High-Efficiency, Long-Term Cardiac Expression of Foreign Genes in Living Mouse Embryos and Neonates

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Background—The development of improved strategies for efficient and reproducible in vivo gene transfer into the murine heart will ultimately allow the intersection of somatic and germline gene transfer strategies to study complex features of cardiac biology and diseases.

Methods and Results—For embryonic gene transfer, an adenovirus vector expressing β-galactosidase was injected in utero into the ventricular cavity of living embryos via microsurgical approaches. The injected embryos were developed to term, and efficient expression of the transgene was detected in all cell types in the heart. For postnatal cardiac gene transfer, adeno virus was injected into the cardiac ventricle of neonatal mice, resulting in efficient expression of the transgene in the outer layer of the myocardium as well as cardiomyocytes in the middle and inner layers of the cardiac wall. Mice examined after 3 weeks displayed a pattern of expression that completely mimicked the pattern seen after 3 days, and gene expression was also found after 6 months. The infected myocytes can be identified by coinfection of an adenovirus expressing green fluorescent protein without affecting their normal physiological function.

Conclusions—We have developed a new strategy to achieve efficient and long-term foreign gene expression in both embryonic and postnatal mouse myocardium via direct intracardiac injection of recombinant adenovirus. The strategy should allow the functional assessment of the expression of dominantly acting exogenous genes, overexpression of wild-type genes, and Cre recombinase–mediated gene ablations at the single-cell level in the context of the intact adult mouse myocardium. (Circulation. 2000;101:178-184.)

Key Words: myocytes ■ viruses ■ proteins ■ myocardium

The development of improved strategies for efficient and reproducible in vivo gene transfer into the murine heart will ultimately allow the intersection of somatic and germline gene transfer strategies to study complex features of cardiac biology and diseases. The mouse is of particular interest, because this species can be precisely genetically engineered and murine cardiac function can be examined at both the organ and the single-cell levels (for overview see Reference 1). High-efficiency, long-term expression in the embryonic, neonatal, and adult mouse heart could be particularly valuable as a strategy for complementation of loss-of-function phenotypes in the growing list of gene-targeted mouse models with developmental defects. In this regard, recombinant adenoviruses have several inherent advantages as gene delivery vectors for cardiac muscle, including high infectivity of cultured neonatal and adult cardiac myocytes with a preservation of relatively normal biological function of the infected cells. As opposed to retroviral vectors, adenoviral vectors do not depend on integration into chromosomal DNA, and they effectively infect nondividing cells. Although the adenoviruses have been used for the expression of foreign genes in porcine, dog, rabbit, and rat models, high-efficiency, tissue-restricted somatic gene transfer to the embryonic and postnatal mouse heart in vivo has not yet been reported. In previous studies, postnatal gene transfer to the myocardium has been complicated by a subsequent inflammatory reaction, with only transient or very-low-efficiency gene expression.

In the present study, we describe an in utero microsurgical approach for direct injection of recombinant adenoviruses into the ventricular cavity of living mouse embryos and a protocol for reproducible and efficient transfection to the postnatal mouse myocardium with long-term gene expression and absence of inflammatory reaction. Our results document the potential to examine the function of any given gene in mouse cardiomyocytes through in vivo gene transfer.

Methods

Construction of Recombinant Adenoviral Vectors

The adenovirus encoding β-galactosidase (β-gal) was generated by use of the methods of Graham and Prevec. The recombinant adenovirus encoding β-gal was injected in utero into the ventricular cavity of living embryos and a protocol for reproducible and efficient transfection to the postnatal mouse myocardium with long-term gene expression and absence of inflammatory reaction. Our results document the potential to examine the function of any given gene in mouse cardiomyocytes through in vivo gene transfer.

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Adenovirus was obtained by cotransfection of the resulting shuttle plasmid DNA and pM17 plasmid DNA in 293 cells, as previously described. Similarly, the adenovirus expressing green fluorescent protein (GFP) was generated by use of cDNAs from pEGFP (Clontech Laboratories, Inc) as reported.

