterization of the animals, 10,000 IE of heparin is applied with a target ACT time of >250 s. During the experiment, especially if time needed for catheterization is prolonged, the ACT should be controlled every 30 min and heparin reapplied if the ACT is <250 s. Controlling the clotting time is utmost important, since a reduced clotting time increases the risk of embolism, especially pulmonary embolism in case of the venous catheterization.
3. *Vessel malformations*
   In rare cases, a vessel malformation, especially on the venous side is observed. These malformations include an unusual branching point of the great cardiac vein itself or a diffuse branching of the AIV. In both cases the animals cannot be used for the SSR-mediated gene therapy and need to be excluded from the study.
4. *Valves in the coronary venous system*
   The venous system contains valves, which might hamper the coronary venous catheterization. In pig hearts, there often occur venous valves at the branching point of the V. azygos and the great cardiac vein. To still introduce the catheter into the great cardiac vein or AIV, the SSR-catheter is placed directly in front of the valve. Then inflate the balloon of the catheter, the catheter is cautiously pushed towards the valve and rapidly deflate the balloon. This procedure might be repeated several times till the SSR catheter is introduced to the great cardiac vein.

5. *Arrhythmias due to arterial occlusion*
   Catheterization and intervention of the coronary arteries carries the risk of arrhythmias (especially in pig protocols), such as ventricular extra beats, ventricular tachycardia or ventricular fibrillation. These events might occur upon coronary artery blockade for retroinfusion. To minimize the risk of these adverse events, anti-arrhythmic medication (magnesium 1000 mg and amiodarone hydrochloride 150 mg) is added to the systemic saline infusion before starting the catheterization. If these arrhythmias still occur, the correct localization of the coronary artery balloon should be verified. In addition, the coronary artery balloon might be inserted more distally, thereby reducing the ischemic area and the arrhythmias.
6. *Shunt veins in the coronary venous system*
   After successful introduction of the SSR-catheter and blocking of the coronary vein and artery an angiography should be performed to estimate the retention time of the solution in the coronary vein. If this retention time is short (direct wash-out of the contrast agent), there might be shunt veins in the coronary venous system. In case the shunt occurs at the proximal part of the AIV the SSR-catheter might be introduced more distally into the vein. If the shunt vein occurs in the distal part of the vein, either the preset pressure of the reservoir and the infusion pressure might be reduced. If this does not improve the washout of the contrast agent and improve the retention time, the selective pressure-regulated retroinfusion for gene therapy cannot be performed efficiently and the animal should be excluded from the study.
7. *Hemorrhagic damage in the coronary veins*
   Hemorrhagic damage of the coronary vein may occur through the catheterization itself or an over-pressure in the SSR-balloon. To prevent any injury and hemorrhagic damage of the coronary vein, the catheters and wires should be handled with care avoiding too much pressure. The balloon of the SSR catheter should be inflated with maximum 1.5 ml air, until an increased resistance of the syringe used for blocking is observed. In case of an uncomplete occlusion of the AIV, the balloon should be deflated, the 0.018″ wire should be reintroduced and the SSR-catheter moved more distally into the AIV. After the procedure, before removing the SSR-catheter the integrity of the vein should be verified by injecting contrast agent into the infusion line of the SSR-catheter. If a dissection of the coronary vein is observed, the animal should be excluded from the study, since an effective application of the gene therapy is not assured.

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Chapter 19

Cardiac Gene Delivery Using Recirculating Devices

Melissa J. Byrne and David M. Kaye

Abstract

Cardiac gene delivery has become an important issue following the emergence of gene therapy for the possible treatment of heart failure. Despite many advances in the management of heart failure (HF), treatment options for many patients with advanced HF remain limited. At a cellular and molecular level, many of the fundamental alterations that contribute to the pathogenesis of HF are becoming better understood and this has resulted in the discovery of new therapeutic targets in animal models of HF, in particular in the area of gene therapy.

Numerous small animal and preclinical studies have examined the efficacy of delivering genes targeting various signaling pathways that are affected as the heart fails. However, the translation of this work into the clinic has been difficult due to the requirement for large scale targeted delivery of the gene. This methods chapter describes a percutaneous method of recirculation that we have employed to successfully deliver potential therapeutic agents, including genes, to the heart.

Key words Gene delivery, Cardiac, Recirculation, Percutaneous, Targeted

1 Introduction

Despite pharmacologic advances in the treatment of heart failure (HF), mortality and morbidity remain unacceptably high. Moreover, for patients with end stage heart failure therapeutic options are limited to heart transplantation and possibly long-term mechanical circulatory support. However, these options are not appropriate for many patients with advanced HF when associated with other comorbid diseases. Accordingly there has been a growing interest in the exploration of alternative approaches for the management of the end-stage HF patient. In particular, the development of a detailed understanding of the cellular and molecular pathophysiology of HF has led to a resurgence and growth in the potential role of gene and cellular therapies for HF.

The failing myocardium is characterized by progressive changes in a complex array of genes related to excitation contraction coupling, myocardial hypertrophy and cardiac fibrosis. While the use of gene transfer approaches to increase the expression of other

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dysregulated genes [1, 2] or to inhibit the effects of over expressed genes or proteins appears promising, a number of barriers exist in the translation of these potential therapies to the clinic. In particular, the development of a broadly applicable approach for gene delivery that provides the maximal potential for local delivery together with the use of viral vectors that can provide an appropriate balance between myocardial tropism, cellular transgene expression and safety, remains a challenge. Moreover, the optimization of these approaches for each therapeutic transgene will be an important determinant of their therapeutic success.

While both small and large animal studies provide firm support for the potential use of gene therapy in the management of clinical HF, several major issues require adequate resolution and optimization during clinical translation. One example of this is the recent completion of a Phase2b clinical trial examining the efficacy of adeno-associated virus serotype 1 carrying sarcoplasmic reticulum Ca^{2+}-ATPase 2a (AAV1.SERCA2a) delivered via intracoronary infusion to heart failure patients (CUPID2, [3]), which did not meet its primary or secondary endpoints.

Two complementary components, the choice of delivery vector together with the mode of delivery are important factors in determining the level of cardiac gene transfer. This is a particularly important consideration in the context of vector dose in the translation from small animal gene delivery outcomes to preclinical large animal and human studies. As such, a key aim is to optimize myocardial transgene expression while limiting systemic expression, and where possible limiting the vector dose required to provide optimal hemodynamic benefit.

A number of percutaneous mechanical methods for the delivery of genes have been described, including intracoronary (antegrade) [4] or retrograde infusion [5], and intramyocardial injection [6]. In addition, a surgical method of delivering genes during cardiopulmonary bypass has been developed (molecular cardiac surgery with recirculating delivery; MCARD, [7]). Each of these techniques encompasses a portfolio of advantages and risks which have been reviewed by Ishikawa and colleagues [8].

For the management of heart failure, the ideal delivery mode should provide global and prolonged exposure to the transgene that safely maintains the homeostasis of the heart. On this basis we previously compared the functional benefit of cardiac AAV-SERCA delivery, administered either via the intracoronary or recirculation route. In that study we showed that only AAV-SERCA recirculation improved cardiac function, consistent with the fact that the myocardial transit time following simple intracoronary injection is typically of the order of 10–15 s [9].

The recirculating delivery technique described in this methods chapter employs a percutaneous technique for recirculating gene

delivery to the heart called the V-Focus system (Osprey Medical Inc, Minnetonka, MN). By producing a catheterization based recirculation pathway for gene delivery we aimed to increase the exposure time of the agent to the myocardium in order to increase transgene expression and where possible to limit off-target exposure.

We have previously employed this approach in the large animal failing heart to deliver gene therapy directed towards restoration of SERCA2a and phospholamban [10, 11], each affording functional benefit. The V-Focus system has been designed to substantially isolate the coronary circulation from the general circulation, with minimal disturbance to the existing cardiac function and in doing so it allowing agents to be introduced into the heart circulation. The system is intended to minimize exposure of the therapeutic agent into the systemic circulation and to maximize myocardial uptake. The only unavoidable loss from the circuit occurs via the Thebesian veins, which communicate directly with the cardiac chambers and not with the coronary sinus. In most patients, the blood flow to these vessels is less than 10% of the total coronary circulation.

This method can be used in large animal models of heart failure including sheep [10, 11], pigs, and dogs, and while this chapter describes the use of the circuit for administration of gene therapy, it has also been employed for cell delivery to the heart [12] and antibiotic delivery to the lower limb [13].

2 Materials

2.1 Surgical/ Procedural Setup

1. Large animal (>30 kg) model of heart failure.
2. Anesthesia: induction and maintenance relevant to the animal model used.
3. Analgesia and antibiotics relevant to the animal model used.
4. Chlorhexidine surgical scrub and sterile drapes for incision site preparation.
5. Vector of interest.
6. Sterile surgical instruments as per vascular procedure.
7. Fluoroscopy equipment.
8. Introducer guide sheath kits: 9Fr and 11Fr.
9. Suture: 4.0 taper, 2.0 cutting.
10. Elastic vessel loops.
11. Heparin: dose relevant to the animal model used.
12. Contrast.

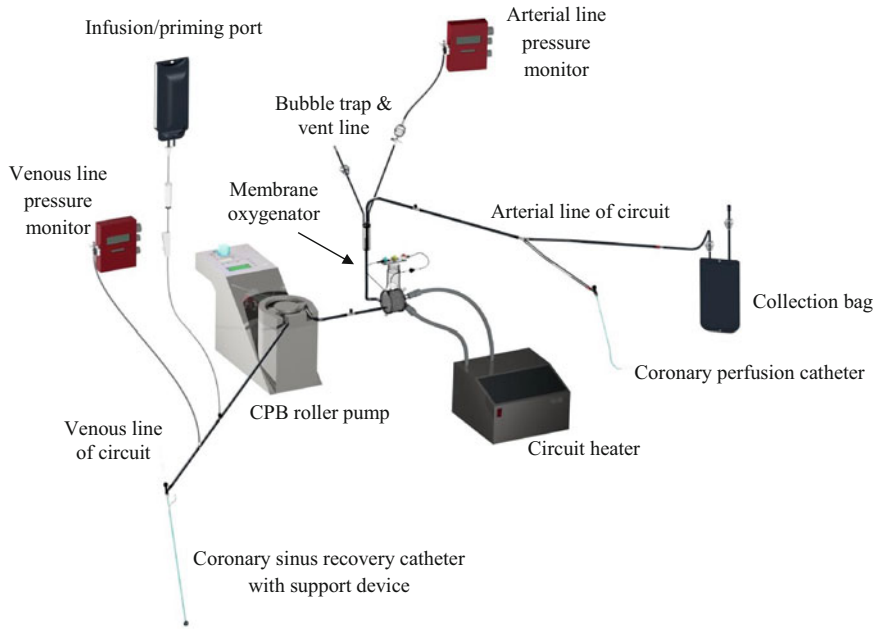

Fig. 1 Diagram of circuit components

2.2 Recirculation

The recirculation system requires the materials listed below and Fig. 1 indicates the completed circuit:

1. Cardiopulmonary circuit kit with venous recovery and arterial perfusion lines. The arterial perfusion line includes a bubble trap, a port for administering the therapeutic agent and a connector to the collection bag, which is used to collect the perfusate following the procedure. The venous recovery line incorporates a port for priming the circuit and subsequent infusion of fluid and a connection for the circuit inlet pressure monitor. In addition, the kit contains required high flow stopcocks, IV giving set, and collection bag.
2. Coronary sinus recovery catheter.
3. Coronary sinus support device.
4. Standard cardiopulmonary bypass (CPB) components required include a pediatric oxygenator with infusion manifold, a perfusion roller pump, circuit pressure monitors, and blood temperature control module and fluid heater.

To complete the recirculation additional components required include:

5. Tubing clamps × 3.
6. Viaflex bags of saline (1 l) × 2.
7. Tuohy Borst connectors × 2.
8. Interventional catheter for perfusion of the left coronary artery, minimum of 7Fr.

9. Guide wires: 0.035″ and 0.014″.
10. 3 stopcock × 2.
11. Source of oxygen.

3 Methods

3.1 Initial Setup

1. Induce anesthesia, intubate, and stabilize a large animal model in the lateral position to enable positioning of introducer sheaths in the jugular and carotid artery.
2. The perfusion system can be set up following the equipment Instructions for Use (IFU) (*see* **Notes 1**–**3**) while the animal is being anesthetized and stabilized and the perfusion catheters are being introduced.
3. Cut down and isolate the carotid artery using the elastic vessel loops and place a purse string suture within the vessel using 4.0 ticron.
4. Using the seldinger technique position the 9Fr introducer sheath within the artery.
5. Repeat this process in the jugular vein, positioning the 11Fr introducer.

3.2 Catheter Introduction

1. Following standard procedures and based on the animal model used, administer heparin (sheep model requires 15,000 U) to achieve an activated clotting time (ACT) adequate for extra-corporeal support (>250 s).
2. Tuohy Borst hemostasis valves are connected to both the recovery and perfusion catheters to minimize bleeding. Use a high-flow stopcock on the side arm of the hemostasis valves, which connects to the circuit venous and arterial lines.
3. Prime the coronary sinus recovery catheter balloon (via a 3-way stopcock) according to standard interventional procedures and decompress the balloon.
4. Cannulate the coronary sinus utilizing standard interventional cardiology techniques (*see* **Note 4**).
5. Introduce the coronary sinus recovery catheter into the coronary sinus until the catheter tip is at least 15 mm into the coronary sinus or proximal end of the balloon is positioned approximately 2–4 mm into the ostia (*see* **Note 5**).
6. Perform a contrast venogram of the coronary sinus to establish size and catheter position.
7. Inflate the occlusion balloon to occlude and check for contrast leakage. (*Note*: Inflate the balloon using diluted contrast medium (50% contrast)).

8. Under fluoroscopic guidance, insert the coronary sinus support device through the coronary sinus recovery catheter. The coronary sinus support device will self deploy (expand) into the coronary sinus creating a scaffold around the tip of the catheter (*Note*: The proximal end of the device should remain flush with the distal tip of the coronary sinus recovery catheter).
9. The 7Fr coronary artery catheter is advanced via the catheter introducer sheath in the carotid artery following standard interventional cardiology procedures, to the coronary artery ostium and the 0.014″ positioned in the left anterior descending (LAD) artery to stabilize the catheter.
10. Once the two catheter systems are in place they are linked via the perfusion system.

3.3 Connection of the Catheters into the Circuit

1. Open the sterile manifold package and hand lines into the sterile field.
2. Apply tubing clamps to both arms of the red arterial line and to the blue venous line and discard manifold.
3. Attach blue venous line to the coronary sinus recovery catheter; take care to ensure that connection is air free.
4. Remove tubing clamp from the venous line.
5. Attach one arm of the arterial line to the coronary artery perfusion catheter in the left coronary artery. Again ensure the connection is air free and do not remove the clamp.
6. Attach second arm of arterial line to the collection bag and remove clamp.
7. Zero the venous pressure monitoring transducer.
8. Turn gas flow on to 500 ml/min.
9. Switch pump on.
10. Slowly increase the pump flow up to 50 ml/min.
11. Check to see that blood is entering the venous line and verify that the oxygenator is filling with blood and the blood exiting is bright red (oxygenated).
12. Continue transfusing until all lines are blood filled and the priming saline enters the collection bag. During this time, optimize the flow rate to maintain an appropriate negative pressure within the circuit.
13. When the blood has back filled the circuit, inflate the recovery catheter balloon (and azygous catheter balloon if used, *see* **Note 5**), close off the collection bag and open the coronary artery catheter.
14. Inflate coronary sinus recovery catheter balloon and azygous catheter balloon if in use—the circuit is now closed and the patient's blood is recirculating through the coronary vasculature.

15. Slowly increase the pump flow to 100 ml/min.
16. Monitor venous inlet pressure until pressure stabilizes. Ensure pressure is negative and does not exceed –100 mmHg. (If negative pressure exceeds –100 mmHg, check position of coronary sinus recovery catheter and support device and lower the flow rate if necessary) (*see* **Note 6**).
17. Monitor the electrocardiogram for changes.
18. Recirculate for 1 min to ensure circuit and animal stability.
19. Once confirmed that flow rate is optimal in relation to negative pressure within the circuit add the agent to be delivered at the port distal to the oxygenator at a rate that matches the flow rate of the pump—this prevents blowing out of the fluid and/or movement of the distal tip of the catheter that can occur due to abrupt changes in pressure.
20. Follow agent with an additional 5 ml of saline to clear the line at the rate matching pump flow rate.
21. Following delivery of the agent, recirculate for 10 min (or as protocol for the particular agent dictates). If negative pressure increases beyond –100 mmHg during recirculation, open thumb wheel on IV administration set and deliver bolus of 20 ml of saline or optionally, reduce follow rate, or small boluses (5–10 ml) of saline may be administered as required.

3.4 Procedural End

1. At the end of recirculation, capture of the circuit volume of blood (200 ml), to further minimize systemic exposure of the agent, can be achieved clamping the arterial line and re-diverting the delivery of blood to the collection bag while maintaining a lower pump flow of ~50 ml/min.
2. Once the circuit volume has been collected, the pump can be turned off, recovery balloon deflated and the wire, support device and catheters removed from the heart following standard interventional cardiology procedures.
3. Introducer sheaths can then be removed and vessels sealed with the purse string suture and the incision closed (2.0 cutting).
4. While the incision is closed, remove the circuit from pump and discard according to institutional waste protocols.

4 Notes

1. During priming of the circuit it is essential that all air is removed from the system and that the height of the oxygenator is maintained throughout the procedure to prevent the drawing in of air from the oxygenator due to gravity.
2. Therefore, it is important to run the closed circuit following priming with saline to confirm circuit patency.

3. This initial recirculation of saline will also allow the temperature of the circuit to be stabilized at ~37 °C prior to connection to the catheters.
4. To position the large coronary sinus catheter recovery catheter, commercially available catheters, sheath and/or guide wire can be used to facilitate cannulation. We use a 5Fr guide catheter over an 0.035″ j-tip wire within the catheter. Coordination of both the wire and the guide sheath facilitates locating and engagement the CS ostium.
5. In both sheep and pigs the left azygous (hemiazygous) vein drains directly into the coronary sinus (rather than emptying into the superior vena cava as seen in dog and human hearts). As such, it is necessary to position the distal tip and inflation balloon are past azygous vein entrance. This will ensure that the blood collected comes from the coronary circulation. If, due to anatomical variations, it is not possible to get past this point, a second balloon catheter (via a second jugular introducer sheath) can be positioned in the vessel to prevent contribution of blood to the circuit. This balloon catheter should be inflated at the same time as the recovery catheter balloon.
6. During recirculation it is important that there is no change in catheter position. Changes can alter the pressure within the circuit, in particular: movement up against a vessel wall and advancement of catheters too far into the lumen of the vessels can increase negative pressure which can result in cavitation of the tubing; and movement of the catheters out of the vessel ostium can lead to loss of agent to systemic circulation. Therefore, prior to connecting the circuit ensure that both introducers and catheters are secure and stable.

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Chapter 20

Molecular Cardiac Surgery with Recirculating Delivery (MCARD): Procedure and Vector Transfer

Michael G. Katz, Anthony S. Fargnoli, Andrew P. Kendle, and Charles R. Bridges

Abstract

Despite progress in clinical treatment, cardiovascular diseases are still the leading cause of morbidity and mortality worldwide. Therefore, novel therapeutic approaches are needed, targeting the underlying molecular mechanisms of disease with improved outcomes for patients. Gene therapy is one of the most promising fields for the development of new treatments for the advanced stages of cardiovascular diseases. The establishment of clinically relevant methods of gene transfer remains one of the principal limitations on the effectiveness of gene therapy. Recently, there have been significant advances in direct and transvascular gene delivery methods. The ideal gene transfer method should be explored in clinically relevant large animal models of heart disease to evaluate the roles of specific molecular pathways in disease pathogenesis. Characteristics of the optimal technique for gene delivery include low morbidity, an increased myocardial transcapillary gradient, esxtended vector residence time in the myocytes, and the exclusion of residual vector from the systemic circulation after delivery to minimize collateral expression and immune response. Here we describe myocardial gene transfer techniques with molecular cardiac surgery with recirculating delivery in a large animal model of post ischemic heart failure.

Key words Gene therapy, Surgical gene delivery methods, Myocardial infarction, Ischemic heart failure, Molecular cardiac surgery with recirculating delivery, Cardiopulmonary bypass vector transfer

1 Introduction

Cardiovascular diseases are still the leading health problem in industrialized countries in terms of morbidity and mortality [1]. The promise of gene therapy in the treatment of acquired and congenital heart and vascular disorders remains very high [2]. In the last decade significant progress was achieved in various areas of gene therapy research including therapeutic angiogenesis, cystic fibrosis, cardiac arrhythmias, heart failure, and ischemic heart disease [3]. However, many unresolved issues limit the clinical application of gene therapy. In general the effectiveness of gene therapy

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is determined by a combination of gene delivery to the target organ, transfection of the genetic material into specific cell types, and the expression level of the transfected gene. Inadequate delivery and transfection efficiency has been one of the major limitations of gene therapy [4]. This efficiency can be improved with the development of better gene-delivery methods and vectors [5]. To this date, no delivery system or viral serotype has optimized cardio tropism without collateral expression in other tissues [6]. A great number of gene delivery techniques have been researched in cardiovascular gene therapy. Two major conclusions should be drawn from the data: the physical route of gene delivery is no less important than the vector system, and gene delivery should be organ targeted with minimal or optimally zero collateral expression. Gene delivery strategies to the heart have included interventions using either an intravascular approach through the coronary arteries or direct delivery to cardiac muscle [7]. There is still no consensus on which method of gene transfer is better: intramyocardial or intracoronary. Each of these methods has its positive and negative aspects. In our view, both of them will be used in the future, though perhaps for differing applications.

