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A comparison of intravenous and indirect intracoronary delivery of AAV5, AAV6 and AAV9 for cardiac gene transfer in mice.

A Thesis submitted in partial satisfaction of the requirement for the degree Master of Science

in

Biology

by

Hongfei Fang

Committee in charge:

H. Kirk Hammond, Chair
Dong-Er Zhang, Co-Chair
Lakshmi Chilukuri
Ngai Chin Lai
David M. Roth

2011

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University of California, San Diego

2011

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ABSTRACT OF THE THESIS

A comparison of intravenous and indirect intracoronary delivery of AAV5, AAV6 and AAV9 for cardiac gene transfer in mice

by

Hongfei Fang

Masters of Science in Biology

University of California, San Diego, 2011

Cardiac gene transfer is a potentially useful treatment for patients with heart disease. The adeno-associated virus (AAV) is a frequently used vector in preclinical gene transfer, but the optimal AAV serotype and route of delivery has not been established. I examined the relative efficacy of three AAV serotypes (AAV5, AAV6, AAV9) and two vector delivery methods (indirect intracoronary, intravenous) to

determine the combination that would provide the highest level of cardiac transgene expression in mice. Using enhanced green fluorescent protein (EGFP) as a reporter to quantify transgene expression, AAV5.EGFP, AAV6.EGFP, and AAV9.EGFP [5 x 10^{11} genome copies (gc)] were delivered via indirect intracoronary or intravenous injection. Three weeks later hearts were removed and EGFP expression was quantified using fluorescent microscopy and immunoblotting. AAV DNA copy number was measured by quantitative PCR. Within each serotype, indirect intracoronary delivery was superior to intravenous delivery. Indirect intracoronary delivery of AAV9 provided the highest level of cardiac gene expression. This vector and delivery method should be used in preclinical studies in which a high level of transgene expression is required.

1. Introduction

Cardiovascular disease affects millions of subjects and is the leading cause of death in the United States (Heron et al., 2009). Congestive heart failure (CHF) affects over 5 million subjects in the US, and is the only cardiovascular disease that is increasing in prevalence. Current treatment for CHF includes the use of β-adrenergic receptor antagonists, angiotensin converting enzyme inhibitors, angiotensin receptor antagonists, and aldosterone antagonists. However, despite these pharmacological therapies and other measures, severe CHF is associated with 50% mortality 3-4 years after diagnosis. Thus cardiac gene transfer is being tested as a possible treatment for heart failure, to address this unmet medical need.

Cardiac gene transfer requires three elements: 1) a gene of interest that will accomplish a desired effect; 2) a vector to express the transgene; 3) and a method to safely and effectively deliver the vector.

1.1 Selection of Transgene

Gene transfer for CHF requires a gene to increase cardiac function. This could be accomplished using an angiogenic gene to increase blood flow, or by using a gene to increase contractile function directly. In paracrine-based gene transfer, a transgene

encodes a protein that is released from target cells, allowing it to interact with multiple cells throughout the body. Paracrine-based gene transfer enables attaining an effective treatment with less efficient gene transfer, on balance, compared with non-paracrine-based gene transfer. Angiogenic genes, such as fibroblast growth factor-4 and hepatocyte growth factor, have beneficial effects in animal models of heart failure (Gao et al., 2004; Jayasankar et al., 2003). In non-paracrine gene transfer, the extent of cardiac gene transfer is more important, as effectiveness will reflect the amount of cardiac transgenic expression in the heart. Adenylyl cyclase type 6 is a non-paracrine protein whose expression has beneficial effects on the failing heart via increasing cardiac contractility (Lai et al., 2004; Roth et al., 2004). In all instances, the extent of cardiac gene transfer is dependent on the choice of vector and delivery method.