**Examination of Whole Embryos With β-Gal Assay**

Assays for β-gal of embryonic tissue were performed by a variation of standard techniques. Briefly, whole embryos were dissected such that communications were made throughout multiple body cavities, including the pleural, pericardial, intraventricular, and peritoneal spaces. The embryos were quickly rinsed twice in ice-cold PBS, permeabilized, and stained with X-gal. Paraffin sections were photographed under dark-field microscopy with a Nikon Optiphot-2.

**Examination of Isolated Neonatal Cardiomyocytes After In Vivo Cotransfection With β-Gal and GFP**

The cells were plated overnight on laminated glass coverslips with etched grids and lettering (Bellco Glass Inc). Localization of the cardiomyocytes expressing GFP was recorded. Subsequently, the cardiomyocytes were fixed and incubated in X-gal solution. The percentage of cardiomyocytes previously noted as green, which also expressed β-gal, was calculated.

**Isolation and Functional Analysis of Infected Adult Cardiomyocytes**

For isolation of rod-shaped cardiomyocytes, the heart was excised 3 weeks after infection. The aorta was cannulated under a microscope while submerged and was mounted in a modified Langendorff perfusion system fitted with heating coils. The heart was perfused at 37°C for 5 minutes at 2 mL/min with a modified Joklik’s minimum essential medium. Subsequently, 150 U/mL of type II collagenase (Worthington) was added, and perfusion continued for 20 minutes. The ventricles were cut and gently triturated. After filtration, cells were resuspended in modified Joklik’s medium, and calcium concentration was increased to 1 mmol/L.

For functional measurements, isolated myocytes were transferred to a temperature-controlled perfusion chamber (HCB-101, Crescent Electronics) located on the stage of an inverted microscope (Nikon Diaphot TMD). Cells expressing GFP were identified by use of a fluorescent light source mounted on the microscope. Images of contracting myocytes were obtained with a CCD camera (FTM 800 NH/PH, Philips). Shortening and relaxation were measured in GFP-negative (n=12) and GFP-positive (n=8) cells with a video edge-detection system (Crescent Electronics).

**Statistics**

Data are presented as mean±SEM. Unpaired 2-tailed t test was performed to assess differences between 2 groups. A value of $P<0.05$ was considered statistically significant.

**Results**

**Development of Microsurgical Technology for the Delivery of Recombinant Adenoviruses Via Direct Intracardiac Chamber Injection in Living Murine Embryos**

In utero manipulations were developed on the basis of methods described by Muneoka et al. A midline laparotomy was performed on pregnant dams at 10 days postcoitum, taking care to stay on the linea alba and away from the inferior epigastric vessels (Figure 2a). Then bilateral, full-length antimesenteric hysterotomies were performed to expose the embryos (Figures 2b and 3b). The 2 embryos closest to the cervix on each side were removed (Figure 3c), and the uterus was loosely closed. The abdomen was then flooded with warm lactated Ringer’s solution (Baxter), and the distal uterus containing the remaining embryos was positioned in the right lateral decubitus position with sterile, saline-soaked cotton fragments.

Crafted pulled-glass micropipettes (World Precision Instruments) were developed with a model 730 pipette puller (David Kopf) and a model MF-79 microforge (Narishige). A needle with a $10\times20-\mu$m side hole (Figure 3a) was found to give the best results. Under submerison, a loosely placed 10-0 monofilament purse-string suture was placed through the membranes incorporating both the yolk sac and amnion. Through a small tear in the membranes (Figure 2c), the needle containing the viral solution could be introduced into the ventricular cavity of the embryo. A retrograde flash of...
blood in the barrel of the needle confirmed the right position before the viral solution was injected. The preplaced purse-string suture was gently approximated (Figure 2d). Hemostasis was a critical portion of the procedure. The abdomen was closed with a running suture, and the mother was allowed to recover. Embryos were delivered either by cesarean section or by maternal euthanasia and use of a foster mother.