Direct intramyocardial injection of viral vectors is a technique that can support sufficient myocardial delivery in different animal models. The efficacy of this mode of cardiac gene delivery is limited by the accessibility of myocardial tissue and the areas injected [8]. This mode of gene delivery is clinically acceptable only during procedures where the myocardium is readily accessible. Unlike the transvascular route, during direct delivery, transgene enters the extracellular matrix and somatic cells, bypassing the blood compartment which includes plasma proteins, blood cells, and neutralizing antibodies which substantially inactivate the vector. Moreover, this method allows for the application of high concentrations of transgene directly at the target site. This approach has been successfully applied in animal models of ischemia, heart failure and cardiac arrhythmias as well as several clinical trials to induce therapeutic angiogenesis in coronary artery disease [9].

Intravenous administrationis the least invasive and simplest route for gene transfer. It finds its application in the treatment of systemic diseases such as hypertension and hyperlipidemia [10]. However, significant limitations of this technique include first-pass pulmonary and hepatic uptake of vector as well as fast dilution in blood circulation. The efficacy of intracoronary administration is much better and this delivery is obviously clinically relevant and appealing, although it cannot escape systemic leakage [11]. The use of retrograde intracoronary transfer through the coronary sinus is undoubtedly a step forward compared with a selective antegrade intracoronary technique due to several factors [12]. First, it can be more easily applied in patients with coronary artery disease. Second, it allows for prolonged adhesion time of the vector to the cardiac endothelium, and overcomes the resistance of precapillary sphincters

proximally located on the arterial side of the capillary bed. Finally, it can reduce myocardial reperfusion injury. Intracoronary delivery has shown limited transduction and varied results with systemic leakage because of the single pass transvascular gene transfer with very fast dilution of vector concentration in the circulating blood with subsequent gene and vector dissemination to collateral organs [13]. The parameters need to be improved are: the contact time of vector in coronary circulation, intravascular flow rate and perfusion pressure, composition of perfusate and endothelial permeability [14]. This limitation encouraged researchers to develop closed-loop recirculatory systems like MCARD, which allowed separation of the coronary vascular bed from the systemic. The principal strength of this technology includes a dramatic (>100-fold) increase in transduction efficiency, the extension of vector residence time, the ability to manipulate endothelial permeability, the avoidance of an immune response to the vector and the ability to washout the vector post gene delivery limiting collateral organ exposure [15].

In this chapter, we describe molecular cardiac surgery with recirculating vector delivery to the ovine heart in a large animal model of post ischemic heart failure.

2 Materials

2.1 Large Animals, Sheep

All animal studies must follow National Institute of Health Guidelines (*Guide for the Care and Use of Laboratory Animals*, NIH publication No. 85–23, revised 1996) and be approved by the appropriate Institutional Animal Care and Use Committee.

2.2 Strain, Age, Weight, Gender

Dorsett male sheep, 0.8–1.4 years old, weighing 35–55 kg (*see* **Note 1**).

2.3 Anesthesia and Perioperative Medications (Table 1)

1. Preoperative analgesia and anesthesia: IV ketamine 10 mg/kg, IV midazolam 0.2 mg/kg, IV propofol 1–8 mg/kg, IM buprenorphine 0.01 mg/kg.
2. Maintenance analgesia and anesthesia: intratracheal isoflurane 1–3 %, fentanyl patch 75 or 100 μg patch. Lacri-lube eye lubricant, lidocaine gel/lubrication, IM Naxcel 2.2 mg/kg.
3. *Additional devices*: anesthesia mask, laryngoscope, endotracheal tubes #7–8Fr, pulse oximeter, thermometer, rumen tube, heating pad, ECG pads.

2.4 Infarct Creation by Direct Ligation of Coronary Arteries

1. *Preoperative analgesia and anesthesia*: bupivacaine 2 mg/kg (intercostal block and incisional wound), IV phenylephrine (inotrope) 0–100 μg/min, IV lidocaine (antiarrhythmic) 1–4 mg/min, IV epinephrine (inotrope) 1–4 μg/min, IV amiodarone 100–300 mg, IM enrofloxacin (baytril) 5 mg/kg, IM buprenorphine 0.01 mg/kg, IM morphine 5 mg, IM banamine 1 mg/kg, IM glycopyrrolate 0.02 mg/kg.

Table 1
Drugs and inductions agents for anesthesia

Drug name	Frequency	Dose (mg/kg)	Route	Concentration
Propofol	Before and during surgical procedures	1–8 mg/kg	IV	10 mg/ml
Ketamine	Before surgical procedures	10 mg/kg	IV	100 mg/ml
Midazolam	Before surgical procedures	0.2 mg/kg	IV	5 mg/ml
Isoflurane	During surgical procedures	1–5 %	inhalation	1–5 %

2. Surgical set: ribs spreaders, blades, knife handle, army navy retractor, DeBakey forceps, Mayo and Metzenbaum scissors, regular hemostats, mosquito hemostats, needle holders, Kelly clamp, Schnidt hemostat. Sutures: #2-0 silk pop-off on taper needle, #4-0 prolene on taper needle, #0 silk ties, #1 vicryl on taper needle, #2 vicryl on taper needle.
3. *Additional devices*: electrocardiogram machine 12-lead, arterial line monitor, connection apparatus and pressure tubing, 24–28Fr angle chest tubes, Pleur-evac.

2.5 Molecular Cardiac Surgery with Recirculating Delivery (MCARD). Procedure and Vector Transfer

1. Personnel. Surgeon, assistant of surgeon, anesthesiologist, cardiovascular perfusionist (operates a heart–lung machine).
2. Blood. Since bleeding in cardiopulmonary bypass cases can occasionally be rapid, unpredicted and life threatening, we will draw 1 U of blood from 1 donor sheep routinely. If there are significant bleeding concerns, we will draw 2–3 U. At the end of bypass, we will evaluate a blood gas and if the hematocrit is greater than 15 % then we will make a clinical judgment, based on ongoing bleeding, arterial blood gases, electrolytes and hemodynamic stability whether or not to transfuse each animal. If the hematocrit is less than 15 % then we will typically transfuse the sheep. We use 3–4 sheep as blood donors for the MCARD procedures. These donor sheep undergo no surgeries; their only involvement is as a blood donor for transfusions for sheep undergoing MCARD. Blood draws will be performed under sedation on the day of MCARD (two sheep undergo draws for each MCARD, ~400 ml drawn for each sheep).
3. Drugs. Drugs and agents administered other than anesthesia including analgesics, antibiotics, hypnotics, sedatives, and tranquilizing medications (Table 2).

Table 2
Drugs and agents administered other than anesthesia

Drug name	Frequency	Dose (mg/kg)	Route	Concentration
Bupivacaine	Once. During MCARD	up to 2 mg/kg	Nerve Block	0.50%
Epinephrine	After CPB	1–4 μg/min	IV	1 mg/ml
Lidocaine (local anesthetic)	After sedation	max 100 mg	SC	1–2%
Lidocaine (antiarrhythmic)	After CPB	1–4 mg/min	IV	0.80%
Heparin	Prior to CPB	130 U/kg	IV/SQ	1000 U/ml
Phenylephrine	After CPB	0–100 μg/min	IV	10 mg/ml
Amicar (Aminocaproic Acid)	After CPB	5 g	IV	250 mg/ml
Dopamine	As needed	2–10 μg/kg/min	IV	250 cm^3 bag
Dobutamine	As needed	3–10 mg/kg/min	IV	250 cm^3 bag
Digoxin Injection	As needed	1–2 ml	IV	100 μg/ml
Metoprolol tablets	As needed	15–25 mg	PO SID	15–25 mg
Metoprolol IV	As needed	1.25–5 mg every 6–12 h	IV up to 15 mg	See instructions
Esmolol	As needed	15–200 μg/kg/min	IV	See instructions
Nitroprusside	As needed	0.5–7.0 μg/kg/min	IV	50 mg/2 ml
Papaverine	As needed	0.1–1.0 mg/kg	IV	20 mg/ml
Amiodarone	After CPB	100–300 mg	IV	150 mg/3 ml
Magnesium	After CPB	0.5 mg–3 g	IV	0.5 mg/ml
Potassium	After CPB	20–40 mEq/l	IV	15%
Sodium Bicarbonate	After CPB	10–50 mEq	IV	7.5–8.4%
Furosemide injectable	As needed	10–50 mg	IM/IV	See instructions
Mannitol	As needed	12.5 mg	IV	12.5 mg vial
Albuterol Inhalant	As needed	2–3 puffs	Inhalant	
Fentanyl	Before and after MCARD	1–3 μg/kg/h	IV	5 mg/ml
Gadolinium	During MRI	12–20 ml	IV	529 mg/ml
Propofol	During MCARD	1–8 mg/kg	IV	10 mg/ml
Cefazolin	Prior and after MCARD	1 g/10 ml Syringe	IV	1 g/10 ml
Naxcel (Ceftiofur Na)	Prior to MCARD	2.2–6.6 mg/kg	IM	1 g/20 ml

(continued)

Table 2
(continued)

Drug name	Frequency	Dose (mg/kg)	Route	Concentration
Vancomycin	During MCARD	5 g	Sternum	1 g/vial
Penicillin	Before and after MCARD	22,000 IU/kg	IV, IM	
Combi-Pen-48 (penicillin G benzathine + penicillin)	After MCARD	10,000 IU/kg	SQ once	150,000 U/ml
Combi-Pen-48 (penicillin G benzathine + penicillin)	In case of Infections after MCARD	20,000 IU/kg	SQ every 3 days	150,000 U/ml
Gentamicin	Before and after MCARD	6.6 mg/kg	IV, IM	50 mg/ml
Enrofloxacin (Baytril)	Before and after MCARD	5 mg/kg	IM 3–5 days	100 mg/ml
Tulathromycin (Draxxin)	In case of lung Infections	2.5 mg/kg	SQ/IM once	100 mg/ml
Covexin 8 (clostridium Types C and D Bacterin-Toxoid)	Vaccine before surgery	5 ml primary dose	SQ	-
Fentanyl patch	Before MCARD	75–100 μg/h	topical	75–100 μg patch
Buprenorphine	Before and after MCARD	0.01 mg/kg	IM	0.3 mg/ml
Morphine	Before MCARD	5–7.5 mg	IV/IM	5 mg/ml
Flunixin meglumine (Banamine)	Before and after MCARD	1 mg/kg	IM	50 mg/ml
VEGF	During CPB	15–60 μg	IV	-
Atipamezole	Before MCARD	0.05 mg/kg	IV	5 mg/ml
Ketamine	Before MCARD	10 mg/kg	IV	100 mg/ml
Diazepam	Before MCARD	0.5 mg/kg	IV	5 mg/ml
Thiamine injectable	As needed	2 ml	IM	50 mg/ml
Isoflurane	During MCARD	1–5%	Inhalant	
Solu-Medrol	As needed	125–500 mg	IM	250 mg/ml
Cimetidine	During MCARD	300 mg	IV	200 mg/ml
Benadryl	During MCARD	50 mg	IV	50 mg/ml
Lasix	As needed	2–4 mg/kg	IV	10 mg/ml
Protamine	After CPB	1.5–3 mg/kg	IV	10 mg/ml
Glycopyrrolate	Before MCARD	0.02 mg/kg	IM	0.2 mg/ml

(continued)

Table 2
(continued)

Drug name	Frequency	Dose (mg/kg)	Route	Concentration
Calcium	As needed	500 mg–1 g	IV	10 ml syringe
Methylene blue	During MCARD	1 mg/kg	CPB circuit	1 mg/ml
Plegisol Infusion	During MCARD	As needed	CPB circuit	1000 ml bag
Hetastarch 6%	During MCARD	1000 ml	CPB circuit	500 ml bag
Del Nido Solution	During CPB	15 mg/kg	CPB circuit	1000 ml bag

CPB cardiopulmonary bypass

4. Surgical set: ribs spreaders, blades, knife handle, army/navy retractor, DeBakey forceps, Mayo and Metzenbaum scissors, regular hemostats, mosquito hemostats, needle holders, Kelly clamp, Schnidt hemostat, weitlaner retractor, tubing clamps, suture forceps, right angle, wire cutters.
5. Sutures: #2-0 silk pop-off on taper needle, #4-0 prolene on taper needle, #4-0 nonpledgeted prolene on taper needle, #0 silk ties, #1 vicryl on taper needle, #2 vicryl on taper needle, #2-0 nylon on cutting needle, #5 steel sternal wires.
6. Additional devices: cannulas/catheters. Perfusion equipment—13Fr Terumo retrograde catheter, 26Fr right angle cannula, 26Fr straight cannula, 12 and 14Fr arterial catheters, 9Fr DLP aortic root cannula, centrifugal pump, ¼′ siliconized tubing, Bentley bubble oxygenator, siliconized polycarbonate connectors. Echocardiogram machine. Arterial line monitor, connection apparatus and pressure tubing. 12Fr–16Fr angio-catheter. 26–28Fr right angle chest tubes, Pleur-evac. Heating system.
7. Intraoperative monitor with ECG, arterial blood pressure, central venous pressure, cardiac output, rectal temperature.
8. Blood gas analyzer.
9. Large animal ICU with postoperative recovery: continuous oxygen supply, vacuum system for chest drainage, ECG, infusion pump with 2–3 lines.

3 Methods

3.1 Preoperative Care and Preparation

1. Anesthetize with 10 mg/kg ketamine and 0.2 mg/kg Midazolam, delivered IV.
2. Transport the sheep from the colony to the preparation room and weigh.
3. Maintain anesthesia by isoflurane gas administration via mask.

4. Position the sheep in sternal recumbence on the preparation table.
5. Intubation: Using a long straight-edge laryngoscope and endotracheal suction (for clearance of secretions where necessary), visualize the vocal cords. Once vocal cords are in adequate view, insert the deflated, regular-cuffed 8Fr endotracheal tube (or alternate size depending upon sheep's laryngeal space and vocal cord aperture), lubricated with viscous lidocaine, into endotracheal passage between vocal cords. Inflate the balloon cuff with 20 ml of air. Confirm placement via auscultation and connect endotracheal tube to the mechanical ventilation apparatus with 2.5–3.5% isoflurane. Depth of anesthesia is monitored by vital signs, pupillary response to light, and response to physical manipulation.
6. Insert the rumen tube, lubricated with lidocaine gel/KY jelly, by feeding the tube gently, but firmly, over the base of the tongue into the esophagus and down into the rumen tract. Confirm placement via the presence of rumen fluid.
7. Administer 0.01 mg/kg buprenorphine IM, 0.02 mg/kg of glycopyrrolate IM, 1 mg/kg banamine IM, and 2.2 mg/kg naxcel IM.
8. Place fentanyl 100 μg patch onto the groin area and lubricate eyes with Lacri-lube.
9. Clip the sheep and cleanse the surgical areas with chlorhexidine scrub followed by chlorhexidine solution.
10. Place EKG pads on clipped forelimbs and secure in place with silk tape.

3.2 Infarct Creation by Direct Ligation of Coronary Arteries (Fig. 1)

1. Connect the sheep to the anesthesia machine with 1.5–2.5% isoflurane.
2. Connect the ECG leads to the ECG machine and connect supportive IV lines.
3. Using the Seldinger technique, place a 7Fr triple lumen catheter in the left jugular vein, which can be used intraoperatively for medication delivery and central venous pressure monitoring (*see* **Note 2**).
4. Place the animal in the supine position. Using sterile technique with a 7Fr catheter, place a carotid or femoral arterial line for invasive blood pressure monitoring and Millar catheter measurements. Following that, position the animal in a right lateral decubitus position. Then prep and drape the left chest in sterile fashion, using chlorhexidine scrub and solution.
5. One hour prior to creation of the infarct, we initiate I.V. amiodarone (75 mg/h), lidocaine (35 mg/h), and phenylephrine (6–25 mg/h) to maintain systemic blood pressure >100 mmHg (*see* **Note 3**).

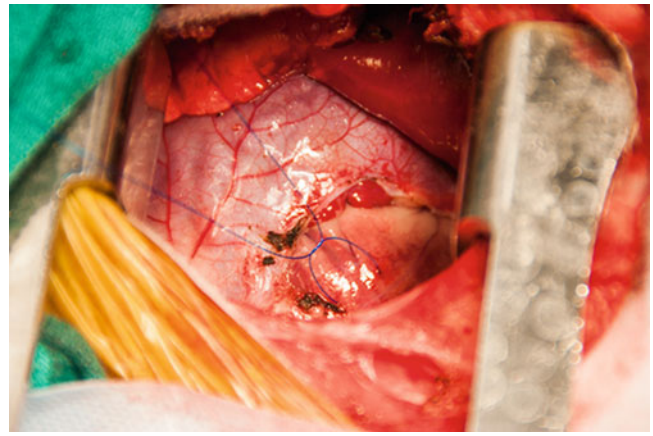

Fig. 1 Infarct creation by direct ligation of coronary arteries. Coronary artery ligation. Left anterior thoracotomy is usually performed through the fifth intercostal space. After opening the pericardium, the coronary anatomy is inspected. Obtuse marginal 1 and obtuse marginal 2 arteries (main branches of the circumflex artery) are ligated with Prolene 4-0 suture

6. Enter the chest via a 5–7 cm left mini-thoracotomy incision at the IV–V intercostal space, reflect the left lung posteriorly, and incise and reflect the pericardium superiorly and laterally. Next, identify and ligate the proximal portion of the first two branches of the obtuse marginal artery with 4-0 prolene sutures, with close attention to EKG changes. Acute ST-elevation and surrounding tissue discoloration confirm the creation of an infarct.
7. Inspect places of coronary artery ligation for residual hemorrhage.
8. Flash the pericardial space with Ringer-Lactate 3–5 times to prevent future adhesions. Loosely approximate the pericardium with 3-0 silk.
9. Place a 28Fr thoracostomy angle tube and connect to low, continuous wall suction via a Pleur-evac for 30 min. The thoracotomy incision is repaired in usual fashion. Discontinue the thoracostomy tube and extubate in sequential fashion. Finally, transport the animal to a recovery room for routine postoperative care.

3.3 Molecular Cardiac Surgery with Recirculating Delivery (MCARD) (Fig. 2)

1. *Preparing for cardiopulmonary bypass.* Position the sheep in supine position and secure all four limbs. Prep and drape the sheep in sterile fashion from neck to knees after induction of anesthesia. Place sterile large drapes and cut holes over chest, right groin and right carotid artery (*see* **Note 2**).
2. Surgically expose the right femoral artery via cut-down procedure in standard fashion. Insert a 16 G angiocatheter into the artery and connect to arterial line apparatus and monitor via

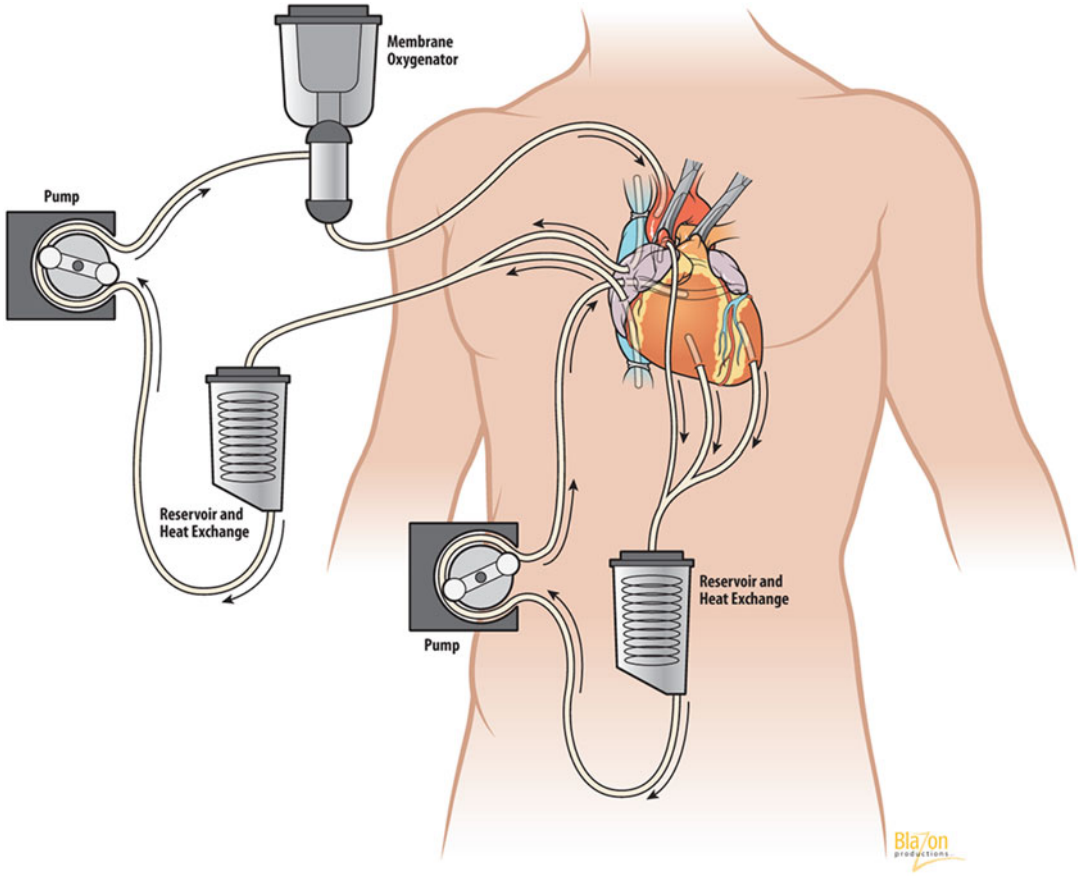

Fig. 2 Molecular cardiac surgery with recirculating delivery (MCARD). Cardiopulmonary-based closed-loop recirculatory system (MCARD). The system includes two separate circuits that allows for complete cardiac isolation and provides recirculation only in the coronary vasculature. Other advantages are increased time of recirculation, possibility for washing out the vector, and retrograde transcoronary sinus gene delivery

pressure tubing. Another option is percutaneous cannulation the right brachial artery or femoral artery for blood pressure monitoring using arterial line kit. Once the arterial line is placed and appropriate waveform and BP are confirmed, secure the line using a 2-0 silk pop-off suture.