1.2 Selection of Vector

The three vectors currently used in cardiac gene transfer include lentivirus, adenovirus, and adeno-associated virus (AAV) (Edelstein et al., 2007). Because cardiac gene transfer involves the infection of terminally differentiated cardiac myocytes, lentivirus is the only suitable retrovirus vector that can be used. Lentivirus provides long-term gene expression, a consequence of integration of the transgene into the host genome. The drawback of this is the risk of insertional mutagenesis. Indeed, the use of a retrovirus vector to treat subjects with X-linked severe combined immunodeficiency resulted in acute lymphoblastic leukemia in two of the nine

subjects treated (Bonnetta, 2002). Lentivirus cannot be delivered effectively to cardiac myocytes via vascular delivery (Fleury et al., 2003). Therefore, its only application would be direct intramuscular injection into the wall of the heart, a method which is difficult to apply in clinical settings. Adenovirus and AAV vectors are able to transfect non-dividing cells such as cardiac myocytes. Adenovirus vectors, in contrast to retroviruses, provide episomal transgene expression. Although this trait limits the vector's potential for long-term expression, it eliminates the risk of insertional mutagenesis. However, virus proteins encoded by adenovirus vectors are recognized as foreign antigens by the host immune system and, depending on route of delivery and targeted organ, can incite an inflammatory response. A clinical study that used very high doses of adenovirus through hepatic artery infusion to treat partial ornithine transcarbamylase deficiency resulted in a patient's death, possibly from an overwhelming inflammatory response (Raper et al., 2003). AAV is a DNA parvovirus that has appealing attributes for gene transfer due to its non-pathogenic nature and its ability to provide long term expression (Kaplitt et al., 1996). Recombinant AAV vectors are created by the replacement of the wild type *rep* and *cap* genes with a gene of interest between the two inverted terminal repeats (ITRs) flanking the virus genome (Figure 1). AAV has been used for gene transfer in lung (Limberis et al., 2009), liver (Favaro et al., 2011), brain (Taymans et al., 2007), and heart (Chu et al., 2003; Kaspar et al., 2005; Zincarelli et al., 2008). The drawbacks of AAV include its limited packaging space (~5 kb) (Wu et al., 2010), relatively inefficient gene transfer with vascular delivery, and arduous manufacturing requirements.

1.3 Delivery Method

The route of delivery of the vector is critically important. The primary impediment to success in cardiovascular gene therapy is in obtaining sufficiently high cardiac transgene expression to provide a beneficial biological effect. For cardiac gene transfer, the choice of virus vector often dictates the delivery method. Lentivirus vectors, because of their inability to cross endothelial cells and gain access to the cardiac interstitium (Fleury et al., 2003), cannot be used effectively by intravascular delivery. Though effective, direct intramyocardial injection, which could be used with lentivirus, adenovirus, or AAV, provides limited cardiac transgene expression, evident only adjacent to the needle tract and, in addition, induces a dose-dependent inflammatory response. Three effective delivery methods for cardiac gene transfer, all vascular-based, include direct intracoronary, indirect intracoronary, and intravenous. Indirect intracoronary delivery is required in mice because the coronary arteries are too small to enable direct catheter insertion for intracoronary infusion. Indirect intracoronary delivery involves cross-clamping the aorta and pulmonary artery and delivery of the vector into the left ventricular (LV) chamber with subsequent indirect delivery into the coronary arteries. This method has been used to deliver adenovirus and AAV vectors in mice and rats. Co-administration of pharmacological agents such as histamine (Lai et al., 2000; Kaspar et al., 2005), nitroprusside (Lai et al., 2004; Roth et al., 2004), sildenafil (Donahue et al., 2000), and substance P (Iwanaga et al., 2004) increase gene transfer efficiency with adenovirus and perhaps AAV vectors. Unlike

adenovirus, systemic venous delivery of AAV vectors (especially AAV6, AAV8, and AAV9) have been used with some success in cardiac gene transfer (Inagaki et al., 2006; Miyagi et al., 2008; Pacak et al., 2006; Zincarelli et al., 2008). AAV5, which provides substantial gene transfer following indirect intracoronary delivery, is less efficient when delivered intravenously (Wang et al., 2005; Palomeque et al., 2007; Zincarelli et al., 2008). However, it has a theoretical advantage in that pre-existing AAV antibodies directed against AAV5 are less common in human subjects than those against AAV8 and AAV9 (Boutin et al., 2010).