Efficient Transduction of the Embryonic Murine Myocardium With Adenovirus

A recombinant adenovirus encoding β-gal was injected into the ventricular cavity of midgestational embryos. Embryos were allowed to develop for 24 hours before death. When $10^7$ to $10^8$ pfu of viral particles were used, we were able to effectively transduce the full thickness of the myocardium (Figure 4a). All cell types in the heart could be effectively transduced (Figure 4b), as determined by light microscopy. The success of the in utero viral transduction approach was confirmed in a series of 54 consecutive embryonic manipulations, resulting in an overall survival of 73%.

Efficient Gene Transfer of the Day 1 Neonatal Mouse Myocardium

A viral solution containing $10^9$ particles of adenovirus encoding β-gal was injected into cardiac ventricles of mouse neonates from the first (n=6), third (n=6), and fifth days (n=6) after birth. The entire outer layer of the myocardium expressed the transduced gene when the viral solution was injected the first day after birth (Figure 5a). Injection at a later postnatal period (day 3 to 5) resulted in a much less efficient expression of the transgene (Figure 5b).

Sections of hearts injected the first day after birth (n=6) were examined 3 days after administration of virus, documenting transgene expression in the nuclei of the cardiomyocytes (Figure 6a). The infection was most efficient in the outer layer of the myocardium, but Figure 6a shows that cardiomyocytes in the trabecular network and the middle and inner layers of the myocardium were also infected. The cardiomyocytes in all parts of the atrial myocardium were efficiently infected, as demonstrated in Figure 6b. No inflammatory cells were found in any of the sections from the atria or ventricles examined after staining with hematoxylin and eosin (Figure 7). The regions of myocardial sections used to examine any inflammatory response were those with highest efficiency of β-gal expression.

The percentage of cardiomyocytes expressing β-gal was estimated by counting positively stained cardiomyocytes isolated from hearts of 6 animals. In some animals, 27% of the cardiomyocytes expressed β-gal. The average percentage of cells expressing the gene was 10.9 ± 3.5%. Little expression of β-gal was found in other organs, with the exception of the liver, because staining with X-gal showed numerous cells positive for β-gal in the liver (Figure 8).

Long-Term Gene Expression Is Achieved When Adenovirus Is Administered in 1-Day-Old Neonatal Mice

To examine the persistence of gene expression, hearts from groups of animals were examined 3 days, 3 weeks, 6 weeks, and 6 months after injection of adenoviral constructs the first day after birth. Figure 6c shows highly efficient gene expression in atrial cardiomyocytes 3 weeks after injection. The pattern of β-gal expression was virtually identical to that found in animals examined after 3 days (Figure 6b). A similar pattern of expression was also found 6 weeks after injection of the virus. When examined after 6 months, the β-gal was still expressed in the infected regions, and there was no evidence of an inflammatory reaction.
Cotransfection and Identification of Viable Infected Cardiac Myocytes

The ability to coinfect cardiomyocytes in vivo with a second virus was examined by intracavitary injection of a mixture of adenovirus encoding GFP and adenovirus encoding β-gal in a group of 6 neonatal mice. In Figure 9a, the bright-field image shows a cardiomyocyte isolated 3 days after adenovirus injection. A cell located on an F on the coverslip expresses GFP, as shown in the dark field under a fluorescent source (Figure 9b). After fixation and staining with X-gal solution, the same cell is blue (Figure 9c), indicating that the expression of β-gal resulted from successful in vivo cotransfection. When cardiomyocytes from all 6 hearts in the group were examined, 93% of the cells expressing GFP also expressed β-gal, suggesting a high fidelity of cotransfection.