3. Shift to the right carotid artery. Expose the artery via cut-down procedure as it was done with the right femoral artery in standard fashion in preparation for introduction of the carotid catheterization to facilitate cardiopulmonary bypass. After securing the delineated right carotid artery cover until bypass configuration begins.

4. Advance the bone saw onto the field and proceed with creating the median sternotomy, starting superiorly at the level of the sternal notch and proceeding distally to at least two finger breadths above the inferior aspect of the sternal plate, taking care not to divide the xiphoid. Open the chest and use Arista to control sternal bleeding.
5. Reflect the lung parenchyma and enter the pericardium, exposing the midline positioned cardiac window. Place pericardial stay sutures.
6. Give Amicar, 5 g. Perform a transepicardial echocardiographic examination to assess initial function of the heart (pre-cardiopulmonary bypass period).
7. Make sure a small basin with the sheep's own serum a 10 and 1 cm^3 syringe and two 4-way stopcocks are on the field. Flush each syringe and the stopcocks with the sheep's own serum.
8. Place a pledgeted 4-0 Prolene purse string using a rosette of pledgets around the right atrial appendage for superior vena cava (SVC) cannulation. Doubly place two #0 silk heavy sutures around the superior vena cava and connect to tourniquets. Ensnare the ascending aorta using umbilical tapes. Place a purse string on the right atrium adjacent to the atrioventricular groove. This will become the cannulation site for the coronary sinus catheter. Place one other purse string, also a rosette of Teflon pledgets, on the right atrium near its junction with the inferior vena cava (IVC) to accommodate IVC cannulation.
9. *Give heparin* (130 U/kg) and check active coagulation time (ACT). Ensure ACT is greater than 400 before proceeding. Bring the systemic lines onto the surgical field. Cannulate the right carotid artery using a 14Fr cannula.
10. Using pericardial pledgets and 4-0 Prolene suture, a horizontal mattress, place a pledgeted suture on the ascending aorta, approximately 1 cm distal to the aortic root. Place the aortic cannula, which is a DLP cardioplegic cannula (containing a vent limb). Cannulate the superior vena cava using a 26 French right angle cannula. Place the retrograde catheter into the coronary sinus. Connect the SVC cannula to a Y connector (with one limb clamped) and connect to the venous limb of the pump circuit. Bring the in-flow lines from the coronary reservoir circuit and out-flow lines up to the table. Recirculate the coronary reservoir solution through a stopcock with care to avoid entry of the solution into the systemic circulation or onto the field. Ligate the right azygous (hemiazygous) veins with a 2-0 silk suture making sure there are no other veins draining into the SVC (*see* **Note 4**).
11. *Partial cardiopulmonary bypass is initiated.* Cannulate the inferior vena cava using a 26Fr right angle cannula.

12. *Full cardiopulmonary bypass is initiated*. Dissect out the heart further as needed. *Cool the heart* to 30° with bypass heat exchanger. Doubly snare the IVC with a double loop of 0 silk suture. Using pericardial pledgets and a 4-0 Prolene suture, place a horizontal mattress pledgeted suture on the ascending aorta approximately 1 cm distal to the aortic root. Place the aortic cannula, which is a DLP cardioplegic cannula (containing a vent limb). Place a stopcock onto the DLP aortic root cannula. Connect the parallel arm of the stopcock to one limb of the Y connector of the cardioplegia line. The other limb will be connected to the coronary sinus catheter. Clamp the cardiac inflow limb (will later connect to the retrograde catheter). Connect the inflow to the Quest system (cardioplegia/cardiac circuit inflow) and de-air. Connect the right arm of the 1/4, 1/4, 1/4 Y connector to the aortic root vent (DLP aortic root vent arm). Connect the root of the Y connector to the yellow vent. Clamp the left arm of the Y connector.
13. The heart begins to *fibrillate*. Reduce aortic flow.
14. *Cross clamp the aorta*. Give 15 cm^3/kg Del Nido *cardioplegia* via the aortic root cannula in antegrade fashion.
15. Dissect out and ligate the left azygous vein (*see* **Note 5**). Then place a purse string 4-0 Prolene suture in the apex of the left ventricle. Volume is left in the heart. Make a stab in the middle of the purse string, and place a cannula into the left ventricular cavity and clamp. Place a pledgeted purse string 4-0 Prolene suture in the right ventricular out-flow tract. Then palace a cannula into the right ventricle, clamp and snare the purse string.
16. *The cardiac circuit is constructed*. The aortic DLP vent remains clamped. Connect the LV and RV vents and aortic root vents via a "Y" connecter to the venous limb of the cardiac circuit. The other limb of the Quest system inflow Y connector is left clamped. This will later be connected to the coronary sinus catheter. Connect the aortic root vent limb of the cardiac circuit to the left arm of the 1/4, 1/4, 1/4 Y connector. Connect the outflow limb of the cardiac circuit to the cardiac venous return. Put a clamp on the outflow limb of the cardiac circuit (directing all vents to the yellow vent for decompression). Connect the arterial limb of the cardiac circuit to the coronary sinus catheter (*see* **Note 6**).
17. *Give a second dose of cardioplegia* with Plegisol 15 cm^3/kg via the aortic root cannula in antegrade fashion.
18. *Isolate the coronary circuit* by tightening the superior vena caval snares, tightening the inferior vena caval snares (make sure these snares are tight. This is the largest source of dilution of the cardiac circuit), and cross clamping the pulmonary artery. Infuse the coronary sinus catheter with only 3 cm^3 to avoid

rupture of the coronary sinus. Connect the cardiac inflow limb to the retrograde catheter. Unclamp the cardiac circuit inflow limb and clamp the cardioplegia limb. Remove all excess volume and air from the circuit by decompressing using the yellow vent and infusing this volume systemically into the pump once the heart is decompressed. Resume flow until coronary sinus driving pressure equals 100–110 mmHg. Inject solution of Methylene blue (1 ml in 10 ml Saline) into the coronary sinus to confirm good cardiac isolation. Clamp the yellow vent and remove the clamp from the cardiac outflow limb directing all cardiac venous return to the cardiac circuit not to the yellow vent. Sinus pressure usually must be kept less than 80 mmHg with driving pressure of 100–110 mmHg.

19. Inject the Nitroglycerine diluted in 10 cm^3 Normal saline. Wait 2 min. *Then inject the vector solution* rapidly to simulate the measured flow rate. Restore flow over 1 min until coronary sinus pressure equals 90–100 mmHg and recirculate the remaining reservoir solution for another 15 min. Periodically decompress the heart into the pop off reservoir at 10 cm^3 per minute. This volume will be discarded.
20. Once the *15 min recirculation* interval is done, unclamp the yellow vent and place the clamp on the cardiac outflow limb directing all cardiac venous return to the yellow vent. Decompress the heart and tell the perfusionist that the yellow vent is "waste". Inject Methylene blue again to confirm isolation. Remove the coronary sinus catheter. Unclamp the cardioplegia limb and clamp the cardiac circuit limb of the Quest system inflow. Clamp the aortic root vent of the DLP cannula to avoid the flush bypassing the heart.
21. The coronary circuit is *flushed* antegrade with approximately 1000 cm^3 of Hespan infused with 100 mg of Solu-Medrol, 50 mg of Benadryl and 300 mg of cimetidine. Note that the aortic root vent must be clamped and the RV and LV effluent must be discarded (since it has viral solution in it). Decompress the heart again (into the yellow vent) and discard excess volume.
22. After flow has been reduced, the *remove the aortic and pulmonary cross clamps* and restore flow. Convert the yellow vent to the systemic vent.
23. Initiate *Rewarming*. Remove the IVC and SVC snares. Remove the aortic root DLP cannula. Defibrillate the heart to restore normal sinus rhythm. The IVC and SVC cannulae is removed. Remove the RV cannula. The LV cannula may now be converted to systemic vents. Administer Epinephrine at 10–20 ml/min (1–2 μg/min). Administer a 50 mg Lidocaine bolus and an infusion at 1 mg/min. Give 5 mg Amicar. Once the heart is contracting well, the LV cannula is removed.

24. *Wean from bypass* after 20 min of reperfusion. Once off bypass, give protamine and check ACT. Blood samples during CPB are obtained at various intervals approximately once every 30–45 min to assess for the animal's serum electrolyte levels, ACT, pH, pCO_2, and pO_2 levels (*see* **Note 7**).
25. After the sheep has been weaned from bypass, obtain another trans-epicardial echocardiogram to grossly determine heart function after bypass. Place bilateral chest tubes to get rid of pneumothorax, and remove these 24–48 h postoperatively. The thoracostomy tubes should subsequently be connected to wall suction through a multiliter Pleur-evac.
26. Give cell saver intravenously to the sheep. Place topical hemostatic agents on the heart, sternum, and/or incision to aid in stoppage of any bleeding from any of these sites. Close the sternal incision with seven to eight 2-0 steel wires in standard fashion.
27. Repair subcutaneous and skin layers with 1 Vicryl and 2-0 Nylon suture, respectively. Remove the femoral arterial line and repair the artery, primarily using 7-0 Prolene.
28. Prior to closure of the right neck incision, place a central venous catheter in the jugular vein to allow for CVP monitoring postoperatively. This catheter will be used for the administration of IV fluids and IV antibiotics.
29. When extubating the animal, care is taken to observe jaw motion, eye movement, and axial motion. Position the animal in sternal recumbency to prevent aspiration and to encourage recovery. Transfer the sheep to the postoperative care area once the animal has been taken off the ventilator.

3.4 Postoperative Care

1. Survival in large animal surgery with CPB depends highly on the quality of immediate critical care. Yet only a small body of literature is devoted to large animal ICU management, tissue perfusion, blood transfusion, or inflammatory response, especially after surgery with CPB. The primary component of acute postoperative care is the maintenance of adequate oxygen delivery to tissues. Appropriate monitoring provides valuable information on the animal's viability. We have created a postoperative protocol (Table 3). A key aspect of this protocol is the constant supervision of the animal for the first 24–36 h after surgery.
2. After the animal recovers from anesthesia, extubate while in sternal recumbency. Ability to maintain the animal in sternal recumbency until standing has been critical to recovery.
3. Affix a nasal cannula to the animal's rostrum with biological glue and connect the cannula to humidified oxygen. Initiate oxygen supplementation at 5 l/min until the sheep is active and able to maintain a pulse oximetry value of 94% or greater.

Table 3
Postoperative care protocol: first 24–36 h

Monitoring	Medication	Fluids and Feed
• Vital signs every 4 h	• O_2: nasal 3–5 l/min, decrease when stable $SpO_2 > 95\%$	• Hetastarch, IV
• SpO_2 monitoring until stable	• Antibiotics	• Normosol-R, IV + Dextrose + KCl until active
• CVP every 4 h	• Furosemide if CVP >10–15 cm H_2O	• Water when alert
• EKG every 4–8 h	• K, Ca, Mg depending on ABG/VBG	• Hay 6 h postop
• ABG or VBG every 2–4 h	• HCO_3 for metabolic acidosis	• Add 5% Dextrose to fluids if still NPO next morning, care for blood glucose level
• Chest tube drainage every hour	• Inotropes in case of low BP	
• Urination	• Amiodarone IV in case of arrhythmia	
	• Normosol-R, IV 50–80 ml/h if CVP <4 cm H_2O	

SpO2 arterial oxygen oxygenation, *CVP* central venous pressure, *K* potassium, *Ca* calcium, *Mg* magnesium, *EKG* electrocardiography, *ABG* arterial blood gases, *VBG* venous blood gases, *HCO3* bicarbonate, *BP* blood pressure, *KCL* potassium chloride, *NPO* nil per os

4. Monitor the animal's physiologic parameters, beginning with measurement of its temperature, pulse, respirations (TPR), heart rate, SpO2, etc.—obtained every 2–4 h and documented in the animal record.
5. Monitor central venous pressure (CVP) via triple lumen catheter in the jugular vein with a CVP manometer, which may remain in place and functional for up to 3 days without infection if properly flushed with heparin. CVP monitoring (target: 4–10 mm H_2O) provides important information regarding blood volume which may allow for life saving interventions with Lasix or fluids. We have found that CVP catheters provide the best balance of safety and monitoring compared to a Swan-Ganz catheter which presents a danger if left in an active animal.
6. Obtain an arterial blood gas sample via the auricular artery of either ear if possible every 2–4 h for the first 12 h to evaluate acid-base level and electrolytes—especially potassium, calcium and magnesium. Correct metabolic acidosis or alkalosis, hypokalemia, hypocalcemia, and hypomagnesemia with IV supplementation. In the absence of arterial access, venous samples from the jugular CVP catheter are used instead.

7. Rewarm the animal with warming blanket to prevent hypothermia (>100 F).
8. Our first antibiotic regime includes procaine penicillin G 22,000 IU/kg IV and gentamicin 6.6 mg/kg IV, given 1 h prior to incision. Postoperatively, provide procaine penicillin G every 6 h and gentamicin every 24 h for 4–5 days. If a second line treatment is required, we use ceftiofur sodium 2.2 mg/kg IM and enrofloxacin 5 mg/kg IM daily for 5 days.
9. In the early postoperative period, we administer a fluid mixture composed of half crystalloid Normosol-R and half colloidal hydroxyethyl starch at a rate of up to 100 ml/h depending on CVP.
10. Perform laboratory analyses of WBC count, electrolytes, liver and kidney function daily for 3 days after surgery and whenever animals appear weak.
11. Assessment of pain in the postoperative period is very important. For pain care buprenorphine and a fentanyl patch will be used for the first 3 days with the dose adjusted based on the animal's activity and vitals. Give the first dose of each after induction of anesthesia. Place the fentanyl patch of 100 μg/h following clipping. At this time 2–5 μg/kg buprenorphine will be given IV. The following signs will be used for pain distress: poor oral intake, increased heart rate/tachypnea, reluctance to move, pawing at the surgical site and inability to stand in the recumbent position and hyperesthesia.
12. Observe and record urine and excrement production and chest thoracostomy tube output. If postoperative blood loss from the chest thoracostomy tubes is greater than 100 cm^3/h, activated coagulation time, hemoglobin, and hematocrit levels are checked immediately. One must then consider a surgical source of bleeding or coagulopathy.
13. Remove the thoracostomy tubes 24–36 h postoperatively. The proper use of thoracostomy tubes helps reduce the incidence of pleural effusions. However, delayed pleural effusions may still occur, requiring insertion of new thoracostomy tubes for 24 h.
14. Observe each sheep daily for evidence of surgical site infection, pain, and cardiovascular or respiratory compromise. This observation includes a physical exam, observation of the surgical site, assessment of attitude, activity, appetite, and fecal production, measurement of temperature, pulse rate, respiratory rate, thoracic auscultation, and oxygen saturation via pulse oximetry. When necessary, perform blood pressure measurements, electrocardiography, or echocardiography.
15. Begin nourishment with water when the sheep is alert, and hay may be given when the animal is standing, but neither is offered prior to 6 h postoperatively.

4 Notes

1. Consistent coronary arterial anatomy in sheep, similar to that of humans, and a lack of collateral vessels provides substantial advantages for creation of a clinically relevant ischemic heart failure model with a predictable myocardial infarct size in this species. The coronary venous systems are also similar between sheep and humans, with the exception of the left azygous vein, which drains the dorsal part of the left chest. This physiologically appropriate large-animal model, involving ligation of the circumflex artery's first two branches is accompanied by almost zero mortality and is highly suitable for demonstrating ventricular remodeling after gene therapy.
2. The anesthesiologist should closely monitor the animal's cardiovascular stability during the surgical procedure to avoid systemic vasodilatation with afterload reduction or arrhythmias, and to attempt to preserve sinus rhythm. Intraoperative monitoring of all essential physiologic parameters is imperative for a successful outcome. These measuring devices include both invasive and noninvasive parameters: pulse oximetry, invasive blood pressure, echocardiography, temperature, and ECG.
3. A variety of drugs are available to prevent ventricular arrhythmias in a myocardial infarction model. From our point of view the best protocol includes receiving IV the two antiarrhythmic drugs amiodarone and lidocaine 1 h before ligation of coronary arteries, with this treatment continued 60–120 min afterward. In cases of low blood pressure we add phenylephrine to this combination.
4. During the MCARD procedure attention should be devoted to the aortic root purse string because sheep have a very short ascending aorta. For the same reason, care must be taken to observe adequate space for placement of the aortic cross-clamp. In the sheep model, the descending aorta begins with an acute angle only after a few centimeters of the ascending aorta. Care not taken in this placement will result in concomitant cross-clamp of both the ascending and descending aorta.
5. Ligation of the left hemizygous vein confirms complete cardiac isolation and prevents systemic loss of viral particles.
6. A standard adult retrograde cannula is used for the cannulation of the coronary sinus for vector delivery. Care must be taken to inspect and confirm that the catheter in positioned properly in the coronary sinus. The best results have been received when the catheter tip was placed in a midway position after the deviation of the posterior descending vein. Placement is confirmed with injection of methylene blue and evaluated by its distribution throughout the major cardiac veins in the right and left ventricles.

7. Prior to stopping CPB, the animal is rewarmed to 36–37 °C, the heart is defibrillated and the lungs are ventilated ensuring that intraoperative atelectasis has resolved. Cardiac rhythm is monitored; acid–base status and plasma electrolytes are reviewed. Inotropic drugs are started at low flow rates. After weaning, cardiac filling and contractility is monitored by echocardiography. When cardiac performance is stable, cardiac inotropic support is ceased. Protamine can be administered in half or full doses until ACT is appropriate.

Acknowledgments

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Part V

Targeting Arrhythmia

Chapter 21

Gene Delivery for the Generation of Bioartificial Pacemaker

Patrick K.W. Chan and Ronald A. Li

Abstract

Electronic pacemakers have been used in patients with heart rhythm disorders for device-supported pacing. While effective, there are such shortcomings as limited battery life, permanent implantation of catheters, the lack of autonomic neurohumoral responses, and risks of lead dislodging. Here we describe protocols for establishing porcine models of sick sinus syndrome and complete heart block, and the generation of bioartificial pacemaker by delivering a strategically engineered form of hyperpolarization-activated cyclic nucleotide-gated pacemaker channel protein via somatic gene transfer to convert atrial or ventricular muscle cardiomyocytes into nodal-like cells that rhythmically fire action potentials.

Key words Bioartificial pacemaker, Heart rhythms, Sick sinus syndrome, Complete heart block, Gene transfer, Hyperpolarization-activated cyclic nucleotide-gated (HCN) channel, Adenovirus, Adeno-associated virus

1 Introduction

Normal cardiac rhythms originate in the sino-atrial node (SAN), a specialized cardiac tissue consisting of only a few thousands nodal pacemaker (Pm) cardiomyocytes (CMs) that resides within the right atrium [1, 2]. The SAN initiates and controls heart rate by spontaneously generating rhythmic action potentials (APs) that propagate through the atria to the atrioventricular (AV) node and subsequently to the ventricles for coordinated chamber contractions and blood pumping. Diseases and aging may lead to malfunction of PmCMs which results in rhythm generation disorders (e.g., bradycardias, sick sinus syndrome (SSS)), necessitating the implantation of electronic pacemaker, with shortcomings such as limited battery life, permanent implantation of catheters, the lack of autonomic neurohumoral responses, risks of lead dislodging (particularly to pediatric patients due to somatic growth) [3–7], etc. As such, the engineering of bioartificial pacemaker or bio-SAN as an alternative or supplement to electronic devices has been pursued.

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The process of pacemaking involves the complex interplay of an array of ionic channels and pumps. Among the key players, the hyperpolarization-activated I_f (f for funny) or the so-called pacemaker current [8] is robustly expressed in PmCMs but absent in healthy adult ventricular (V) CMs. This depolarization current I_f is encoded by the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel gene family [9–13]. Despite the structural similarity with the classical depolarization-activated voltage-gated K^+ (K_v) channels whose fundamental building blocks are monomers consisting of six transmembrane domains (S1–S6), HCN channels are activated by hyperpolarization rather than depolarization, and they permeate Na^+ and K^+ nonselectively. To date, four isoforms (HCN1–4) have been identified, with HCN1 being most abundantly expressed in the brain and substantially in the SAN [14, 15]. Unlike HCN2 and HCN4 that are found in both the central nervous system and the heart, HCN3 is absent in the heart. Of note, native I_f is heteromeric made up of multiple HCN isoforms in different stoichiometries, making their reproduction in any heterologous or other systems extremely difficult because of such complex molecular identity. In response to neural inputs, I_f generated by HCN channels modulates heart rate by regulating the rates of cellular depolarization to AP threshold and thereby the firing frequency. Indeed, mutations in the human HCN gene have been reported to underlie certain forms of familial sinus node dysfunction [16, 17]. In our earlier structure–function studies of HCN constructs, we discovered that their opening and closing are highly dependent on the length of the linker between the S3 and S4 transmembrane segments and several charged amino acid therein [18–20]. In general, long and short linkers shift the steady-state activation of HCN channels in the negative and positive directions, making them energetically more difficult and easier to open, respectively. In particular, a recombinant, hHCN1ΔΔΔ, whose S3–S4 linker residues 246–248 (EVY) have been deleted, displays biophysical properties similar to the native heteromeric I_f current. Such findings led to the possibility of engineering HCN activity for programming bioartificial pacing [21].