1.4 Study Rationale

A direct comparison of indirect intracoronary and intravenous delivery of AAV vis-à-vis cardiac gene transfer efficiency has not been performed, but is of pivotal importance to advance the field. Intravenous delivery has the advantage of being simple to apply, but its subsequent diffuse delivery to off-target tissues presents a potential problem and requires higher doses to attain a given level of cardiac transgene expression. Indirect intracoronary delivery enables longer exposure of virus vectors to the heart and is a viable method to achieve efficient cardiac gene transfer (Roth et al., 2004; Kaspar et al., 2005). However, it requires invasive methods that may be cumbersome to apply clinically. Establishing which of these two methods provides optimal cardiac transgene expression is important for preclinical research.

The goal of my work was to compare indirect intracoronary versus intravenous delivery using three AAV vectors (AAV5, AAV6, AAV9).

Previous methods to assess cardiac gene transfer efficiency have measured proportion of heart exhibiting fluorescence using reporter genes such as EGFP (Kaspar et al., 2004; Bish et al., 2008; Prasad et al., 2011). However, this method does not take into account the intensity of EGFP fluorescence and therefore does not accurately measure the extent of gene transfer. In the present study, I have quantified the level of AAV-mediated EGFP fluorescence in the heart by measuring the product of the area and EGFP fluorescent intensity. My hypothesis was that indirect intracoronary delivery of AAV will provide higher levels of cardiac gene expression compared to systemic intravenous injection, regardless of AAV serotype, and that indirect intracoronary delivery of AAV9 would provide the highest level of cardiac transgene expression.

2. Materials and Methods

2.1 Animals

Housing and treatment of animals followed institutional and NIH guidelines, and protocols that were approved by the VA San Diego Healthcare System. Eighty-two 10-12 week old male C57/BL6 J mice (27 ± 3 g) (Jackson Laboratories) were used in these experiments. Mice not injected with AAV vectors were used as controls.

2.2 Vector Production

Self-complementary AAV (scAAV) vectors used in this work were manufactured by Dr. Atsushi Miyanohara at the University of California San Diego Vector Core. Construction of scAAV vectors required that a 20 base-pair deletion be induced in the terminal resolution site in one of the two ITRs of the AAV plasmid (McCarty et al., 2003; Wang et al., 2003; Miyanohara et al., 2005). A CMV-promoter-EGFP-SV40PolyA cassette was isolated by digestion of pEGFP-N1 (Clontech, CA) with Asel-Aflll. The resulting fragment was inserted between the two ITRs of the pAAV backbone to form a new plasmid designated pscAAV-CMF-EGFP (Figure 1).

To purify scAAV vectors, either plasmid pRep2-Cap5, pRep2-Cap6, or pRep2-Cap9 and pAd-Helper plasmid (Xiao et al., 1998) were transiently transfected with HEK293T cells. Plasmids pRep2-Cap5 and pRep2-Cap6 were constructed by

subcloning each Cap gene from pXYZ5 obtained from Dr. Snyder, U. Florida (Zolotukhin et al., 2002) or pDF6 from Dr. Grimm, Stanford U (Grimm et al., 2003). The pRep2-Cap9 plasmid was obtained from Dr. James M. Wilson, U. Penn. Seventy-two hours after transfection, cell lysates were prepared and treated with benzonase. Viruses were then pelleted through 25% sucrose-cushion ultracentrifugation. The resuspended viruses were purified via anion-exchange column chromatography (Q-Sepharose, GE Health Science) (Zolotukhin et al., 2002; Gao et al., 2000) and then concentrated by 25% sucrose-cushion ultracentrifugation. Virus pellets were resuspended in 10mM Tris-HCl (pH 7.9, 1 mM $MgCl_2$, 3% sucrose). Virus genome copies (gc) were quantified using real-time quantitative PCR with virus genome DNA prepared from the purified virus preparations.

2.3 Intravenous Injection

Spontaneously breathing mice were anesthetized (1.5% isoflurane); an incision made at the neck, and the jugular vein was isolated. A 31-gauge needle and syringe containing $5x10^{11}$ gc of AAV5.EGFP, AAV6.EGFP, or AAV9.EGFP (50 μl total volume of each serotype) was injected into the jugular vein. The needle was maintained for 10 seconds after injection to ensure vector delivery. Visual inspection confirmed that the content was delivered. The wound was then sutured with 7-0 Prolene and mice were assessed for gene transfer and expression three weeks later.