Isolation, Identification, and Physiological Assessment of Viable Rod-Shaped Mouse Cardiomyocytes Infected by Adenovirus Expressing GFP

Successfully infected cells were identified by the fluorescent signal at 450 nm (Figure 10). Shortening and relaxation were measured in GFP-negative (n=12) and GFP-positive (n=8) cells. In the adult noninfected mouse myocytes, a shortening of 9.35±0.94% and a shortening duration of 469±29 ms were found. These functional parameters were not different from data obtained in infected cells, which were 9.84±1.46% and 467±34 ms, respectively.

Discussion

In this study, we report a microsurgical technique that will allow us to achieve highly efficient transgene expression in developing mouse embryos by use of recombinant adenovirus vectors. We have also presented a highly efficient and reproducible protocol to achieve in vivo delivery of adenoviral constructs by use of a micromanipulator system. In addition, coinfection with adenoviral constructs harboring GFP allowed identification of isolated adult rod-shaped cardiomyocytes for functional assessment at the single-cell level.

Although previous attempts have been made to transduce the whole embryonic heart in nonmammalian models, high-efficiency and spatially restricted transduction of the mammalian heart has not been established. In mammals, adenoviral vectors have been delivered via intra-amniotic injection to target the pulmonary epithelium for therapy of cystic fibrosis. Transgene expression was found in the proximal airways, but no expression was observed in cardiac tissue. A recent study used intraplacental delivery of recombinant adenoviruses in mice and found that a little less than 20% of the ventricular cardiomyocytes expressed the transgene. However, intraplacental delivery leads to substantial expression of the marker gene in noncardiovascular tissue. In our study, in which the virus was delivered directly to the
heart, little expression of the marker gene was observed in other organs. No inflammatory reaction was observed.

It is intriguing that in vivo administration of adenoviral constructs the first day after birth has significant advantages compared with later delivery. Efficiency, reproducibility, and duration of gene expression all were substantially improved when 1-day-old neonatal mice were used. The virus was most likely trapped in the highly developed neonatal trabecular network of the ventricles and atria as well as within the pericardium. The cells in the midmyocardium, however, were most likely infected through the coronary arteries. In one previous study,13 neonatal mice were injected intravenously with 10⁹ pfu recombinant virus encoding β-gal. However, only ≈0.2% of cardiac cells had undergone gene transfer. In another study performed in neonatal rats,14 adenovirus was injected into the thoracic cavity. Expression of the reporter gene choramphenicol acetyltransferase was found in the heart, but the highest levels were in the lungs and diaphragm, with a substantial degree of variation between animals. The authors explain their findings by the relatively imprecise nature of the thoracic injection. In comparison, the percentage of cardiomyocytes expressing β-gal from the injected neonatal heart averaged ≈11% but could be up to 27% of the cardiomyocytes. Intrathoracic injection performed in neonatal mice in our laboratory gave much lower efficiency and higher lethality than intraventricular injection (G.C., Y.W., and K.R.C., unpublished observation, 1997). Using the cytomegalovirus promoter, we were able to compare the expression of β-gal in several organs after injection into the left ventricle. Very little expression of β-gal was found in the lungs and other remote organs, except the liver. It should be noted, however, that our approach for gene transfer into the embryonic and postnatal heart will be even more powerful when cardiac-specific promoters are used. In adult mice,15 direct

Figure 6. Representative sections of heart from mouse injected with AdCMVlacZ first day after birth. a, Trabecular network and inner layer of myocardium excised, sectioned, and stained 3 days after injection. b, Section from atrium of same heart. c, Sustained strong expression of β-gal in section from atrium obtained 3 weeks after injection.

Figure 7. Representative hematoxylin-eosin–stained sections from hearts obtained 3 days (cross section, left), 3 weeks (atrial trabeculum, middle), and 6 weeks (left ventricle, right) after injection of adenovirus encoding β-gal. No inflammatory cells were observed.
intramyocardial injection has been performed. However, the limited spatial extent of transfection and possible damage of cells surrounding the needle track makes that approach, in our opinion, much less appealing than successfully performed intracavitary injection.