Unlike pacemaker cells, adult atrial and ventricular CMs are normally electrically quiescent unless they are stimulated by electric signals transmitted from neighboring cells that originate from the SAN. This is largely due to the absence or scarce expression of the HCN-encoded I_f and the intense expression of the Kir2-encoded inward-rectifier K^+ current (I_{K1}), which stabilize a negative resting membrane potential at ~−80 mV. Indeed, suppression of I_{K1} expression in normally silent ventricular muscle cells by 80% can induce spontaneous action potential firing in a binary "on-and-off" manner. However, the induced frequency is only one-third of the normal physiological range and therefore practically not suitable for acting as a reliable biological pacemaker [22].

Alternatively, recombinant HCN channels have been employed for the induction of automaticity [21, 23–26]. By expressing exogenous modified HCN1 (whose S3–S4 linker has been shortened to favor channel opening) in the VCMs from adult guinea pig left ventricle via adenovirus (AdV), transduced myocytes displays physiological pacemaking activities that resemble those of native nodal cells, which cannot be observed in VCMs transduced with AdV carrying wild-type HCN or HCN with a lengthened S3–S4 linker [21]. Such induced bioartificial pacing can be inhibited by HCN-specific blocker ZD7288. Interestingly, the ZD7288-silenced cells generate typical ventricular AP phenotype upon electrical stimulation, implying that the HCN-converted pacemaker cells retain their ventricular identity when not functioning an as a bio-SAN—a dual identity that is not seen in native nodal cells.

To test whether HCN-mediated bio-SAN can mimic the native pacemaker, a swine model of SSS has been developed such that clinically relevant procedures and assessments can be performed [26]. Importantly, the anatomical and physiological properties of swine heart are more comparable to those of human, when compared to small animals (e.g., >500 bpm of rodent hearts versus ~70–80 bpm of pig and human hearts). Porcine SSS model can be created by firstly ablating the native SAN using radiofrequency, followed by the implantation of a dual-chamber electronic pacemaker for device-supported cardiac pacing. Focal injection of the recombinant adenovirus AdV-CMV-GFP-IRES-HCN1ΔΔΔ in the left atrium of the SSS swine reproducibly induces a stable in vivo bio-SAN that fires at physiological heart rate, successfully reducing the dependence of electronic pacing (from 80 to 15 %) over a period of 2 weeks. As an alternative, Hu et al. [27] demonstrated a somatic reprogramming of VCMs to pacemaker-like CMs via an ectopic expression of embryonic transcription factor T-box 18 (Tbx18) in a swine model with ablated AV node (complete heart block). Tbx18 is required for the formation of the sinus node head during embryonic SAN specialization. AdV-mediated overexpression of Tbx18 confers electrically quiescent CMs temporary pacemaker activity [28]. The bioartificial pacemaker created also responds to β-adrenergic stimulation and shows minimal reliance on the backup electronic device [27].

Although high titers of viral particles with high in vivo transduction efficiencies can be readily produced, AdV only mediates transient transgene expression without genome integration. Indeed, transgene expression has been shown to peak at ~1 week, plateaus, then decline over a period of ~3–5 weeks after transduction in previous gene-transfer studies. No transgene expression could be detected by week 10 [29–31]. Furthermore, the intense immune response evoked by AdV also significantly hampers its use for investigating long-term efficacy and safety. As such, the replication-deficient and nonpathogenic recombinant adeno-associated virus

(rAAV) has been employed. Like AdV, rAAV can also be generated at high titers for mediating persistent genetic modification. Moreover, rAAV elicits minimal humoral and cellular immune responses in human [32]. Indeed, rAAV-mediated gene transfer has been shown to be highly efficient in the heart as reported previously [33–38].

Building upon our series of previous studies, here we summarize a method to functionally reprogram CMs into PmCMs via AdV- and rAAV serotype 9 (rAAV9, an isoform chosen for cardiac tropism)-mediated overexpression of the engineered human hHCN1ΔΔΔ. The system has been tested in preclinical large animal porcine models of SSS and complete heart block.

2 Materials

2.1 Media

1. HEK-293T/17 Growth Medium: high-glucose DMEM, 10% FBS.
2. Transfection Medium: high-glucose DMEM, 2% FBS.
3. Serum-free high-glucose DMEM.

2.2 Solutions, Buffers, Reagents

1. 0.05% trypsin–EDTA.
2. Polyethylenimine (PEI; linear; 25,000 MW; 1 mg/mL).
3. Lysis buffer: Milli-Q water, 150 mM NaCl, 50 mM Tris–HCl (pH 8.5).
4. Ammonium sulfate.
5. Benzonase I.
6. 40% polyethylene glycol (PEG) solution.
7. 5 M NaCl solution.
8. 5× TD solution: 5× PBS solution, 5 mM $MgCl_2$, 12.5 mM KCl.
9. OptiPrep solution: 60% iodixanol solution.
10. 0.5% phenol red solution.
11. 15% iodixanol layer solution: 14.4 mL OptiPrep solution, 11.5 mL 5 M NaCl solution, 11.5 mL 5× TD solution, 20.2 mL Milli-Q water.
12. 25% iodixanol layer solution: 16.7 mL OptiPrep solution, 8 mL 5× TD solution, 15.4 mL Milli-Q water, 100 μL 0.5% phenol red solution.
13. 40% iodixanol layer solution: 21.8 mL OptiPrep solution, 6.4 mL 5× TD solution, 3.8 mL Milli-Q water.
14. 60% iodixanol layer solution: 32 mL OptiPrep solution, 80 μL 0.5% phenol red solution.

15. Lipofectamine 2000.
16. Cesium chloride (CsCl).
17. 0.22 μm PES filter.

2.3 Plasmids/Vectors

1. pAdV-GFP-IRES-hHCN1ΔΔΔ plasmid (*see* **Note 1**).
2. Ψ5 adenovirus (*see* **Note 2**).
3. pTR-hHCN1ΔΔΔ-T2A-EGFP plasmid (*see* **Note 3**).
4. pDG9 helper plasmid (*see* **Note 4**).

2.4 Animal Procedures

1. Isoflurane.
2. Heparin.
3. Pentobarbital sodium.
4. Formalin.
5. Scalpel.
6. Dressing forceps.
7. Dissecting forceps.
8. Suture forceps.
9. Surgical scissors.
10. 7F electrophysiological catheter.
11. Radiofrequency generator.
12. Dual chamber pacemaker.
13. Syringe.
14. Insulin needle.
15. 2/0 absorbable sutures.
16. 2/0 non-absorbable sutures.
17. Mapping catheters.

3 Methods

3.1 Production of AdV-GFP-IRES-hHCN1ΔΔΔ

1. Maintain Cre8 cells (HEK-293 cells stably expressing Cre recombinase) in T25 culture flasks.
2. Upon 80–90% confluency, co-transfect cells with 2.1 μg of purified Ψ5 AdV and 2.1 μg of purified pAdV-GFP-IRES-hHCN1ΔΔΔ using Lipofectamine 2000.
3. Incubate cells for 5–9 days until cytopathic effects are observed.
4. Harvest cells and supernatant in a 50 mL tube. Freeze-thaw in a dry ice–ethanol bath and a shaking 37 °C water bath.
5. Repeat the freeze-thaw cycle for thrice. Vortex after each thawing.

6. Remove cells and debris by centrifugation at 2095 × *g* for 15 min. Collect the supernatant in a 15 mL tube.
7. Add 2 mL of the supernatant to a 90% confluent T25 of Cre8 cells.
8. Incubate cells until cytopathic effects are observed.
9. Amplify the virus by repeating the procedures for three to four times at which time the virus is analyzed for purity by plaque assays.
10. Concentrate the resulting viral supernatant on a CsCl gradient made by layering three densities of CsCl (1.25, 1.33, and 1.45 g/mL).
11. Ultracentrifugate at 50,000 × *g* for 4 h at 14 °C.
12. Immediately after ultracentrifugation, remove a band of virus at the interface between the 1.33 and 1.45 g/mL layers.
13. Repeat ultracentrifugation at 300,000 × *g* for 4 h at 14 °C.
14. Purify virus by dialysis in phosphate-buffered saline overnight at 4 °C.
15. Determine virus titer by plaque assay and store the remaining purified virus below −80 °C. Avoid freeze-thaw cycle.

3.2 Production of rAAV9-hHCN1 ΔΔΔ-T2A-EGFP

1. Maintain HEK-293T/17 cells in five T175 culture flasks.
2. Upon confluency, add 5 mL 0.05% trypsin–EDTA to detach and replate the cells in six TripleFlasks (each provides a total culture area of 500 cm^2).
3. On the next day, prepare a PEI–plasmid mix containing 700 μL PEI, 50 μg of pTR-hHCN1ΔΔΔ-T2A-EGFP, and 150 μg of pDG9 helper plasmid in 20 mL DMEM. Incubate the mix at room temperature for 15 min.
4. Add the PEI–plasmid mix to 90 mL transfection medium. Replace spent medium in each flask with this transfection mix.
5. Incubate cells in 37 °C incubator supplemented with 5% CO_2 for 3 days.
6. To harvest the virus, detach transfected cells by gently tapping the flasks.
7. Collect cells and supernatant in 250 mL centrifuge tubes.
8. Centrifuge at 200 × *g* at 4 °C for 10 min.
9. Transfer the supernatant to a sterile container. Add ammonium sulfate (31.3 g per 100 mL supernatant) and dissolve the salt completely by shaking vigorously.
10. Incubate on ice for 30 min and centrifuge mixture at 8300 × *g* at 4 °C for 30 min.
11. Discard supernatant and centrifuge again at 8300 × *g* at 4 °C for 30 min.

12. Aspirate remaining supernatant.
13. Resuspend pellet in lysis buffer (3.5 mL per 100 mL supernatant). Label it as "Supe".
14. Resuspend cell pellet previously obtained in lysis buffer (9 mL per TripleFlask). Label it as "Crude Lysate".
15. Freeze-thaw "Supe" and "Crude Lysate" in dry ice bath.
16. Repeat the freeze-thaw cycle for thrice. Vortex after each thawing.
17. Add a final concentration of 1 mM $MgCl_2$ and 1500 units of Benzonase I (per 10 mL solution) to "Supe" and "Crude Lysate".
18. Mix well and incubate at 37 °C for 1 h.
19. Centrifuge at 3400 × *g* at 4 °C for 20 min.
20. Collect supernatant in a sterile container.
21. Concentrate the supernatant from "Crude Lysate" by adding a final concentration of 8% PEG solution.
22. Mix well and incubate on ice for 2 h. Do not freeze PEG.
23. Centrifuge at 2500 × *g* at 4 °C for 30 min.
24. Resuspend pellet in 9.5 mL lysis buffer per 30 mL supernatant.
25. Prepare an iodixanol gradient in a 32.4 mL-ultracentrifuge tube with volume and concentration stated below (*see* **Note 5**): (Top) 7.3 mL of 15%, 5 mL of 25%, 4 mL of 40% and 4 mL of 60% (Bottom).
26. Overlay the viral supernatant on top of the iodixanol gradient.
27. Concentrate virus by ultracentrifugation at 16,500 × *g* for 3 h at 18 °C (maximum acceleration and deceleration), then 25,800 × *g* for 1 min at 18 °C (maximum acceleration but no deceleration).
28. Immediately after ultracentrifugation, collect concentrated virus from the 40% iodixanol (colorless) layer by drawing the virus layer through a 18 G syringe needle (*see* **Note 6**).
29. Purify virus by dialysis using tubing with molecular weight cut-off of 12,000–14,000 Da in lactated Ringer's Injection solution overnight at 4 °C (*see* **Note 7**).
30. Filter virus with 0.22 μm PES filter.
31. Verify (*see* **Note 8**) and quantify (*see* **Note 9**) virus obtained.
32. Store purified virus below 80 °C. Avoid freeze–thaw cycle.

3.3 Porcine SSS Model and Catheter-Based Gene Transfer

All animal procedures are handled in accordance with Institutional Animal Care and Use Committee (IACUC) protocol.

1. At Day 0, anesthetize the animal using isoflurane (1–3%) with intubation and mechanical ventilation.

2. Perform cut down at bilateral external jugular veins and right femoral vein.
3. Administrate heparin (3000 IU) intravenously.
4. Insert a 7F electrophysiological catheter into the right atrium and navigate using X-ray to the SAN located between the junction of superior vena cava and high right atrium.
5. Set a radiofrequency generator in the temperature control mode (50 °C) and the maximum output at 35 W.
6. Deliver radiofrequency energy at sites exhibiting the earliest endocardial activation during sinus rhythm. Repeat the delivery of ablating radiofrequency up to 60 s in duration until stable sinus dysfunction (baseline sinus rate <35 bpm) is achieved.
7. Implant a dual-chamber or epicardial pacemaker with one lead positioned at the high anterolateral wall of the right atrium and another at the right ventricle apex to provide supportive pacing if the mean heart rate drops below 60 bpm.
8. Perform lateral thoracotomy incision to expose the heart.
9. Inject a total dose of 2×10^{10} PFU of AdV or 1×10^{11} vg of rAAV, or control vehicle, into the left atrium appendage via left thoracotomy (*see* **Note 10**).
10. Mark the injection site with sutures (*see* **Note 11**).
11. Close muscles and fascia in a continuous manner using 2/0 absorbable sutures.
12. Close skin in an interrupted manner using 2/0 non-absorbable sutures.
13. Recover the animal from anesthesia.
14. Perform ECG recording and pacemaker interrogation twice a week, until sacrifice.
15. On the day of sacrifice, perform cut down at the right internal jugular vein and left femoral vein.
16. Insert 6–8F sheaths into the veins, then insert coronary sinus and mapping catheters via the sheaths, and position the catheters in coronary sinus, right atrium and right ventricle.
17. Perform electroanatomical mapping of right atrium and right ventricle with pacemaker rate at 60 bpm (Fig. 1).
18. Euthanize the animal with intravenous pentobarbital sodium (300 mg/mL) at 100 mg/kg.
19. Harvest the heart and fix the heart tissue injection site and negative control site specimens in 10% formalin for paraffin embedding, sectioning, and staining.

3.4 AdV/rAAV Injection in Porcine Model with Complete Heart Block

All animal procedures are handled in accordance with Institutional Animal Care and Use Committee (IACUC) protocol.

1. At \Day 0, anesthetize the animal using isoflurane (1–3%) with intubation and mechanical ventilation.

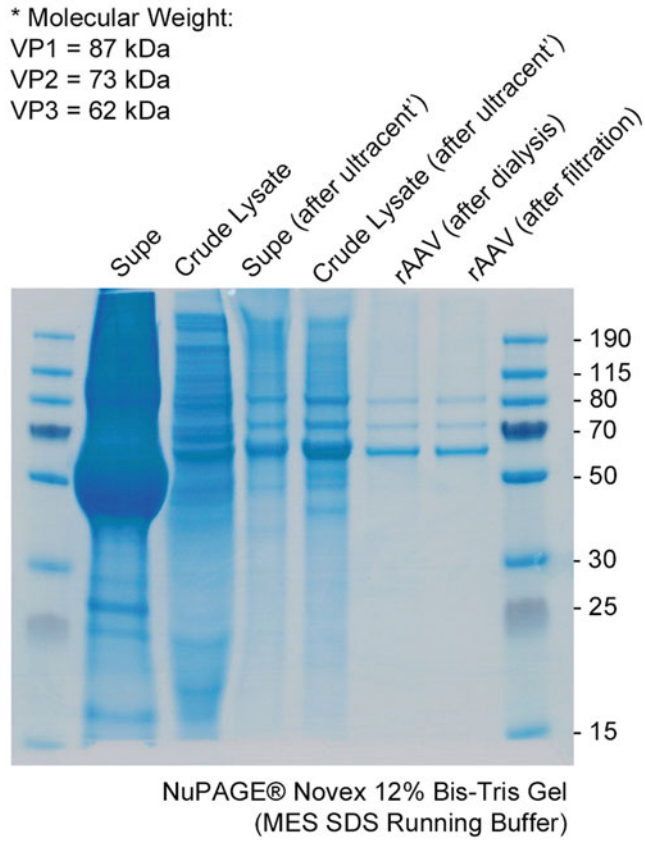

Fig. 1 (**a**) Fluoroscopic image showing the pacing lead and mapping catheter at the right ventricle during electroanatomic mapping. (**b**) Location of pacing lead and mapping catheter on the electroanatomical map

2. Perform cut down at bilateral external jugular veins and right femoral vein.
3. Administrate heparin (3000 IU) intravenously.
4. Insert 6–8F sheaths into the veins, then insert coronary sinus and ablation catheters via the sheaths, and position the catheters in coronary sinus, right atrium and right ventricle.
5. Perform baseline electroanatomical mapping of right atrium and right ventricle.
6. Insert pacing lead via the peel-away sheath and implant it at the right ventricle apex.
7. Implant pacemaker pulse generator subcutaneously at the infraclavicular region to provide supportive pacing if the mean heart rate drops below 60 bpm.
8. Ablate atrioventricular node by radiofrequency ablation catheter until complete heart block is achieved.

9. Perform post-AV node ablation right atrium and right ventricle mapping with pacemaker at 60 bpm (Fig. 1).
10. Perform lateral thoracotomy incision to expose the heart.
11. Inject a total dose of 2×10^{10} PFU of AdV or 1×10^{11} vg of rAAV into the right ventricle via right thoracotomy access (*see* **Note 10**).
12. Mark the injection site with sutures (*see* **Note 11**).
13. Close muscles and fascia in a continuous manner using 2/0 absorbable sutures.
14. Close skin in an interrupted manner using 2/0 non-absorbable sutures.
15. Recover the animal from anesthesia.
16. Perform ECG recording and pacemaker interrogation twice a week, until sacrifice.
17. On the day of sacrifice, perform cut down at the right internal jugular vein and left femoral vein.
18. Insert 6–8F sheaths into the veins, then insert coronary sinus and ablation catheters via the sheaths, and position the catheters in coronary sinus, right atrium and right ventricle.
19. Perform electroanatomical mapping of right atrium and right ventricle with pacemaker rate at 60 bpm (Fig. 1).
20. Euthanize the animal with intravenous pentobarbital sodium (300 mg/mL) at 100 mg/kg.
21. Harvest the heart and fix the right ventricle tissue injection site and negative control site specimens in 10% formalin for paraffin embedding, sectioning, and staining.

4 Notes

1. A bicistronic AdV shuttle vector pAd-CMV-GFP-IRES carrying the recombinant hHCN1ΔΔΔ gene with shortened S3–S4 linker is used for AdV production. The internal ribosomal entry site (IRES) allows the simultaneous translation of GFP and hHCN1ΔΔΔ [21].
2. Adenovirus Ψ5, with packaging signal Ψ flanked by *loxP* sites, serves as a helper virus for replicating and packaging the gutless virus (AdV-GFP-IRES-hHCN1ΔΔΔ) that keeps only 5′ and 3′ inverted terminal repeats and the packaging signal from the wild-type AdV. When the shuttle vector and the helper adeovirus are co-transfected into the Cre recombinase-expression cells, the packaging site of the adenovirus Ψ5 is excised while other viral proteins for AdV generation are retained. As such,

the AdV-GFP-IRES-hHCN1ΔΔΔ is preferentially packaged and can be further amplified and purified by repeated infection in Cre recombinase-expression cells.