2.4 Indirect Intracoronary Injection

Mice underwent endotracheal intubation and subsequently received 1% isoflurane mixed with 100% oxygen for sustained anesthesia. A re-circulating water pad was used to attain hypothermia (24°C, rectal). A thoracotomy was performed between the second and third rib space and the proximal aorta and pulmonary artery were isolated and occluded using a vascular clamp (Roth et al., 2004). A 31-gauge needle and syringe containing serotonin (0.2 μg), PBS, and $5x10^{11}$ gc of AAV.EGFP (100 μL total volume of each serotype) was injected into the LV cavity. The clamp was released 60 seconds after occlusion and the wound was closed with 7-0 Prolene. Mice recovered in their cages on a re-circulating water pad (37°C) and were assessed for gene transfer and expression three weeks later.

2.5 Quantification by Fluorescence

Three weeks after gene transfer, mice were anesthetized (5% isofluorane) using a nose cone and reflexes were checked to determine depth of anesthesia. The chest was then opened and the heart arrested with 1M KCl (35 μl) administered directly into the LV cavity. The heart was removed and the LV free wall separated from the interventricular septum (IVS). The LV free wall and IVS were placed separately between two glass slides. Grey-scale images of the LV free wall and IVS were digitally obtained by a Cool Snap Pro camera at a 10-second exposure time via a

Nikon Eclipse TS100 microscope (20x magnification). The LV epicardium, LV endocardium, and RV and LV sides of the IVS were assessed. Background intensity was obtained from control animals that were not injected with AAV. EGFP fluorescence was quantified using ImagePro Plus analysis software (Media Cybernetics). EGFP fluorescent intensity-area product was determined by measuring the average EGFP fluorescent intensity per surface area of tissue and multiplying this value by the surface area that showed EGFP fluorescence.

$$\frac{\textbf{Flour. Intensity}}{\textbf{Surface area } (\mu m^2)} \times \textbf{Area Fluor. } (\mu m^2) = \textbf{Fluor. Intensity-area product}$$

2.6 Quantification by Immunoblotting

In addition to the evaluation of fluorescent intensity, immunoblotting was used to quantify EGFP protein expression. LV free wall and IVS from each mouse were homogenized. In addition, liver samples (25 mg, left lobe) from each mouse were homogenized for qPCR and immunoblotting.

Samples were placed in 1.5 ml microcentrifuge tubes (Eppendorf) and submerged in liquid nitrogen. Subsequently samples were homogenized with homogenizing buffer (25mM Tris-HCl, 0.5mM EDTA, 0.5mM EGTA, pH 7.4). Samples were diluted (2 μg/μl) and analyzed with a 12.5% SDS-polyacrylamide gel

using 1° rabbit EGFP antibody followed by a 2° goat anti-rabbit IgG horse radish peroxidase antibody. Membranes were then exposed (5 minutes) to X-ray film and protein concentrations were quantified from the ratio of the image optical density (IOD) between the EGFP and GAPDH control bands.

2.7 Quantification of AAV DNA

To analyze vector copy number, LV free wall, IVS, and liver homogenates underwent DNA extraction using a DNeasy tissue kit (Qiagen DNeasy), following the protocol provided by the manufacturer. DNA concentration was determined by Nanodrop spectrophotometry, with 125 ng of DNA used as a template per reaction for quantitative real-time PCR. The primers were complementary to the CMV promoter in all AAV vectors; forward primer, 5'- GGCATTATGCCCAGACATGAC-3'; reverse primer, 5'-CACCATGGTAATAGCG-3'. Real time qPCR was performed by the UCSD Genomic Core. Data are reported as AAV gc per ng total genomic DNA.