Our protocol for delivery of adenovirus to neonates is highly reproducible with regard to gene transfer efficiency, and a high number of animals can be injected within a relatively short time period. For anesthesia, we used rapid cooling, which is possible because of the small body mass in neonates and the lack of thermoregulatory capabilities. By lowering the body temperature, we were able to temporarily arrest the heart, which prolonged the exposure of the heart to the virus. Injection into the minute cardiac chambers of the neonatal hearts required crafted pulled-glass micropipettes mounted on a micromanipulator system. Successful placement of the pipette within the ventricular cavity was achieved in >90% of the animals. Each animal could be anesthetized and injected in <10 minutes.

We found that the expression of the transfected gene was stable for several months after administration of adenovirus to the neonatal mice. Previous studies in adult rats and rabbits have shown only transient expression, and in most studies, adenovirus injection was accompanied by substantial inflammatory reaction. The mechanism for the reduction in gene expression in those studies is not known, but it has been attributed to intracellular degradation of the viral genome, a direct lytic effect of the virus on the cells, inactivation of gene transcription, or immune response against the transduced cells. We speculate that absence of immune response in neonatal mice is the main reason for the long-term expression in these animals. It has been shown that in neonatal mice, B-cell and T-cell responses are impoverished compared with those in adults. These reduced responses are the result of deficient accessory cell numbers and function. Adenovirus-mediated gene expression can be greatly prolonged in nude mice compared with that in immune-competent mice. The long-term stability of the transgene expression and the absence of inflammatory reaction allow physiological studies on cardiac myocytes from adult mice.

Identification of viable, infected cardiomyocytes was easy and reliable with coinjection of adenovirus expressing GFP and adenovirus expressing β-gal. Successful cotransfection of cardiomyocytes in vivo has not been reported previously but has been shown in vitro. By coinjecting virus encoding GFP and a gene of interest, the transfected cardiomyocytes can therefore be
identified and single-cell physiology can be examined. In our study, we showed the feasibility of this approach by measuring physiological function in adult mouse cardiomyocytes isolated 3 weeks after administration of virus encoding GFP. No functional changes were found in GFP-infected cells, which is important for future use of this approach.

Recently, mouse models of cardiac developmental defects,21 cardiac hypertrophy,22–24 and dilated cardiomyopathy25 have been developed by overexpression or deletion of specific genes. Our in vivo strategy for gene transfer into the mouse myocardium will allow examination of the ability to rescue such cardiac phenotypes by introduction of genes with subsequent assessment of function at the single-cell level. Because several of these mouse models develop the disordered phenotype around birth25 or after overload,26 and associated increase in wall stress,26 gene delivery in the late embryonic or early postnatal phase would be expected to rescue the cellular phenotype. Gene transfer by use of our protocols may also partly rescue global function of a disordered phenotype, because a recent study in adult rats27 showed altered cardiac function, although only patchy expression of phospholamban was found and inflammatory reaction occurred. Our strategy also allows genetic targeting of the heart prenatally (P.J.G.; Ross, MD; O’Gorman, PhD; and K.R.C., unpublished observations, 1996) and postnatally and (Chien and K.R.C., unpublished observations, 1997) with temporal and spatial control by adenovirus-mediated delivery of Cre recombinase to mouse engineered floxed alleles.3,15,28,29

In conclusion, we have shown temporally and spatially restricted efficient in utero gene transfer to living mouse embryonic and postnatal hearts. Viable myocytes can be detected from coinfection with adenoviral constructs expressing GFP and analyzed for physiological function at the single-cell level. Our approaches provide a useful strategy to couple somatic and germline gene transfer and modification to explore the functional role of calcium cycling genes in cardiac development and diseases.30

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