3. pTR-hHCN1ΔΔΔ-T2A-EGFP is created by cloning recombinant hHCN1ΔΔΔ and EGFP into the pTRUF backbone. The T2A linker peptide allows simultaneous expression of the two genes. SURE 2 Supercompetent bacterial cells (Strategene/Agilent Cat. # 200152) have to be used for cloning and re-transformation of AAV pTR plasmids. Transformed SURE 2 Supercompetent bacterial cells should be cultured at 30 °C. Restriction enzyme SmaI can be used to ensure the retainment of the inverted terminal repeat region of the AAV: two bands of size 4.1 and 3.1 kb (instead of one 7 kb-band) can be obtained upon electrophoresis.
4. pDG9 is generated by replacing the capsid gene in pDG, the helper plasmid for packaging AAV serotype 2. The resulting replication-deficient rAAV serotype 9 is cardiotropic and is recommended for creating bioartificial pacemaker.
5. Formation of iodixanol gradient is a critical step for rAAV purification. To prevent mixing of the layers during the preparation, a peristaltic pump with controlled flow rate can be used: viral solution is firstly added to the ultracentrifuge tube, followed by sequential underlaying of iodixanol layers through a glass pipette (starting with the 15% iodixanol layer).
6. After ultracentrifugation, rAAV is distributed through the 40% iodixanol layer. Clamp the centrifuge tube firmly to a stand to allow the insertion of 18 G syringe needle at about 1 cm below the 25–40% interphase. Pull the syringe to recover about 4 mL of viral solution. Avoid drawing any solution that contains phenol red.
7. Dialysis allows removal of unwanted materials in the rAAV solution. Replacing the lactated Ringer's Injection solution and allowing dialysis for another 8–10 h is recommended, especially if any colored layer is collected after ultracentrifugation.
8. Virus can be verified by SDS-PAGE with Coomassie blue staining. Signature bands of VP1, VP2, and VP3 could be observed at 87 kDa, 73 kDa, and 62 kDa, respectively (Fig. 2).
9. Quantification of virus can be done by qPCR using sequences within the CMV-ie enhancer region as primers.
10. Concentrated virus is injected using a steerable catheter equipped with a 33 gauge circular injection needle that can be advanced and retracted, at 4 sites within 3 mm of each other. Given the proximity and spread, one macrosite is formed. Multiple injections (~5–6) of smaller volumes to form a single

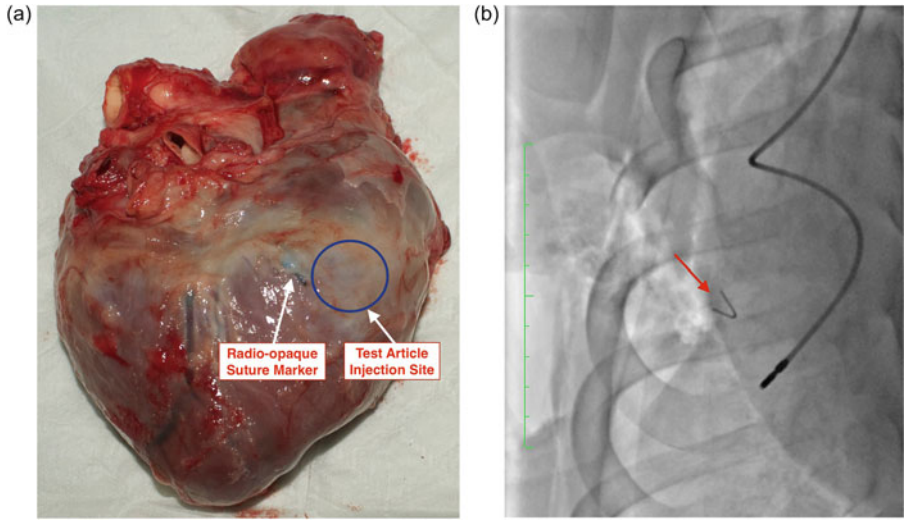

Fig. 2 SDS-PAGE analysis of samples collected at different stages of rAAV production. rAAV samples are separated by 12% Bis-Tris gel and the gel is then stained with Coomassie blue solution. Three distinct bands representing AAV capsid proteins VP1, VP2, and VP3 can be visualized in purified samples

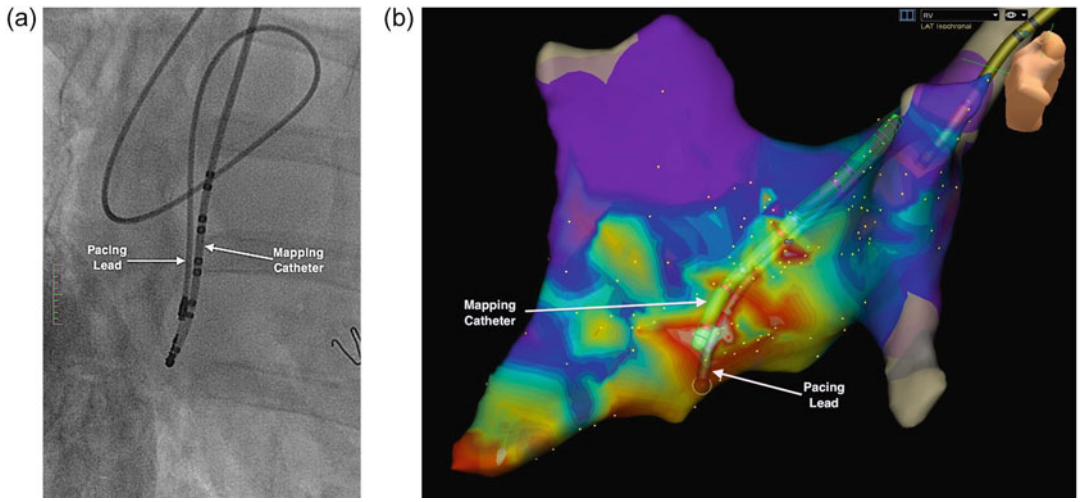

Fig. 3 (**a**) An example of virus injection site and suture marker on pig heart. (**b**) Radiopaque suture marker (*red arrow*) visualized under fluoroscopy imaging

bio-SAN are preferred as one shot of >0.5–1 mL into the relatively thin atrium or right ventricle often leads to significant undesirable swelling and vector spilling.

11. The injection site is marked with radiopaque sutures that can be visualized under fluoroscopy imaging (Fig. 3).

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Chapter 22

Gene Therapy for Post-infarction Ventricular Tachycardia

J. Kevin Donahue

Abstract

Cardiac arrhythmias are a leading cause of morbidity and mortality in the developed world. In particular, cardiac arrest or sudden cardiac death is the leading cause of death in these countries. Death generally results from a ventricular tachyarrhythmia, and pathology data have shown that cardiac arrest victims very frequently have evidence of coronary atherosclerosis with either acute ischemia or healed myocardial infarction. In this work, we describe an animal model that reproducibly has inducible ventricular tachyarrhythmias after healing of a myocardial infarction scar and a gene delivery method that allows gene transfer to the scar and surrounding myocardial tissues. Use of the method allows gene delivery to the arrhythmia model for testing of hypotheses related to ventricular tachyarrhythmia mechanisms and for efficacy testing of proposed gene therapies. To date, all work in this area has been preclinical, but it is our hope that continued development in this area will 1 day allow translation of this method into clinical practice.

Key words Gene therapy, Gene delivery, Arrhythmia, Ventricular tachycardia, Ventricular fibrillation, Myocardial infarction, Cardiac ischemia

1 Introduction

Ventricular tachyarrhythmias (VT) are the leading cause of death in the developed world [1]. Currently available therapies have limited efficacy and measurable toxicity. Antiarrhythmic drugs are able to suppress VT. Other than β-adrenergic blocking drugs, all currently available antiarrhythmic drugs have adverse or at best neutral effects on mortality [2–8]. Even a neutral effect is a marker of toxicity because the positive effect of arrhythmia suppression must be balanced by other negative effects to give the overall neutral result. VT ablation can eliminate arrhythmia circuits, but recurrences are common and VT ablation procedures are long, complex, and, in a small percentage of cases, complicated by adverse events [9]. The current mainstay of VT therapy is the implantable cardioverter defibrillator (ICD). ICDs have been repeatedly shown to save lives [7, 10, 11], but they do not prevent VT. They terminate already occurring VT with pacing or a shock. ICD therapies have been associated with increased mortality [12]. The limitations of

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currently available therapies motivate the search for new preventative or treatment options. Gene therapy has been reported in preclinical models to reduce or eliminate VT [13–15].

In this report, we describe methods for creation of a preclinical model to test post-infarct VT therapies and for delivery of genes to the infarct borderzone to modify the susceptible tissues and impair their ability to generate or sustain VT. The preclinical model is described for pigs because that is the species in which it was developed and validated [13, 16]. Other infarct-VT models have been reported in sheep, dogs, rabbits, rats and mice. We use the pig model because our interest is in arrhythmias occurring in the chronic state after the infarct scar is completely healed. Pig coronary anatomy, cardiac structure and function, and electrophysiology are similar to human. Important differences include transmural Purkinje fibers in the pig (these extend only through the endocardium in humans) and differences in an early repolarizing current (pigs only have I_{to2} but humans have I_{to1} and I_{to2}). We have found smaller mammals difficult to use for translational studies, so we have focused on large mammalian work. In addition, rodent studies have limited value because rodent electrophysiological function is considerably different than that of larger mammals. The dog model has been used primarily in the subacute period when the scar is healing because VT inducibility is significantly reduced after the infarct scar matures [17–19]. Sheep are expensive. With the limitations of these other species, we settled on pigs, and we describe those methods here.

Since our infarct model is porcine, it stands to reason that our gene transfer methods are also developed and validated in the pig [20]. The two principle gene delivery methods for large mammals are direct intramyocardial injection and coronary vascular perfusion. Injection results in dense, local gene transfer, and perfusion allows broader but less efficient gene delivery. We have not yet found a solution for the limited volume of delivery for injection, and we have identified several interventions that increase efficiency of delivery by perfusion, so we have used the perfusion method for our preclinical studies. Details of the major conceptual elements of our perfusion method are described in Sasano et al. [16]. These include use of vasodilators to maximize delivery to the local capillary level; vascular permeability inducers to increase probability of gene transfer vector transit from the vasculature into the extracellular space, and simultaneous arterial and venous perfusion to provide further driving force for exit from the vasculature. In addition, we optimize virus concentration and delivery time as much as possible, and we make every attempt to avoid exposure of the viral vector to anything that might inhibit gene delivery. This strategy resulted from a series of investigations that identified the various relevant components of the delivery system [20–23].

2 Materials

2.1 Drugs and Solutions

- Telazol (tiletamine/zolazepam, stock concentration 50 mg/ml of each component, we dose on a mg/kg of each component basis so 2.2 mg/kg is 2.2 mg tiletamine + 2.2 mg zolazepam per kg animal weight).
- Ketamine 2.2 mg/kg.
- Xylazine 2.2 mg/kg.
- Isoflurane.
- Chlorhexidine scrub sponges.
- Betadine scrub sponges.
- Betadine solution.
- Sterile 0.9% saline (bags for IV administration, bottles for intraoperative use).
- Cefazolin.
- Antibiotic flush solution: Neomycin 40 mg, Polymyxin B 200,000 U in 500 ml of 0.9% saline.
- Radiographic contrast agent: omnipaque, visipaque or similar.
- Dopamine 5–20 mg/kg/min infusion.
- Phenylephrine 50–200 μg bolus, 10–200 μg/min infusion.
- Atropine 0.05 mg/kg bolus.
- Amiodarone 150 mg brought to a total volume of 10 ml with saline.
- Tadalafil 10 mg tablet.
- Pretreatment solution: vascular endothelial growth factor (VEGF) 0.5 μg/ml, adenosine 5 mg/ml, nitroglycerin 250 μg/ml, to a final volume of 10 ml in 0.9% saline.
- Gene transfer solution: 6×10^{10} pfu adenovirus or 6×10^{12} vp adeno-associated virus (AAV), adenosine 5 mg/ml, nitroglycerin 250 μg/ml, to a final volume of 12 ml in 0.9% saline.

2.2 Supplies

- IV catheters: 22–24 gauge size.
- Endotracheal tube (5-0 size for pigs).
- ECG patches.
- Defibrillator patches.
- Surgical towels.
- Surgical drapes.
- Vein pick.
- Syringes and needles.
- 3-way stopcocks (for maintaining pressure in the angioplasty catheters and for building the gene delivery manifolds).

- Sutures and ties: 0 silk ties for vessel isolation and ligation, 2-0 vicryl suture for subcutaneous tissue closure, 3-0 vicryl suture for skin closure. Sutures should be on cutting needles.
- Vascular sheaths: 7 and 8 French size.
- Coronary guide catheters: JL3.5 or JL4, MB2 shapes, 6–7 French size.
- Angioplasty catheters with balloon size of at least 3 mm × 10 mm.
- Guidewires: 0.035″ and 0.014″ width in lengths greater than 90 cm.

2.3 Equipment

- Sterile surgical equipment: towel clips, scalpel with #10 blade, forceps (Debakey, Treves toothed), scissors (Metzenbaum, Iris, Mayo), clamps (right angle, hemostat), needle driver, bowls to contain sterile solutions (saline, radiographic contrast).
- Intubation equipment: laryngoscope with Miller blade, endotracheal tube stylet.
- Vital signs monitor with capacity for ECG, invasive and noninvasive blood pressure, O_2 saturation, and end-tidal CO_2 measurement.
- External defibrillator with connections for patches and paddles.
- Anesthesia ventilator.
- Fluoroscopy table with animal cradle.
- ICD and lead.
- Angioplasty catheter insufflator.
- Electrophysiology recording system with stimulator (We use the St. Jude Medical EPMedSystems workmate because it is the only one we have found that has system configuration and filter settings compatible for monophasic action potential recordings).

2.4 Staff

- Surgeon, sterile-scrubbed surgical assistant, non-scrubbed assistant, recorder (*see* **Note 1**).

3 Methods

3.1 Induction of Myocardial Infarction

1. Sterilize all surgical equipment.
2. Maintain the animal in NPO status overnight.
3. Sedate the animal by intramuscular (IM) injection of a telazol, ketamine, and xylazine mixture, 2.2 mg/kg of each component.

4. Intubate, ventilate, and anesthetize with isoflurane 1.75% (*see* **Note 6**).
5. Clean the skin if needed, shave to remove hair from surgical areas and from ECG and defibrillator patch sites.
6. Insert an intravenous catheter (ear veins are the most accessible in pigs) and maintain vein patency with a slow infusion of 0.9% saline.
7. Apply electrodes for 12-lead ECG to conventional positions, and apply external defibrillator patches in anterior-right sternal and posterior apical positions.
8. Sterilize the skin by scrubbing with a chlorhexidine sponge for 5 min followed by a Betadine sponge for 5 min and then spray the surgical field with Betadine solution.
9. Drape the edges of the surgical field with towels and a surgical sheet, and secure these in place with towel clips.
10. In the right neck, cut down and isolate the carotid artery and jugular vein. In the pig, the external jugular is larger so it more easily accommodates catheters, sheaths and leads.
11. Insert a 7 Fr. sheath into the jugular vein for IV access (later this site will be used to implant the ICD lead); insert an 8 Fr sheath into the carotid artery (for blood pressure monitoring and coronary catheterization) (*see* **Note 7**).
12. Administer heparin (*see* **Note 5**): for pigs, the dose is a 10,000 U initial IV bolus and then 5000 U bolus each hour while catheters are in the artery.
13. Continuously monitor temperature, ECG, BP, ETCO2, and SaO2 monitoring (*see* **Note 8**).
14. Access the left coronary system by placing a JL 3.5 or JL4-shaped coronary catheter into the left main coronary artery; cather placement should be performed over an 0.035″ guidewire (*see* **Note 7**).
15. Image the left coronary system by infusion of nonionic radiographic contrast medium during fluoroscopic recording.
16. Subselect the left anterior descending coronary artery (LAD) using a 0.014″ guidewire placed through the coronary catheter.
17. Catheterize the LAD by insertion of an angioplasty catheter over the 0.014″ wire; generally a balloon size of 3–4 mm × 10 mm is sufficient. For this application, either monorail or over-the-wire catheter is acceptable. Place the catheter so that the entire balloon (visible by radiographic markers) is distal to the second diagonal branch of the LAD (Fig. 1).

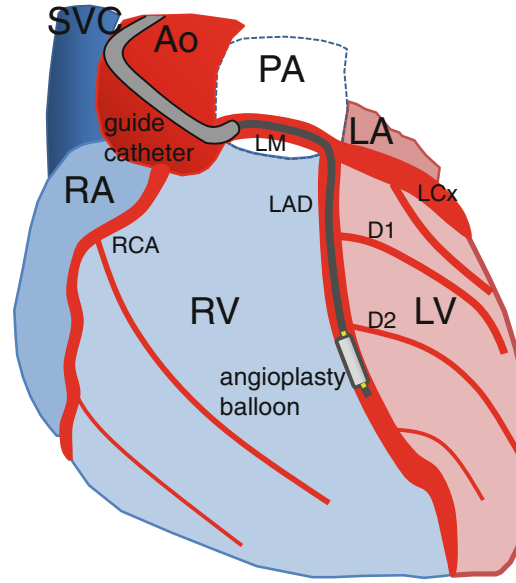

Fig. 1 Schematic of the coronary arterial anatomy with angioplasty balloon position to create the MI. The *yellow boxes* surrounding the angioplasty balloon depict the radiographic markers for the balloon. The catheter is positioned so that the proximal marker is beyond the origin of the D2. *SVC* superior vena cava, *Ao* aorta, *PA* pulmonary artery, *LA* left atrium, *LV* left ventricle, *RA* right atrium, *RV* right ventricle, *RCA* right coronary artery, *LM* left main coronary artery, *LCx* left circumflex coronary artery, *LAD* left anterior descending coronary artery, *D1* and *D2* first and second diagonal branches of the LAD

18. After confirming angioplasty catheter location by repeat infusion of contrast agent through the coronary guide catheter, occlude the LAD by expanding the angioplasty balloon to 3 ATM pressure. After the balloon is inflated, dislodge the coronary guide catheter from left main coronary (*see* **Note 9**).
19. Secure all catheters to the body (*see* **Note 10**).
20. *Immediately* defibrillate/cardiovert any sustained ventricular arrhythmias. Less than 5 s should lapse between onset of the arrhythmia and defibrillation (*see* **Note 2**).
21. If needed, maintain blood pressure with IV saline, dopamine (5–20 μg/kg/min), or phenylephrine (50–200 μg bolus) (*see* **Note 11**). In pigs, we keep the systolic blood pressure greater than 50 mmHg during infarction.
22. If frequent ventricular ectopy (>10 single premature ventricular beats/min or any non-sustained ventricular tachycardia) or any sustained ventricular arrhythmia occurs, administer amiodarone 150 mg in 10 ml saline IV over 10 min (*see* **Note 12**).
23. If hemodynamically significant bradycardia occurs (heart rate < 60 beats per minute with hypotension), administer atropine 0.05 mg/kg IV (*see* **Note 13**).

24. If hypotension is intractable, consider repositioning the balloon to a slightly more distal position in the artery. This issue is generally apparent within 5 min of occlusion and is notable by continual progressive drop in blood pressure. Due to issues of ischemic preconditioning, it is best to use experience as a guide for correct initial balloon positioning and avoid repeated deflation/inflation of the balloon.
25. In the last 30 min of the infarction procedure, if the animal is stable, remove the jugular sheath and use that access to place a defibrillator lead in the right ventricular apex. Attach the defibrillator lead to an ICD, and place the ICD in a subcutaneous pocket in the right neck. Administer cefazolin 1 g IV during ICD implantation and flush the defibrillator pocket with antibiotic solution after ICD implantation for infection prophylaxis.
26. After 150 min, deflate the angioplasty balloon. Confirm that the balloon is fully deflated and then remove all coronary catheters from the sheath.
27. Titrate off pressors. Remove the arterial sheath when stable. Close the neck incision with a 2-0 vicryl running subcutaneous suture and a 3-0 vicryl running skin suture. Dress the wound. Taper off anesthesia. Extubate when the animal is awake enough to protect airway. Monitor the animal until it has recovered enough to be returned to the holding pen.
28. If the defibrillator can be programmed to allow heart rates up to 300 bpm (in pigs), program the shock function on. Otherwise, keep the defibrillator off and use only for weekly assessment of arrhythmia inducibility.

3.2 Weekly Noninvasive Assessment of Arrhythmia Inducibility

1. Maintain the animal in NPO status overnight.
2. Sedate with IM injection of telazol, ketamine, and xylazine, 2.2 mg/kg of each. This dose is generally enough to make the animal unresponsive. Administer supplemental doses of sedation if needed to achieve an appropriate level of consciousness.
3. Clean the skin if needed; shave to remove hair from the ECG and defibrillator patch sites, apply ECG and defibrillation patches.
4. Interrogate the defibrillator using the company-specific programmer.
5. Perform programmed ventricular stimulation through the defibrillator as follows:
   (a) Test basic drive trains of 350, 300 and 250 ms. Use 8 beats per drive.
   (b) Sequentially add premature beats and test to refractoriness or a minimum coupling interval of 140 ms. For each drive train cycle length, test single, double, and triple extrastimuli.

(c) For any induced arrhythmias, obtain a brief 12 lead ECG recording prior to arrhythmia termination.

(d) Burst pace from the ICD to terminate any monomorphic VT. If that fails, cardiovert from the ICD, or if necessary, from the external defibrillator.

(e) Immediately defibrillate any polymorphic VT or VF using the ICD, and if necessary, the external defibrillator.

6. Monitor the animal until awake and return to the holding pen.

3.3 Gene Transfer

1. Sterilize all surgical equipment.
2. Maintain the animal in NPO status overnight.
3. Administer tadalafil 10 mg PO on the morning of the procedure. This can generally be done by pulverizing the pill and mixing it with a minimal amount of carrier (applesauce, wet pig chow, etc.).
4. Sedate the animal by intramuscular (IM) injection of a telazol, ketamine, and xylazine mixture, 2.2 mg/kg of each component (*see* **Note 14**).
5. Intubate, ventilate and anesthetize with isoflurane 1.75% (*see* **Note 6**).
6. Clean the skin if needed, shave to remove hair from surgical areas and from ECG and defibrillator patch sites.
7. Insert an intravenous catheter and maintain vein patency with a slow infusion of 0.9% saline.
8. Apply electrodes for 12-lead ECG to conventional positions, and apply external defibrillator patches in anterior-right sternal and posterior apical positions.
9. Sterilize the skin by scrubbing with a chlorhexidine sponge for 5 min followed by a Betadine sponge for 5 min and then spray the surgical field with Betadine solution.
10. Drape the edges of the surgical field with towels and a surgical sheet, and secure these in place with towel clips.
11. In the right neck, cut down and isolate the carotid artery and internal jugular vein. Insert a 7 Fr sheath into the vein and an 8 Fr sheath into the artery. If the artery was ligated rather than repaired at the time of infarction, extract clot from the vessel to achieve patency.
12. Advance a multipurpose coronary catheter through the jugular venous sheath into the coronary sinus (*see* **Note 15**).

    (a) Place an 0.014″ wire through the multipurpose catheter into the great cardiac vein.

    (b) Advance an over-the-wire angioplasty catheter into the proximal great cardiac vein (Fig. 2).