2.8 Statistical Analysis

Student's t-test (unpaired, two-tailed) was used to detect group differences between intravenous and indirect intracoronary delivery by serotype (GraphPad Prism 5). A 2 x 3 ANOVA was performed to determine the optimal serotype and delivery method among the 6 groups tested (GB-STAT 8.0). Linear correlation analysis was performed to compare EGFP fluorescence and against EGFP by immunoblotting in the

left ventricle (GraphPad Prism 5). A statistical test between related correlation coefficients was used to determine whether assessment of fluorescent intensity-area was superior to the area of fluorescence alone (Meng et al., 1992).

3. Results

3.1 Indirect Intracoronary versus Intravenous Delivery

3.1.1. EGFP Fluorescence

Cardiac transgene fluorescence was evaluated three weeks after indirect intracoronary or intravenous delivery for each of the 3 vectors. Both methods of injection showed variable fluorescence in myocardium (Figures 2-4). Indirect IC delivery was associated with regions of high intensity fluorescence. In contrast, IV delivery was associated with diffuse and homogenous low intensity fluorescence. With both delivery methods, fluorescence patterns were similar in LV free wall and IVS. For all serotypes, indirect IC delivery yielded higher levels of fluorescence compared to IV delivery (Table 1). Indirect IC delivery of AAV5 yielded a 240-fold higher level of fluorescent intensity versus intravenous delivery. The superiority of indirect intracoronary over intravenous delivery was also evident for AAV6 (24-fold) and AAV9 (3-fold). Of the six different groups of serotype and delivery methods, indirect intracoronary delivery of AAV9 provided the highest level of EGFP fluorescent intensity (Table 2, Figure 7A**)**. The superior EGFP fluorescence seen with indirect IC delivery was confirmed by immunoblotting and quantitative PCR (see below).

3.1.2. Immunoblotting

LV free wall and IVS samples underwent immunoblotting to assess EGFP expression (Figure 5). Indirect IC delivery was associated with increased levels of EGFP protein versus intravenous delivery with all AAV serotypes (Table 1). Fold increases (indirect IC versus intravenous) were: AAV5: 28-fold; AAV6: 22-fold; AAV9: 3-fold. When comparing all serotypes and delivery methods, indirect IC delivery of AAV9 provided the highest levels of EGFP (Table 2, Figure 7B).

A test between correlation coefficients was performed to determine whether the use of the intensity-area product to measure EGFP expression was superior to traditional methods, which measure the area of fluorescence alone. In this test, the differences between two dependent correlations from a single sample, EGFP content versus EGFP fluorescent intensity-area product (Figure 9A**)** and EGFP content versus % area EGFP fluorescence (Figure 9B), were compared against the correlation of EGFP fluorescent intensity-area product versus % area EGFP fluorescence. The test showed that the measure of fluorescent intensity-area product was the superior method of assessing EGFP transgene expression in the heart ($p < 0.0001$).

3.1.3. Quantitative PCR Analysis

To determine whether increased EGFP expression and fluorescent intensity were associated with increased AAV vector DNA amounts, qPCR was performed. Indirect

IC injection consistently provided higher levels of AAV DNA than intravenous delivery (Table 1). The fold differences (indirect IC versus IV) were: AAV5: 154-fold; AAV6: 42-fold; AAV9: 3-fold. When comparing across all serotypes and methods, indirect IC delivery of AAV5 provided the highest AAV DNA levels (Table 2, Figure 7C).

3.2 Analysis of AAV Gene Transfer in Liver

Immunoblotting of liver samples revealed no difference between indirect IC and IV delivery within the same serotype (data not shown). However, AAV9, whether delivered by indirect IC or IV injection, showed the highest levels of EGFP expression in the liver (Figure 8A). AAV DNA amounts in liver showed a similar pattern, with no difference between indirect IC and IV delivery within the same serotype (data not shown), but varying levels of copies between different serotypes. AAV9, delivered by indirect IC or IV injection, provided the highest AAV genome copies in the liver, followed by AAV5 and AAV6 (Figure 8B).