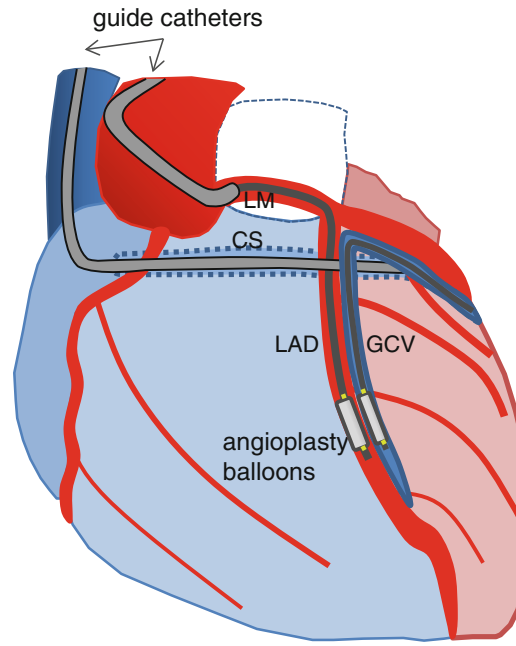

Fig. 2 Schematic of the coronary arterial and venous anatomy with angioplasty balloon positions for simultaneous perfusion of gene transfer solutions through the LAD and GCV. *LM* left main coronary artery, *LAD* left anterior descending coronary artery, *CS* coronary sinus, *GCV* great cardiac vein

13. Advance a Judkins left 3.5–4 guide catheter into the left main coronary artery.
    (a) Infuse nonionic radiographic contrast medium into the left main to visualize the left coronary anatomy. Mark the approximate location of the prior balloon placement from the MI procedure.
    (b) Place an 0.014″ wire through the Judkins left catheter into the mid-portion of the LAD.
    (c) Advance an over-the-wire angioplasty catheter into the LAD and place immediately distal to the second diagonal branch, approximately where the catheter was placed for the MI procedure (Fig. 2).
14. Connect an infusion manifold to each angioplasty catheter (Fig. 3). Connect syringes containing the pretreatment, gene transfer and flush solutions to the manifold (*see* **Note 4**).
15. Gene transfer (best if performed by two people) (*see* **Note 1**):
    (a) Simultaneously expand both angioplasty balloons to 3 ATM.
    (b) Infuse pretreatment solution over 3 min (*see* **Note 3**): 5 ml into each vessel at a flow rate of 1.66 ml/min (slightly faster than 0.5 ml every 20 s) (*see* **Note 16**).

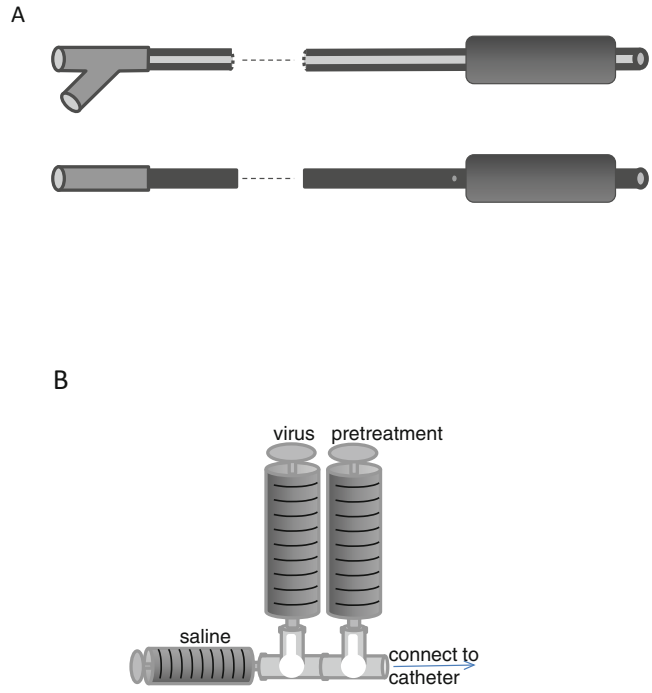

Fig. 3 Schematic of gene therapy equipment. (**a**) Over-the-wire (*top*) vs. monorail (*bottom*) angioplasty catheters. The over-the-wire catheter has a hollow core allowing a wire to pass from the catheter hub to the tip (and thus allowing perfusion through the catheter). The monorail catheter has a solid core and the wire passes from the tip to a port immediately proximal to the balloon. Either catheter is suitable for the infarction procedure. An over-the-wire angioplasty catheter is necessary for perfusion of gene transfer solutions. (**b**) The manifold attaching to each angioplasty catheter hub is composed of two joined 3-way stopcocks. Two 12 ml syringes are attached to the side ports for pretreatment and virus solutions, and a 5 ml syringe is attached to the end port for saline flush. The order of perfusion (pretreatment → virus → flush) causes the last drops of each solution to be flushed through the system by the next solution

(c) Infuse gene transfer solution over 2 min: 6 ml into each vessel at a flow rate of 3 ml/min (1 ml every 20 s).

(d) Infuse saline flush over 10 s (2 ml per vessel).

(e) During and after solution infusions, pace using the ICD if the heart rate decreases a clinically significant amount and defibrillate immediately if sustained ventricular arrhythmias occur. These functions are best automated by programming the ICD appropriately prior to starting the gene transfer procedure.

(f) Deflate the angioplasty balloons.

16. After completion of the infusion protocol, remove the catheters. When the heart rate and blood pressure are stable, remove the sheaths and repair or ligate the vessels.
17. Monitor the animal until awake and return to the holding pen.
18. Transgene functional testing is generally performed 4–7 days after gene transfer for adenovirus, 1–2 months after gene transfer for AAV, or whenever consistent with experimental goals.

4 Notes

4.1 General Notes

1. Adequate staffing of the procedures is essential for animal survival. The infarction procedure requires a surgeon with experience in vessel cut-down and coronary catheterization, a non-scrubbed assistant who can pass equipment and supplies to the surgeon and perform chest compressions if needed, and a recorder who is responsible for anesthesia, drug administration, external defibrillation, rhythm and vital signs monitoring. Noninvasive assessment of arrhythmia inducibility can generally be performed by two people, one to operate the ICD programmer, external defibrillator and electrophysiology recording system and the other to monitor sedation, rhythm, and vital signs. The gene transfer procedure is staffed with individuals playing the same roles as the myocardial infarction procedure, and an additional assistant surgeon is needed to hold catheters in position and infuse the gene transfer solutions through one of the catheters (with the surgeon infusing through the other catheter).
2. Pigs do not tolerate ventricular arrhythmias for longer than several seconds. Close rhythm monitoring and prompt defibrillation is essential for animal survival.
3. Our research has shown that a minimum of 3 min exposure to VEGF and nitroglycerin is needed to activate the intracellular signaling pathways that increase vascular permeability. Permeability effects of low calcium solutions are immediate. Nitroglycerin and low calcium exposure needs to be continuous to maintain the driving force for permeability. VEGF can be discontinued after the initial exposure (which is why it is included in the pretreatment and not the gene transfer solution). Likewise, continuous exposure to adenosine, nitroglycerin and low calcium are needed to maintain vasodilation through the gene delivery period.
4. It is critical to avoid infusion of air bubbles or any debris into the coronary vasculature (particularly the coronary arteries). All solutions should be sterile filtered to remove debris.

Extreme caution should be used to eliminate air bubbles from solutions, particularly during catheter or syringe connections.

5. Adequate heparinization is essential to avoid clot formation on catheters placed in the arterial system or the coronary vasculature. We have seen fairly quick clot formation and adverse animal outcomes when heparin administration has lapsed.

4.2 Specific Procedural Notes

6. For electrophysiology measurements, the level of isoflurane is important because it affects multiple ion channels and produces a concentration-dependent effect on action potential duration [24]. We strive to use a consistent dose of isoflurane in all animals during electrophysiology assessment for consistency during measurement.
7. Flush out catheters and sheaths after initial insertion and prior to use after any prolonged placement in the body. Coronary guide catheters should contain side holes to allow perfusion of the coronary artery while the catheter is in place.
8. ETCO2 assessment needs to consider that low ETCO2 can be due to overventilation but it can also be due to decreased cardiac output. If the ETCO2 is decreasing during infarction, the reason is likely cardiac output and reduction in ventilation is inappropriate.
9. Dislodging the guide catheter after occlusion of the LAD needs to be performed carefully so that the balloon is not dislodged as well. The guide catheter needs to be pulled back at the same time that the angioplasty catheter is pushed forward to move the one catheter without moving the other. This maneuver should be performed under fluoroscopic visualization.
10. The subject will move during defibrillation, so it is critical that all catheters and sheaths (and any other equipment or supply attached to the subject) be secured to avoid dislodgement during defibrillation.
11. Dopamine is preferred during MI because the afterload increasing effects of phenylephrine will increase cardiac workload. We only use phenylephrine when there is obvious vasodilation (warm red extremities), which can sometimes occur as a result of the anesthetics.
12. Monitor blood pressure closely during amiodarone infusion. We dilute the amiodarone to a total volume of 10 ml using 0.9% saline and administer 1 ml boluses of this solution each minute for 10 min.
13. Some protocols advocate prophylactic atropine prior to intubation for pigs. We have never done this, and we have not had any problems. We use atropine only if bradycardia is hemodynamically limiting.

14. If the subject has any significant heart failure, ketamine alone (15–25 mg/kg for pig) is used because the telazol–ketamine–xylazine mixture depresses respiratory drive. We have never seen this level of heart failure with our pig infarct-VT model. We regularly see this level of heart failure in our pig atrial fibrillation-heart failure model [25], and that experience taught us that this is an essential modification for safe induction of anesthesia in heart failure animals.
15. In the pig, cannulation of the distal coronary sinus can be difficult because it continues at an acute angle where the azygous vein inserts. A guide catheter with a broad curve and slight upturn at the lip seems to be most effective. If the multipurpose catheter is not working, other options include hockey stick, Judkins left, Amplatz left, or JCL shapes.
16. Adenosine is difficult to get into solution at room temperature. We heat it to 40–45 °C to make a stock solution of 50 mg/ml and then allow this to cool to room temperature. We then add room temperature VEGF, nitroglycerin, and saline to compose the pretreatment. For the virus solution, we add room temperature nitroglycerin and saline. All solutions are sterile filtered before use. The gene transfer solution has ice-cold virus stock added at the last minute. Never cool adenosine containing solutions of either stock of treatment concentration or it will precipitate. The volume of virus stock is never enough to alter solution temperature sufficiently to be a problem.

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Part VI

Targeting Pulmonary Hypertension

Chapter 23

MicroRNA Delivery Strategies to the Lung in a Model of Pulmonary Hypertension

Lin Deng, Andrew H. Baker, and Angela C. Bradshaw

Abstract

Pulmonary arterial hypertension (PAH) is characterized by enhanced proliferation of pulmonary artery smooth muscle cells and endothelial cells associated with obliteration of small pulmonary arterioles and formation of plexiform lesions. To date, no curative treatments have been identified for pulmonary arterial hypertension. There are various therapeutic options, including conventional medical therapies and oral, subcutaneous, intravenous, and inhalation delivery. We have previously shown that miR-143/145 knockout can prevent the development of chronic hypoxia-induced pulmonary hypertension (PH) in mice. Here, we use chronic hypoxia-induced PH as a disease model to evaluate miR-143/145 inhibition after delivery of antimiRNAs via the subcutaneous or intranasal routes. We use qRT-PCR and immunofluorescence to confirm that both delivery strategies efficiently inhibit miR-143/145 in lung tissue from mice with chronic hypoxia-induced PH.

Key words Pulmonary hypertension, MicroRNA, Anti-miR-143/145, Subcutaneous, Intranasal, Hypoxia, Delivery

1 Introduction

Pulmonary arterial hypertension (PAH) is a progressive disease that leads to substantial morbidity and eventual death. PAH is characterized by marked remodeling of the distal pulmonary arteries, which causes increased pulmonary vascular resistance [1]. One of the major challenges in treatment of PAH is the effective delivery of PAH therapeutics to sites of pathological vascular remodeling (mainly small distal pulmonary arteries) [2]. Our group was the first to report broad dysregulation of miRNA during the development of PAH [3]. Several studies subsequently demonstrated that miRNAs play a critical role in PAH pathogenesis. Therapeutic miRNA delivery to the lung in animal PAH models has been achieved via several routes: (a) subcutaneous injection [4, 5], (b) intravenous delivery [6], (c) intranasal delivery [7], (d) intratracheal delivery [8], and (e) intraperitoneal injection [9].

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Here, we demonstrate that miRNA within lung tissue can effectively be targeted via subcutaneous injection or intranasal administration of antimiRNA locked nucleic acid (LNA) oligonucleotides ("antimiRNAs") to mice. For subcutaneous delivery, two doses of antimiR-143 or antimiR-145 were administered to mice at a concentration of 25 mg/kg on days 1 and 7 during a 14-day hypoxia exposure period. Treatment with antimiR-143 or antimiR-145 prevented the development of chronic hypoxia-induced PAH. Moreover, subcutaneous administration of antimiR-143 at days 14 and 17 of a 21-day chronic hypoxia exposure period effectively rescued PAH in this model. Finally, two 5 mg/kg doses of intranasal antimiR-145 to mice significantly decreased the levels of miR-145 in the lung [4, 5].

2 Materials

All experiments were performed on 8-week-old female (*see* **Note 1**) C57BL/6J wild-type mice purchased from Charles Rivers.

2.1 Subcutaneous and Intranasal Administration

1. Equipment for small animal anesthesia, including isoflurane and O_2.
2. AntimiRNA of target and Scramble antimiRNA dissolved in sterile PBS (*see* **Note 2**); 16-mer oligonucleotides comprising at least nine LNAs with an LNA at both 5′ and 3′ ends and with full phosphorothioate linkages (In collaboration with MiRagen Therapeutics, Boulder, Colorado, USA). For co-localization studies, cy3-labeled antimiRNA (*see* **Note 3**); (MiRagen Therapeutics, Boulder, Colorado, USA) can be used.
3. Insulin syringes for subcutaneous antimiRNA delivery (29G/0.5 in. needle length).
4. 200 μl pipette and sterile tips.

2.2 Hypoxia-Induced Pulmonary Hypertension Model

1. Hypobaric hypoxic chamber.
2. Humidity/Temperature recorder.
3. Clean bedding and food/water.

2.3 Tissue Harvest

1. 25G needle.
2. 5 ml Syringe.
3. Phosphate-Buffered Saline.
4. 10% Formalin.
5. Scissors (Springbow Dissection Scissors, Straight or Curved Blades, and Surgical Scissors).

6. Forceps (Graefe Iris Forceps, Curved, Serrated, 9.5 cm long, 0.7 mm tip width).
7. Dry ice or liquid nitrogen.
8. Laboratory Scales.

2.4 RNA Extraction and qRT-PCR Analysis

1. RNeasy Mini Kit.
2. RNase-free DNase set.
3. RNase-free H_2O and 100% ethanol.
4. RNase-free tips and pipette.
5. TissueLyser II.
6. NanoDrop ND-1000 spectrophotometer.
7. Benchtop microcentrifuge.
8. PCR machine.
9. 96- and 384-well plates.
10. Taqman®MicroRNA Reverse Transcription Kit: 10× RT buffer, dNTP mix w/dTTp (100 M total), RNase inhibitor (20 U/μl), and MultiScribe™ RT enzyme (50 U/μl).
11. 2× TaqMan® Universal Master Mix II, no UNG.
12. 5× RT primers and 20× Taqman Probe designed against your miRNA of interest.
13. 20× U6 snRNA Taqman Probe (*see* **Note 4**).

2.5 Immunofluorescence

1. 6 μm sections of lung tissue on silanized glass slides, cut from tissues embedded in Tissue-Tek OCT Embedding Compound using a cryostat.
2. Humidified chamber for slide incubations.
3. 4% Paraformaldehyde (PFA).
4. Phosphate buffered saline, pH = 7.4 (PBS).
5. 0.1% tween in PBS (PBS-T).
6. Goat serum.
7. Anti-alpha smooth muscle Actin primary antibody.
8. Alexa Fluor 488-labeled secondary antibody.
9. Coverslips and ProLong®Gold Antifade Mountant with DAPI.
10. LSM510 confocal microscope.

3 Methods

3.1 Chronic Hypoxia-Induced Pulmonary Hypertension (PH) Model

1. All animal experiments were performed in accordance with the UK Animal Procedures Act (1986) and to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health.

2. A hypobaric hypoxia chamber is used for the development of hypoxia-induced PH.
3. Mice should be housed in clean cages supplied with ample food and water, at least six mice per group (*see* **Note 5**).
4. For acclimatization, the hypoxic chamber is only depressurized from ~1000 mbar (ambient room pressure) to 750 mbar on the first day (at a rate of 50 mbar/h).
5. The next day, the chamber is depressurized from 750 to 550 mbar (~10% O_2). Pressure is then maintained at 550 mbar for a continuous period of 14 days (*see* **Note 6**). Sustained hypoxia induces pulmonary vasoconstriction leading to the development of pulmonary hypertension.
6. A temperature of 21–23 °C and relative humidity of 30–50% should be maintained in the hypoxia chamber.
7. Bedding, food and water should be changed every 5 days (*see* **Note 7**).

3.2 Subcutaneous Injections of AntimiRNA and Cy3 Labeled AntimiRNA

1. All injections must be performed using sterile needles and syringes, with fresh needles and syringes used for each mouse (*see* **Note 8**).
2. The antimiRNA solution should be brought to room temperature prior to administration, and diluted to the appropriate dose. The effective dose for each animal is 25 mg/kg, for example, an 8-week old female mouse will weigh ~20 g so the actual dose required per animal will be in the region of 0.5–0.625 mg (*see* **Note 9**), diluted in 200 μl PBS.
3. No anesthesia is required for subcutaneous administration. The animal should be restrained by scruffing on a level surface. Applying light downwards pressure to the animal, insert the needle of the syringe into the loose skin fold over the neck that is held between the fingers in the scruff (Fig. 1). Inject the antimiRNA solution slowly into the neck fold. Larger volumes (>50 μl) will leave a visible rounded pouch beneath the skin. Animals can then be returned to their cages.
4. For our pulmonary hypertension studies, we administer two successive doses of 25 mg/kg antimiRNA subcutaneously, at days 1 and 7 of a 14-day period of chronic hypoxia.
5. The design of the subcutaneous injection regimen is shown in (Fig. 2).
6. Following sacrifice and tissue harvest, immunofluorescence can be used to detect Cy3-labeled antimiRNA and qRT-PCR can be used to evaluate the extent of miRNA repression after treatment. An example from antimiR-143 treated animals is shown in (Fig. 3).

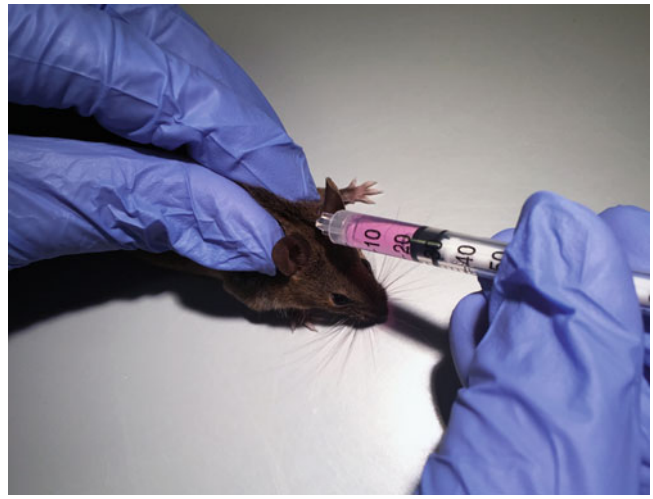

Fig. 1 Subcutaneous injection technique. Restrain the mouse by the scruff method. Using thumb and forefinger, handle the mice to make a tent of skin over the scruff. Then insert the needle parallel to the skin directed toward the posterior of the mice at the base of the tented skin

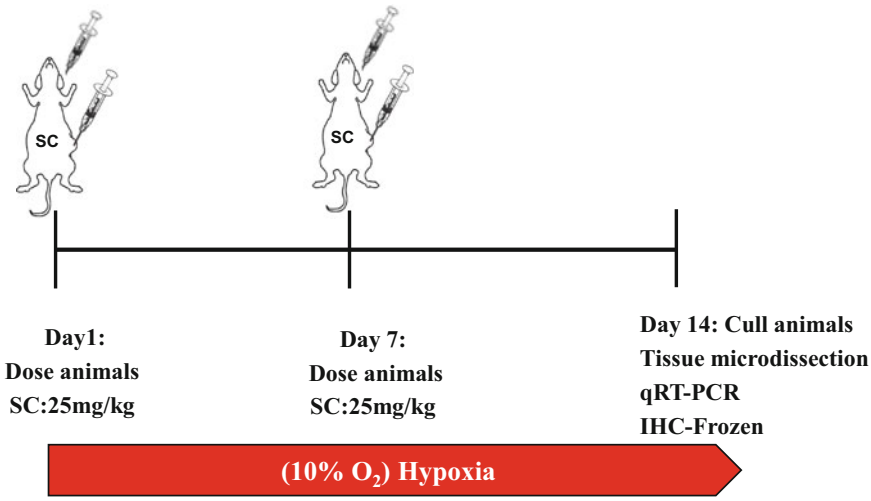

Fig. 2 Design of antimiR-143 and Cy3 labeled antimiRNA subcutaneous dosing regimen. 25 mg/kg antimiR-143, Scramble control, Cy3-labeled antimiRNA or PBS were administered subcutaneously to female C57/Bl6J mice at days 1 and 7 of a period of chronic hypoxia. Animals were sacrificed on day 14 for tissue harvest

3.3 Intranasal Delivery of AntimiRNA

1. All intranasal instillations must be performed using 200 µl sterile pipette tips, with fresh tips used for each mouse.
2. Prior to intranasal instillation, the mice should be anesthetized via exposure to 3% (v/v) isoflurane in O_2 (*see* **Note 10**). Once the animals are under shallow anesthesia, mice should be held as shown (Fig. 4), with the body tilted at a 45° angle. Using a 200 µl pipette, gently administer 25 µl dropwise to each nare,

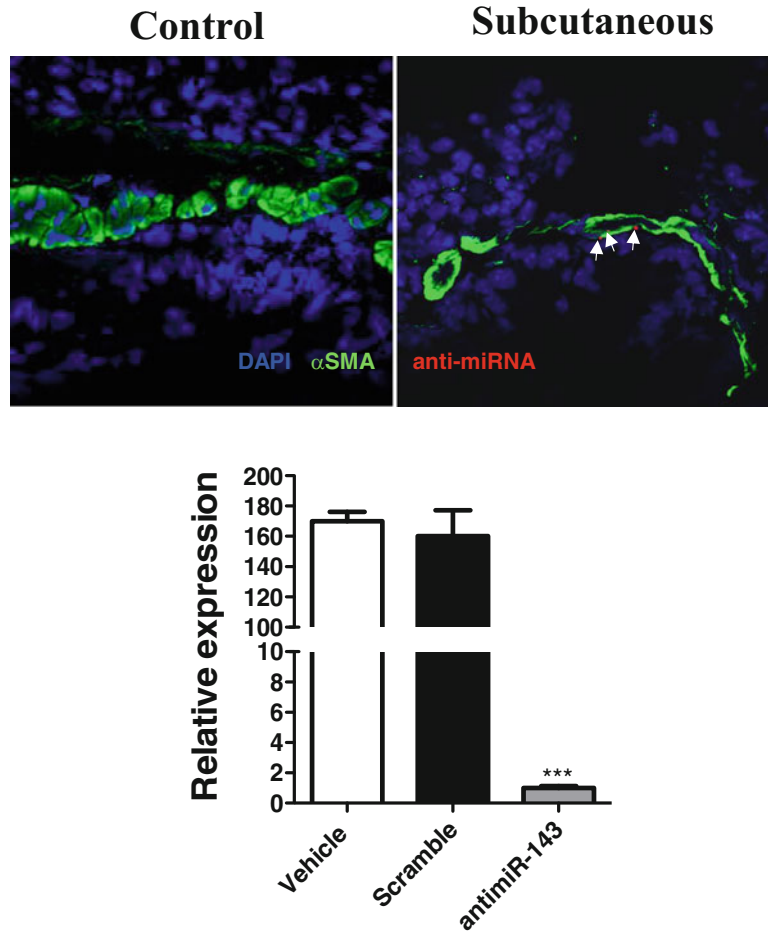

Fig. 3 Detection of Cy3-labeled antimiRNA and evaluation of miR-143 expression in total lung after subcutaneous administration of antimiRNA. (**a**), Immunofluorescence shows the Cy3-labeled antimiRNA (*red*) in α-SMA$^+$ smooth muscle cells (*green*) in lung tissue after subcutaneous administration of Cy3-labeled antimiRNA to mice. (**b**), q-PCR analysis of miR-143 expression in lung homogenates of mice after subcutaneous administration of anti-miR 143 or a scramble control. Data expressed as fold change ± SEM and analyzed by a one-way ANOVA followed by a Tukey's post hoc test, $n = 6$. ***$p < 0.001$

waiting between drips to ensure the mouse has inhaled the liquid. The total administration volume should not exceed 50 μl (25 μl/nare). Animals can then be returned to their cages to allow them to recover from anesthesia.

3. The design of the intranasal injection regimen is shown in (Fig. 5).
4. Following sacrifice and tissue harvest, immunofluorescence can be used to detect cy3-labeled antimiRNA and q-PCR can be used to evaluate the extent of miRNA expression after treatment. An example from antimiR-143 treated animals is shown in below (Fig. 6).

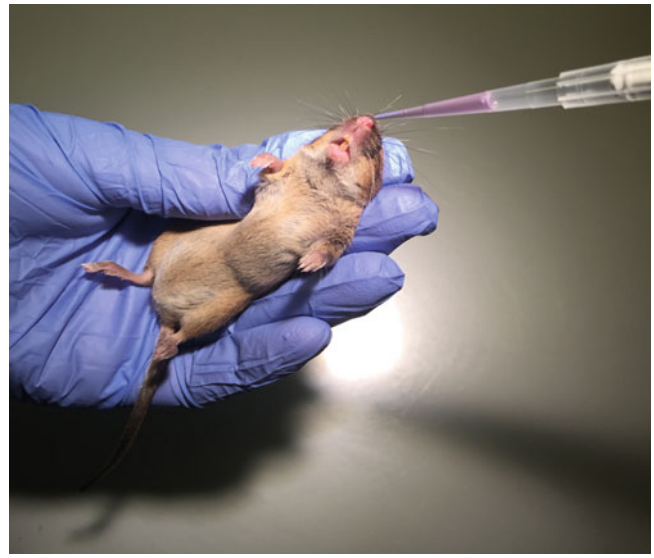

Fig. 4 Positioning of mice for intranasal delivery of antimiRNA. Mice should be held under shallow inhalational anesthesia. Anesthetized mice should be gently held at a 45° angle during intranasal administration. Using a 200 μl tip, gently administer 25 μl of antimiRNA solution dropwise to each nare

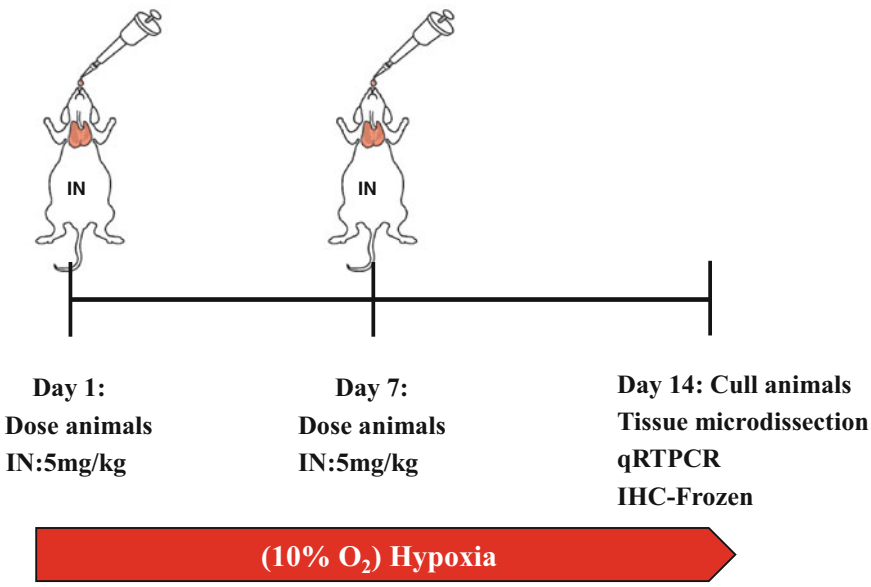

Fig. 5 Design of AntimiRNA-145 and Cy3 labeled antimiRNA intranasal dosing regimen. 5 mg/kg antimiRNA-145, Scramble, Cy3-labeled antimiRNA or PBS was administered intranasally to C57BL/6J mice on days 1 and 7. Mice were sacrificed on day 14 for tissue harvest

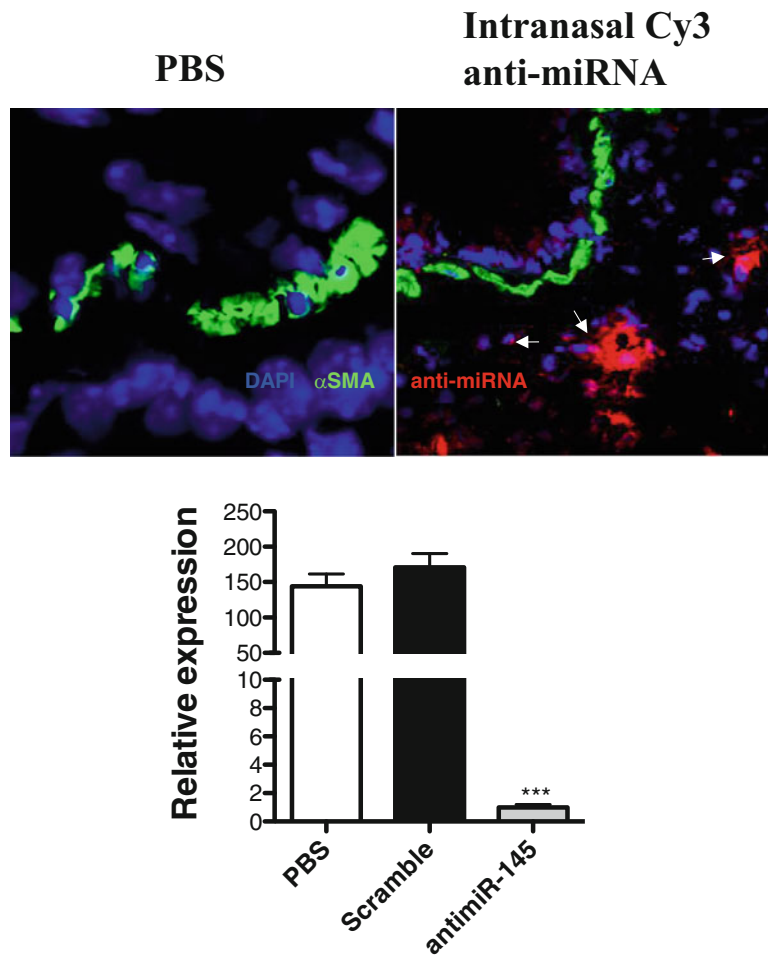

Fig. 6 Detection of Cy3-labeled antimiRNA and evaluation of miR-145 expression in total lung after intranasal administration of antimiRNA. (**a**), Immunofluorescence shows the Cy3-labeled antimiRNA (*red*) in α-SMA$^+$ smooth muscle cells (*green*) in lung tissue after intranasal administration of Cy3-labeled antimiRNA to mice. (**b**), q-PCR analysis of miR-145 expression in lung homogenates of mice after intranasal administration of antimiR 145 or a scramble control. Data expressed as fold change ± SEM and analyzed by a one-way ANOVA followed by a Tukey's post hoc test, $n = 6$. ***$p < 0.001$

3.4 Tissue Harvest

For pulmonary hypertension studies, tissues harvested include lung, pulmonary arteries and heart.

1. Sacrifice the animal using a Schedule I method, for example cervical dislocation.
2. After excision of sternal skin using surgical scissors and opening of the peritoneal cavity, cut away the diaphragm by grasping the xiphisternum with forceps and puncturing the diaphragm using the scissor tips. This will deflate the lungs and allow careful excision of the diaphragm around the base of the ribcage.

3. Open the chest cavity along the midline by excising the sternum longitudinally, cutting up to the neck.
4. Expose the lungs by cutting away the ribcage from the sternal incision (*see* **Note 11**).
5. Flush the pulmonary vasculatures with PBS (*see* **Note 12**) by grasping the apex of the heart with toothless forceps and injecting 5 ml PBS with 5 ml syringe into right ventricle with a small gauge needle (25 G) (*see* **Note 13**).
6. Excise the whole lungs and heart, including the aortic arch and pulmonary arteries, weigh on the scale, and then place in cold PBS.
7. The pulmonary artery begins at the base of the right ventricle, which branches into left and right pulmonary arteries. Cut all the fat and superficial tissues surrounding the heart and pulmonary artery, and remove the aorta and bronchus artery carefully without damaging the pulmonary artery. Using blunt forceps to hold the pulmonary artery branch and cut with scissors at the right ventricle and left and right lung lobes.
8. Collect individual lung lobes using scissors and forceps. We fix the left lung lobe overnight in 10% formalin for paraffin embedding, the right superior lobe and post-caval lobe are snap-frozen in liquid nitrogen or on dry ice for use in protein extraction, the right middle lobe is snap-frozen for RNA extraction, and the right inferior lobe is embedded in OCT for cryo-sectioning. To facilitate sectioning, lungs can be cryoprotected after fixation in 4% PFA by incubating in a 30% sucrose solution at 4 °C overnight before embedding in OCT (*see* **Note 14**).

3.5 Right Ventricle Hypertrophy Assessment

1. Right ventricle hypertrophy is assessed after dissecting the whole heart.
2. Remove the atrial, large vessels and pericardial fat by careful dissecting with forceps and scissors under a dissecting microscope.
3. The right ventricle free wall (RV) is separated from the left ventricle plus septum (LV + S) by careful dissection using Springbow microsurgical scissors and forceps.
4. RV: LV + S ratios are calculated after weighing both dissected RV and (LV + S); the ratio of RV/(LV + S) can be used as an index of PH.

3.6 Evaluation of miRNA Expression in Tissue Samples by qRT-PCR (See Note 15)

1. Snap-frozen lung, pulmonary artery and right ventricular tissues are disrupted and homogenized using the QIAGEN TissueLyser II system, with 5 mm stainless steel beads with 700 µl QIAzol lysis reagent. Total RNA is then extracted using the QIAGEN RNeasy Mini Kit together with DNase treatment following manufacturer's instructions.

2. Total RNA is quantified using a NanoDrop 1000 spectrophotometer (ThermoScientific). RNA purity can be evaluated using the A_{260}/A_{280} ratio, with a ratio of ~2.0 accepted as good RNA purity.
3. For miRNA qRT-PCR, cDNA is synthesized using stem-loop reverse transcription primers as per the Taqman microRNA assay protocol (Applied Biosystems, Paisley, UK). RNA should first be diluted to a concentration of 2 ng/μl concentration.
4. Add the miRNA-RT reaction as shown in Table 1 (*see* **Note 16**).
5. The thermal cycler program for miRNA-RT is in Table 2.
6. Taqman qPCR Reaction as in Table 3: add 20.5 μl RNase-free H_2O to the RT product, and mix thoroughly (*see* **Note 17**).
7. For each probe tested, a negative reverse transcription control should be run alongside a nuclease-free water control. All qPCR experiments are performed in triplicate wells using the 7900HT sequence detection system (Applied Biosystems). Thermal cycling conditions begin with a 10 min incubation period at 95 °C, then followed by 40 cycles of 15 s at 95 °C and then 60 s at 60 °C.
8. Calculate the mRNA expression relative to the control sample using the $-2^{\Delta\Delta Ct}$ method. For miRNA expression analyses in mouse tissue, we use U6 as a housekeeping gene.

Table 1
Two step miRNA reverse transcription reaction (RT) mix

	1×
10× RT buffer	0.075 μl
MultiScribe™ RT enzyme	0.5 μl
dNTP	0.075 μl
RNase Inhibitor	0.095 μl
RNase-free H_2O	2.08 μl
5× RT Primer	1.5 μl
Diluted RNA Samples	2.5 μl
In total:	7.5 μl

Table 2
Thermal cycling parameters for RT reactions

16 °C for	30 min
42 °C for	30 min
85 °C for	5 min (Inactivation of reverse transcriptase)
Hold at	4 °C

Table 3
Quantitative real-time PCR reaction mix (per well)

	1×
Taqman master mix (2×)	6.25 µl
20× Primer/Probe mix	0.625 µl
RNase-free H_2O	3.125 µl
MiRNA-RT Product	2.5 µl
In total	12.5 µl

3.7 Immunofluorescence

1. Microscope slides of 6 µm OCT lung sections should be removed from −80 °C storage 20 min prior to use and allow them to thaw at room temperature (*see* **Note 18**).
2. Using a wax pen, draw rings around sections (*see* **Note 19**), then fix by incubating in 4% PFA for 10 min at room temperature.
3. Wash sections twice with PBS for 5 min, then permeabilize tissues by incubating with 0.1% Tween20 (in PBS; PBS-T) for 10 min (*see* **Note 20**). All washes should be carried out at room temperature.
4. Wash sections three times with PBS for 5 min, then block nonspecific reactive sites by incubating with 10% goat serum diluted in PBS-T for 30 min at room temperature.
5. After one 5-min wash with PBS, add primary antibodies to sections (made up in 1% goat serum diluted in PBS-T) and incubate in a humidified chamber overnight at 4 °C (*see* **Note 21**). For our experiments, we used an α-SMA antibody diluted 1:200 to 1 µg/ml.
6. The next day, sections should be washed three times in PBS for 5 min prior to incubation with Alexa Fluor-conjugated secondary antibodies raised in goat (in the example presented in (Figs. 3 and 6), an Alexa 488-conjugated goat anti-rabbit antibody is used). These antibodies are used at a 1:500 dilution in PBS at an effective concentration of 4 µg/ml. Sections should be incubated with secondary antibodies for 1 h at room temperature, in a darkened humidified chamber.
7. Wash sections three times in PBS for 5 min in the dark, before mounting sections using ProLong Gold antifade mountant containing DAPI. Slides should be allowed to cure at room temperature overnight in the dark before visualizing by confocal microscopy (*see* **Note 22**).

4 Notes

1. Pulmonary hypertension occurs more frequently in females, so female mice are more commonly used in studies of chronic hypoxia-induced PH, except in cases where gender differences in PH susceptibility or development are being evaluated. We have not observed any gender-specific differences in antimiRNA delivery efficacy.
2. AntimiRNA should be prepared and diluted in sterile PBS. AntimiRNA solutions can be aliquoted into smaller volumes (we suggest aliquoting into single-use doses) and stored at −80 °C until required.
3. For Cy3-labeled miRNAs, you can buy the antimiRNA, premiRNA, or miRNA mimic and the label kit (Silencer® siRNA Labeling Kit with Cy™3 dye) to label the miRNAs.
4. Stability of housekeeping gene expression between treatment groups should be verified for each experiment. If U6 is not stable, alternative housekeeping gene candidates are snoRNA202 or snoRNA234.
5. Generally, researchers consider six mice per group as an adequate sample size for PAH studies on mice using the chronic hypoxia model. Here, we recommend a group size of ten mice, to cover attrition rates during the hemodynamic measurements.
6. The atmospheric pressure in the chamber should remain stable at 550 mbar. We recommend checking pressures each day to avoid discrepancies due to pressure fluctuations.
7. To ensure animal safety when opening the hypoxic chamber to replace cages, food and water, increase pressure to 1000 mbar at a rate of 100 mbar/h. After changing cages, food and water, return the hypoxic chamber pressure to 550 mbar by depressurizing at a rate of 100 mbar/h.
8. For subcutaneous injection, it is not usually necessary to sterilize the skin, but you can prepare the injection site with 70% ethanol if required. The syringe needle should be inserted between the fingers grasping the neck skin and should be directed toward the posterior of the mice.
9. Excess antimiRNA volumes should be prepared for each injection, to ensure that each mouse receives a full dose.
10. Mice should be maintained under shallow anesthesia, as to ensure the antimiRNA solution is inhaled spontaneously. Delivery should take no more than 30 s, however if the mice regain consciousness during the procedure they can be re-anesthetized to finish the delivery.

11. When cutting away the diaphragm, be careful to avoid puncturing the lungs using the dissecting scissors.
12. The pulmonary vasculatures are flushed with PBS to remove blood from the lung and heart and to ensure that remnant antimiRNA in the systemic circulation does not affect tissue analyses.
13. It is necessary to cut the liver or the abdominal aorta to let the perfusate flow out when flushing the pulmonary vasculature.
14. Dissected tissues that will be used for RNA/protein extraction and OCT blocks should be stored at −80 °C until they are processed.
15. When using Taqman primers and probes to assess the expression of miRNAs, reagent usage can be minimized by using 384-well plates. We perform our assays in triplicate to reduce variation due to technical pipetting errors.
16. When preparing Taqman Mastermixes, include excess volume (2–5 wells) in your calculations.
17. In order to avoid pipetting errors, we dilute the RT product with 20.5 μl RNase-free H_2O before adding 2.5 μl to each well of the 384-well plate.
18. Storage and cutting of cryostat sections can be made easier by using disposable base molds for embedding tissue samples in OCT. Different tissues require varying temperature settings on the cryostat: for lung tissue, we typically cut at −16 °C.
19. Drawing rings around sections with a hydrophobic (wax) pen reduces the volume of reagents required for immunofluorescence.
20. When only a few slides are being processed for immunofluorescence, washes can be carried out in a Coplin jar to minimize the volume of buffer required.
21. A humidified chamber is required in order to prevent evaporation from sections, which will cause uneven or false staining.
22. If sections need to be visualized immediately by confocal microscopy, clear nail lacquer applied around the edges of coverslips can be used to prevent the liquid mounting medium from leaking out onto the microscope objectives.

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Chapter 24

Inhaled Gene Transfer for Pulmonary Circulation

Jaume Aguero, Lahouaria Hadri, Nadjib Hammoudi, Lauren Leonardson, Roger J. Hajjar, and Kiyotake Ishikawa

Abstract

Chronic pulmonary hypertension (PH) is associated with right ventricular failure and high mortality regardless of the underlying disease. Currently, therapies can improve clinical outcomes in specific subsets of patients, but have little impact on the progression of pulmonary vascular remodeling. Upon new advances in vector development and delivery techniques, gene therapy is a novel strategy in this field with the potential of overcoming the main limitations of approved drug therapies: modulation of novel anti-remodeling targets and selective pulmonary vasculature targeting with minimal systemic effects. In the recent years, several reports have shown that gene transfer to the pulmonary vascular system is feasible in rodent models of PH. Our group has focused on the translation of airway delivery of viral vectors in small and large animals. Here, we describe a procedure to achieve vector transduction at the distal vasculature in animal models of PH and the methods to evaluate the outcomes of this intervention as a promising new approach in pulmonary vascular diseases.

Key words Airway delivery, Gene therapy, Pulmonary hypertension, Large animal model, Adeno-associated virus, Right ventricular failure, Vascular remodeling, Pulmonary vascular disease

1 Introduction

Pulmonary vascular disease (PVD) is defined by the development of vessel wall remodeling changes in the distal pulmonary vasculature, and is a consequence of a heterogeneous group of clinical conditions [1]. In the clinical setting, PVD is characterized by progressive dyspnea and exercise intolerance, and diagnosis relies on the detection of pulmonary hypertension (PH) upon right heart catheterization. PH is defined by a mean pulmonary artery (PA) pressure above 25 mmHg [1], and based on available clinical studies, underlying PVD is suspected by additional hemodynamic abnormalities in the pulmonary circulation, such as increased pulmonary vascular resistance (PVR), transpulmonary gradient or diastolic to PA wedge pressure differences [1–3]. The main determinant of prognosis in patients suffering PH is the impact of sustained high afterload on right ventricular function, leading to premature heart failure and death [4].