4. Discussion

Identifying the optimal vector and delivery method for cardiac gene transfer is critically important for both preclinical and clinical applications. The purpose of this study was to compare the efficiency of three commonly used AAV vectors using two delivery methods. My hypothesis was that indirect intracoronary delivery would provide higher levels of cardiac transgene expression than would intravenous delivery, regardless of serotype. The most important findings of these experiments were: 1) indirect IC delivery of all three vectors (AAV5, AAV6, AAV9) showed higher levels of cardiac gene transfer compared to intravenous delivery of the same vectors (Figure 6); 2) the optimal vector and delivery method was indirect intracoronary injection of AAV9; and 3) the evaluation of extent of gene transfer using an area-intensity product is superior to traditional methods.

Differences in the distribution of gene expression as determined by fluorescent microscopy were observed between the two delivery methods. For example, intravenous delivery of AAV9 provided homogenous but low intensity fluorescence throughout the myocardium, but indirect intracoronary delivery was associated with reduced homogeneity but greater fluorescent intensity. This observation was taken into consideration when levels of gene transfer were quantified. Traditional methods of EGFP fluorescence expressed data as a percentage of the LV showing fluorescence (Kaspar et al., 2004; Bish et al., 2008; Prasad et al., 2011). This method does not take into account the intensity of the transgene signal and therefore provides an inadequate

assessment. Indeed, although the area of fluorescence is substantial after IV delivery, the fluorescent intensity is quite low (Figures 2-4). My approach, for the first time, took into account both the area showing EGFP fluorescence as well the intensity of the signal and showed the superiority of indirect IC over IV delivery. If one's goal is to obtain the highest possible level of LV expression with the lowest possible dose of AAV, our data indicate that indirect IC delivery should be selected over IV delivery.

My results confirm that intravenous delivery of AAV9 is superior to IV delivery of AAV5 and AAV6. Other groups have reported extensive cardiac gene transfer using IV delivery of AAV6 only after co-administration of vascular endothelial growth factor (Gregorevic et al., 2004), which increases microvascular permeability (Roberts et al., 1995). I did not use this method because of the high cost of recombinant VEGF and because I wanted to compare AAV vectors and delivery methods directly without confounding factors.

Although indirect IC and IV delivery provided different levels of cardiac gene transfer, this same pattern was not seen in the liver, where similar levels were seen regardless of route. AAV9 provided the highest level of EGFP expression and AAV DNA copies in liver. I assessed AAV DNA and transgene expression in liver for two reasons. First, it was important to evaluate expression in an organ associated with AAV tropism. Second, IV delivery of AAV vectors and subsequent liver transgene expression might be exploited to assess AAV vectors for paracrine-based cardiac gene

transfer. In this application, the transgene is released into the blood and has beneficial cardiac effects. The liver's large size and AAV tropism make it a suitable target for paracrine-based gene therapy. High levels of AAV gene transfer in the liver has been previously described (Zincarelli et al., 2008; Nathwani et al., 2011).

Indirect IC delivery of AAV5 provided substantial levels of cardiac gene transfer, which differs from some reports (Zincarelli et al., 2010). These discrepancies may reflect differing methodologies used between this study and previous ones (cross clamping, hypothermia to increase dwell time, serotonin). Indirect IC delivery of AAV5 was associated with higher vector genome copies than AAV6 or AAV9, but provided somewhat lower levels of fluorescent intensity and EGFP expression (Table 2). I speculate that AAV5 may undergo different intracellular trafficking, with consequent variation in nuclear entry. AAV2 vectors enter murine NIH 3T3 fibroblasts in vitro but are less efficient in entering the nucleus in order to promote gene expression (Hansen et al., 2000). Even so, indirect IC delivery of AAV5 provided superior levels of cardiac gene transfer than did IV delivery of any AAV vector.

4.1 Implications

I have shown that indirect IC delivery of AAV vectors provides significantly higher levels of cardiac transgene expression than IV delivery. This finding is

important for researchers using gene transfer in animal models of human diseases. It also provides important data for translation to clinical studies. Namely, IV delivery of AAV vectors is likely to be inferior to indirect IC delivery vis-à-vis obtaining the highest degree of cardiac transgene expression per AAV amount delivered. Translation of these findings to clinical applications would require an approach that preserves intracoronary delivery but avoids aortic and pulmonary artery cross-clamping, which would be impractical in clinical settings. One solution would be to limit AAV-mediated cardiac gene transfer to those subjects undergoing coronary artery bypass graft surgery or valve replacement - procedures that require cardiopulmonary bypass, in which the heart is arrested and perfused by an independent circulation for 1-3 hours. This would enable continuous bathing of the heart alone with AAV, providing ample dwell time to enable maximal cardiac gene transfer. Indeed, previous studies in sheep using a similar system have shown substantial AAV-mediated cardiac gene transfer (White et al., 2011).