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Current therapeutic options for chronic PH are largely dependent on the clinical classification for each patient undergoing the diagnostic process [1]. Advances in the cellular and molecular mechanisms involved in Group 1 PH (also designated as pulmonary arterial hypertension, PAH) have led to novel drug developments targeting the main pathways including endothelin receptor antagonists, prostacyclin analogs and activators of the soluble guanylate cyclase (sGC)/cGMP axis (phosphodiesterase five inhibitors, and more recently, sGC activators) [1]. Clinical trials for these drugs have focused on Group 1 PH, while some benefit may be present in other groups with novel agents [5, 6], in particular groups 2 (PH due to left heart disease) and 4 (chronic thromboembolic PH).

Limitations for the widespread use of current vasodilator drugs include the frequency of systemic, undesired side effects, lack of long-term sustained clinical benefits, as well as a high economic cost of these treatments [1]. In addition, during the past few years, the unraveling of novel molecular mechanisms involved in PH have set growing interest in developing more specific, target-driven therapeutic strategies. In this regard, gene therapy may overcome some of the limitations of current treatments, by selectively modulating novel pathways that are not targeted by any drug at present [7]. Recently, several studies by independent groups have shown the potential therapeutic benefit of modulating a variety of molecular targets using gene therapy [7, 8]. For instance, endothelial NOS, prostacyclin synthase or BMPR2 have been successfully modulated in the pulmonary vasculature leading to improved hemodynamics in animal models of PH [9–11].

In order to develop gene therapy strategies for PH, vectors that efficiently target the pulmonary vasculature and provide sustained expression of the gene of interest are needed. In this regard, advances in viral vector technology have made available the recombinant adeno-associated viruses (AAV)s that allow different tissue tropisms based on the capsid proteins composition, while eliciting minimal immune response. In addition, delivery methods that preferentially transduce the lung vasculature with minimal exposure of the vector to off-target tissues are essential to guarantee the feasibility, safety, and translatability of this strategy for PH patients [12].

The purpose of this protocol is to describe a novel airway delivery method of vectors in large animal models of PH that efficiently transduces the distal pulmonary circulation and elicits improvements in vascular remodeling and hemodynamics.

2 Materials

2.1 Animal Preparation and PH Model Creation

For Anesthesia Induction and Maintenance

1. Telazol (tiletamine/zolazepam).
2. Isoflurane.

3. Propofol.
4. Fentanyl patch.
5. Prophylactic antibiotics.
6. Respirator suitable for swine with adjustable inspiratory oxygen concentration.
7. ECG and pulse oxymetry monitor.

For Surgical PH Model Creation in Swine

8. Surgical suite and sterile drapes.
9. Standard surgical tools: Scissors, forceps, scalpel. Bioabsorbable and Nylon sutures, Silicone Thoracic Drain 20Fr., Gauzes.
10. Cotton Umbilical Tape 1/8″ × 18″.
11. A 3.5-mm diameter plastic cylinder.
12. Furosemide.

2.2 Functional Evaluation of PH in Large Animals: Hemodynamic and Echocardiography Assessments

1. Procedure room equipped with a fluoroscopy system (C-arm).
2. Standard cath pack for sterile percutaneous angiographies (syringes, towels, bowls, gauze).
3. Sheath introducer 8 French.
4. Swan-Ganz Catheter 7 French.
5. Capnograph.
6. Blood gas analyzer.
7. Pressure transducers.

2.3 Airway Gene Delivery

1. Procedure room equipped with a fluoroscopy system (C-arm).
2. MicroSprayer® Aerosolizer and accompanying syringes (Model IA-1B, Penn-Century, Inc.) customized for large animal experiments (in 20–40 kg Yorkshire swine, a 50 cm-length tip is optimal, but needs to be designed according to the animal species and the proximal airway to carina distance).
3. Multipurpose coronary diagnostic catheter shortened to fit the Sprayer (7 Fr).
4. Viral vector encoding reporter gene (lacZ or GFP) or the therapeutic gene of interest.
5. Ambu bag.
6. Airway filter with high filtration efficiency.
7. Personal protective equipment (PPE) including gloves, masks, gown, and eye protection.

2.4 Evaluation of the Transduction Efficiency Using β-Galactosidase Expression

1. O.C.T. Compound.
2. Glass slides.
3. Cryotome.
4. X-Gal staining kit.

5. Neutral buffered 10% formalin solution.
6. Primary antibodies against reporter gene or gene of interest.
7. Brightfield and confocal microscope.

3 Methods

3.1 Animal Preparation and PH Model Creation

1. Fast the animals overnight. Administer prophylactic antibiotics prior to surgery.
2. Anesthesia is induced with intramuscular administration of 6.0 mg/kg Telazol (tiletamine/zolazepam). Orotracheal intubation is performed first by trained personnel, and peripheral oxygen saturation and heart rate are continuously monitored (*see* **Note 1**). A peripheral ear vein access is subsequently obtained.
3. For surgical procedures (surgical PH model creation), inhaled isoflurane (1–3%) is adjusted according to animal sedation status. Analgesia after the procedure is provided using postoperative 25–50 μg/h fentanyl patch. During the procedure, oxygen saturation, heart rate, and systemic blood pressure are continuously monitored. Animals are given prophylactic antibiotics twice daily for 5 days after the thoracotomy.
4. Surgical creation of the PH model in swine. A left lateral thoracotomy at the fifth intercostal space is performed under sterile conditions (*see* **Note 2**). Remove the lung from surgical site by applying a wet gauze and squeeze (*see* **Note 3**). Obtain a good view of the left atrial posterior wall where pulmonary veins enter. Widen the incision if necessary.
5. The superior left pulmonary vein and the common inferior pulmonary vein are carefully dissected in the extrapericardial space close to the left atrium. Consistent degree of venous stenoses are achieved by placing a cotton umbilical tape around a 3.5-mm diameter plastic cylinder that is subsequently removed once the tape is tightly secured (*see* **Note 4**).
6. Close the chest in layers, making sure that all air is evacuated using a drainage chest tube. Furosemide 4 mg/kg is given after the procedure to prevent acute pulmonary edema [13, 14].

3.2 Functional Evaluation of PH in Large Animals: Hemodynamic and Echocardiography Assessments

1. Follow the same anesthesia induction as in Subheading 2.2. Use intravenous propofol 8–10 mg/kg/h for hemodynamic evaluation (*see* **Note 5**).
2. Transthoracic echocardiography can be used for right ventricle (RV) noninvasive imaging and is convenient to perform within the animal facility. Apical views of the RV can be obtained with the animal on right lateral recumbency by placing the probe in the sub-xiphoid position. Modified apical views of the RV and

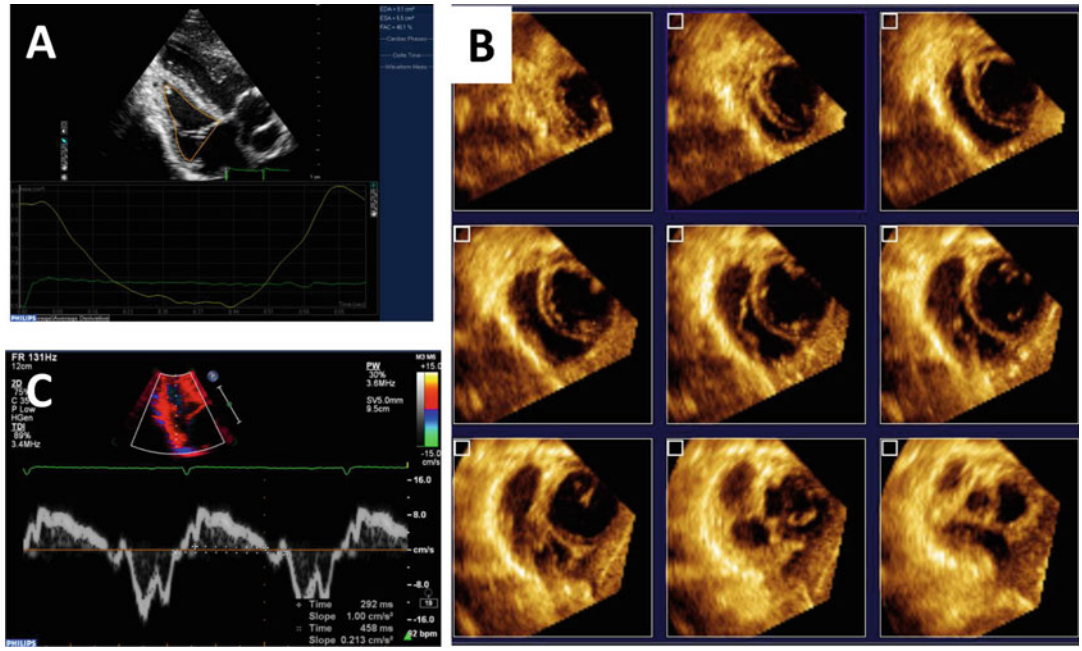

Fig. 1 Right ventricular noninvasive characterization using echocardiography allows evaluation of the PH model and the changes induced by therapeutic gene transfer. Two- (**a**) and three (**b**) dimensional datasets can be obtained from modified apical views in swine models, as well as the Doppler spectral signal (**c**) for time intervals and Tei index

3D datasets can be acquired with ECG gating (Fig. 1). Offline analyses provides quantitative information of RV dimensions and performance [13].

3. Under sterile conditions, a femoral vascular access is obtained using the Seldinger technique. For right heart catheterization with a 7 French Swan-Ganz catheter, an 8-Fr sheath is placed in the femoral vein (*see* **Note 6**). As an alternative, the jugular veins can also be accessed in swine but care must be taken to avoid undesired carotid artery punctures.
4. Positioning of the Swan-Ganz catheter to obtain right side hemodynamics is achieved using fluoroscopic guidance (C-arm). Calibrate pressure sensor carefully. This is particularly important when measuring right side pressures as they are often much lower than left side pressures. Before measurements are obtained, hemodynamic stability must be obtained and the catheter locations should be confirmed for every measurement (*see* **Note 7**).

3.3 Airway Gene Delivery

1. Therapies should be preceded by hemodynamic measurements to obtain baseline values. Gene delivery can be performed subsequently. Connect an airway filter to the tracheal tube.
2. Research personell in the operating room should take appropriate precautions for vectors (*see* **Notes 8** and **9**).

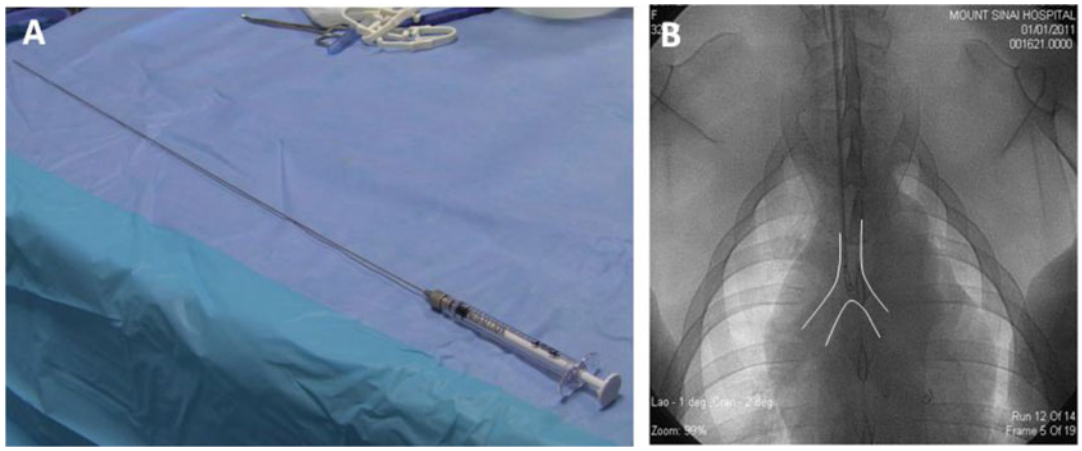

Fig. 2 Intratracheal gene delivery requires a MicroSprayer device (**a**) that is placed near the carina guided by fluoroscopy (**b**)

3. The vector is prepared in the injection syringes (*see* **Note 10**).
4. The aerosolizer device is inserted carefully through the endotracheal tube and the position of the tip determined using fluoroscopic guidance (*see* **Note 11**). By inserting the aerosolizer tip in a 7 Fr multipurpose coronary diagnostic catheter, cut to fit the Sprayer inside, the device can be advanced in the trachea while avoiding undesired damage in the tracheal mucosa. Once the tip of the aerosolizer is 2–3 cm proximal to the tracheal bifurcation, the multipurpose coronary diagnostic catheter is gently pulled back a few cm (Fig. 2).
5. The total vector dose is split into three equal aliquots that will be injected in the dorsal, right, and left lateral recumbent positions, allowing at least 5 min between each injection (*see* **Note 12**).
6. Injection of the vector solution should be coordinated to the inspiration phase. Also, pre-injection alveolar recruitment maneuvers using the Ambu will facilitate a more even and distal distribution of the vector.
7. After vector delivery is completed, mechanically ventilate for an additional 20–30 min with continuous monitoring of the EKG, hemodynamics, and respiratory parameters.
8. Once the observation period is finished without complications, the animal is recovered. The vector leak from the airway is minimal; however, keep the precaution materials on throughout the procedure.

3.4 Evaluation of the Transduction Efficiency

1. Humanly euthanize the animals after an appropriate time post-gene delivery to allow transgene expression. Through a median sternotomy, lung tissue from both side of the lungs at different lobes are collected. Remove blood from the tissue specimens by perfusing the vessels with PBS (*see* **Note 13**).

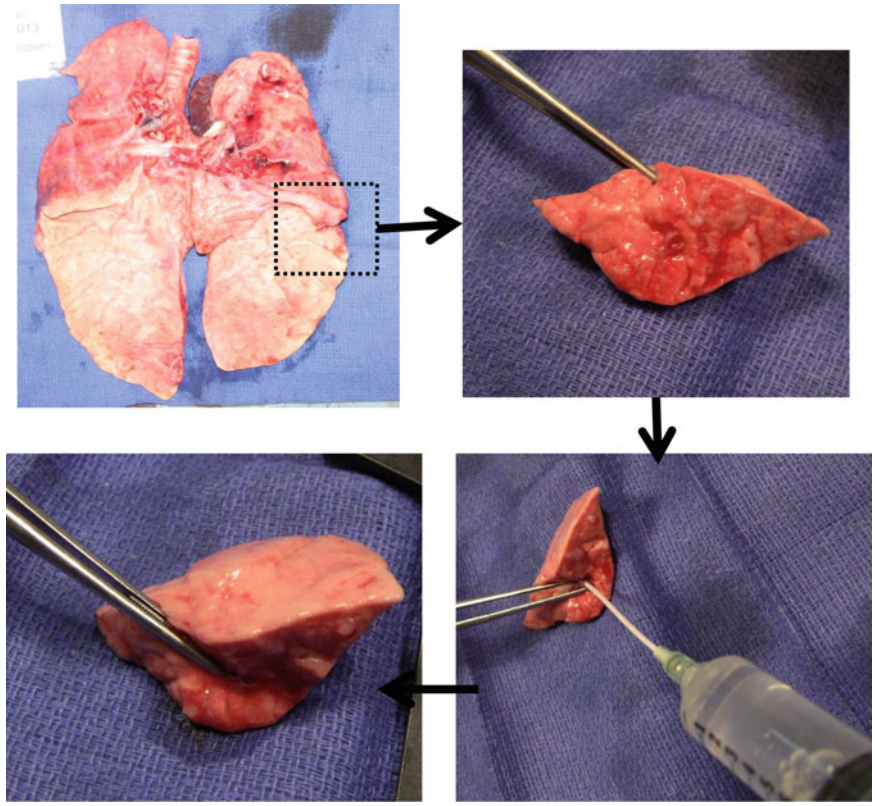

Fig. 3 Lung tissue is inflated with OCT for easier handling and ulterior staining techniques

2. For lung tissue fixation, gently insufflate the airway with 50% OCT in PBS, embed in labeled OCT molds and freeze the blocks. Using a cryotome, sections (8–10 μm) are prepared on glass slides for subsequent staining (*see* **Note 14**) (Fig. 3).
3. The staining technique to detect transgene expression depends on the reporter or protein of interest. For vectors with the lacZ reporter, the product of β-galactosidase activity can be detected in brightfield microscopy using the X-Gal staining kit. However, detection of β-galactosidase using specific primary antibodies is more sensitive and can be more precisely localized using confocal microscopy. For specific proteins of interest, primary antibodies that are well validated in swine tissue are needed. Colocalization with specific cell type markers such as alpha-smooth muscle actin (for smooth muscle cells) or endothelial NOS (endothelial cells) allows a more clear identification of preferential cell type of gene expression (*see* **Note 15**) (Fig. 4).

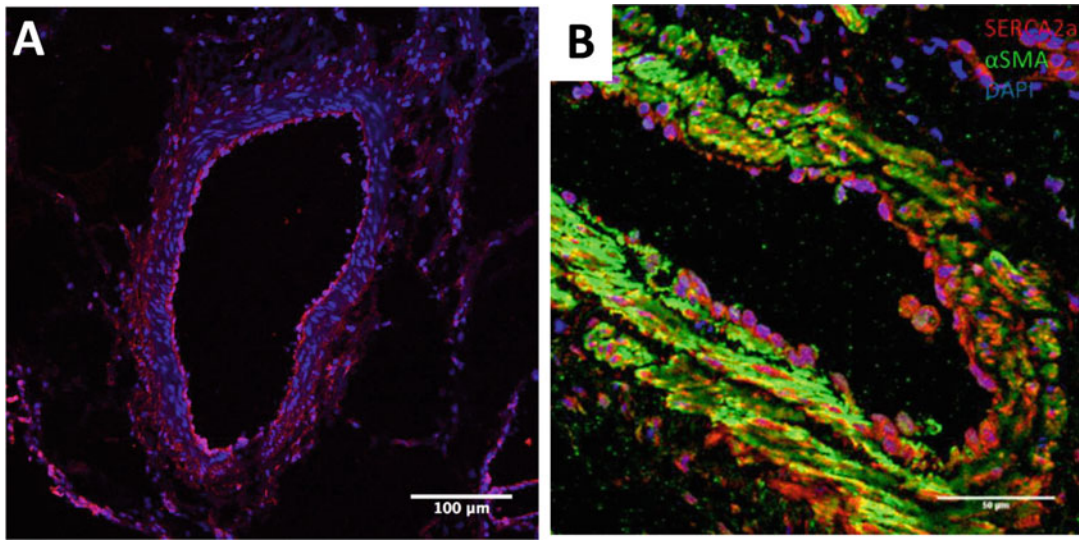

Fig. 4 Assessment of transgene expression can be performed using immunofluorescent staining. Four weeks after the delivery procedure, β-galactosidase protein is found in the pulmonary vasculature (*pink*, **a**). Upon delivery of the AAV1.CMV.SERCA2a vector, overexpression of SERCA2a protein (*red*, **b**) colocalizes with smooth muscle cells (*green*, **b**)

4 Notes

1. In animals with moderate to severe PH, hypoxia can significantly worsen the hemodynamics and animals can easily die from brief hypoxia. Oxygen should be supplied continuously during the preparation, and rapid intubation and immediate ventilation is necessary to prevent prolonged hypoxemia that may influence the stability of the hemodynamic evaluation.
2. When entering the pleural cavity, use caution not to injure the lung. Cutting the pleural membrane during the expiration will reduce the risk of accidental injury.
3. Lungs can be inflated using an Ambu bag after banding the pulmonary veins.
4. The optimal degree of vein stenosis depends of the animal growth rate. Applying too tight of a stenosis can cause subacute lung edema that develops 2–3 h after surgery. The data provided applies to female Yorkshire swine with BW 10–13 kg.
5. Some anesthesia drugs such as isoflurane have a strong vasodilatory effect and can mask mild PH.
6. Guidance of the percutaneous puncture using vascular echography minimizes the number of attempts and vascular injury, and is advisable if repeated right heart catheterization procedures to monitor hemodynamics overtime are planned.

7. Appropriate ventilation parameters are set depending on the investigators needs. The following parameters provide stable and reproducible conditions under general anesthesia in our experience: oxygen inspiratory fraction 40%, 10 ml/kg tidal volume at 15 respirations per minute to maintain an end-tidal CO_2 between 35 and 45 mmHg as determined by capnography. A portable blood gas analyzer provides a detailed blood gas profile that can be particularly informative in diseased animals. Cardiac output is determined by thermodilution.
8. When testing vectors that can affect research personnel, personal protective equipment including gloves, gowns, shoe covers, respirators, face shields and safety glasses must be worn during the viral vector delivery procedures and animal necropsies. The risk of infection by exposure to an infectious aerosol must be minimized by primary containment and multiple secondary barriers such as specialized ventilation systems, air treatment systems to decontaminate or remove agents from exhaust air, and controlled access zones.
9. We have previously used AAV vectors using this delivery method [12]. According to the NIH Guidelines, recombinant AAVs in which the transgene does not encode either a potentially oncogenic gene product or toxins, and are produced in the absence of a helper virus can in most cases be handled at Biosafety Level 1. Decontamination of working areas is recommended. Experiments must be performed in accordance with the Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee for the use of AAV in these animals.
10. If the vector should be kept cold to maintain its activity, vector preparation should be done by a second operator after placing the Sprayer in the appropriate position.
11. An L-shape connector with a hole for inserting the sprayer will facilitate the procedure and reduce vector leak from the animal.
12. Raising the head by 20 cm will facilitate a more distal deposition of the injected solution.
13. Lung adhesions at the site of surgical manipulation is frequent in these animal models. This may limit the integrity of certain regions of the lung parenchyma.
14. Cryosectioning of the lung OCT block may be challenging especially when incomplete airway insufflation is present. As an alternative, prior fixation in 10% formalin can be used. In formalin-fixed paraffin blocks, deparaffination and the antigen retrieval step with sodium citrate pH 6.0 may yield good immunostaining results for many of the antibodies.
15. Due to cellular turnover, the gene expression signal detected using different techniques may fade overtime.

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