4.2 Conclusions

In conclusion, indirect intracoronary delivery of AAV serotypes provides superior cardiac transgene expression compared to intravenous delivery regardless of AAV serotype. Moreover, the fluorescent intensity-area product provides a more accurate measurement of the extent of gene transfer compared to methods assessing

fluorescent area alone. In settings where the maximal extent of cardiac gene transfer is desired, indirect intracoronary delivery of AAV9 is the optimal approach

5. Appendix

Table 1: Data entries denote mean±SE. Probability values from Student's t-test are for group differences between indirect intracoronary (IC) and intravenous (IV) delivery by serotype.

Table 1. Indirect Intracoronary vs. Intravenous Delivery by Serotype				
Serotype	**Analysis**	**Delivery Method**		**p**
		IC	**IV**	
AAV5	**Fluorescent Intensity / 10^6**	81 ± 16 (n = 14)	0.34 ± .03 (n = 12)	0.0001
	Immunoblotting	1.1 ± 0.1 (n = 6)	0.04 ± 0.01 (n = 6)	< 0.0001
	AAV DNA (gc)	2000 ± 420 (n = 8)	13 ± 5 (n = 6)	< 0.002
AAV6	**Fluorescent Intensity / 10^6**	88 ± 20 (n = 12)	3.6 ± 0.2 (n = 13)	0.0003
	Immunoblotting	1.4 ± 0.1 (n = 6)	0.07 ± 0.01 (n = 6)	< 0.0001
	AAV DNA (gc)	106 ± 32 (n = 6)	3 ± 0.5 (n = 7)	< 0.005
AAV9	**Fluorescent Intensity / 10^6**	160 ± 16 (n = 14)	63 ± 6.9 (n = 13)	< 0.0001
	Immunoblotting	2.2 ± 0.3 (n = 6)	0.9 ± 0.1 (n = 6)	< 0.002
	AAV DNA (gc)	143 ± 14 (n = 8)	51 ± 9 (n = 7)	< 0.005

Table 2: Data entries denote mean±SE. Probability values from 3x2 ANOVA followed by Dunn's Bonferroni Correction for post-hoc analysis. IC, indirect intracoronary; IV, intravenous

Table 2. Optimal AAV Serotype & Delivery Method by Method of Assessment					
Analysis	**Serotype**	**Delivery Method**		**Optimal Vector / Method**	**p**
		IC	**IV**		
Fluorescent Intensity / 10^6	**AAV5**	81 ± 16 (n = 14)	0.34 ± .03 (n = 12)	AAV9 IC	< 0.01
	AAV6	88 ± 20 (n = 12)	3.6 ± 0.2 (n = 13)		
	AAV9	160 ± 16 (n = 14)	63 ± 6.9 (n = 13)		
Immunoblotting	**AAV5**	1.1 ± 0.1 (n = 6)	0.04 ± 0.01 (n = 6)	AAV9 IC	< 0.05
	AAV6	1.4 ± 0.1 (n = 6)	0.07 ± 0.01 (n = 6)		
	AAV9	2.2 ± 0.3 (n = 6)	0.9 ± 0.1 (n = 6)		
AAV DNA (gc)	**AAV5**	2000 ± 420 (n = 8)	13 ± 5 (n = 6)	AAV5 IC	< 0.01
	AAV6	106 ± 32 (n = 6)	3 ± 0.5 (n = 7)		
	AAV9	143 ± 14 (n = 8)	51 ± 9 (n = 7)		

Figure 1: Structure of the pscAAV.CMV.EGFP. Enhanced green fluorescent protein (EGFP) was placed under control of the immediate early gene human cytomegalovirus promoter (CMV) with a polyadenylation termination signal from the human β-globin gene (Poly A) flanked by inverted terminal repeats (ITR).

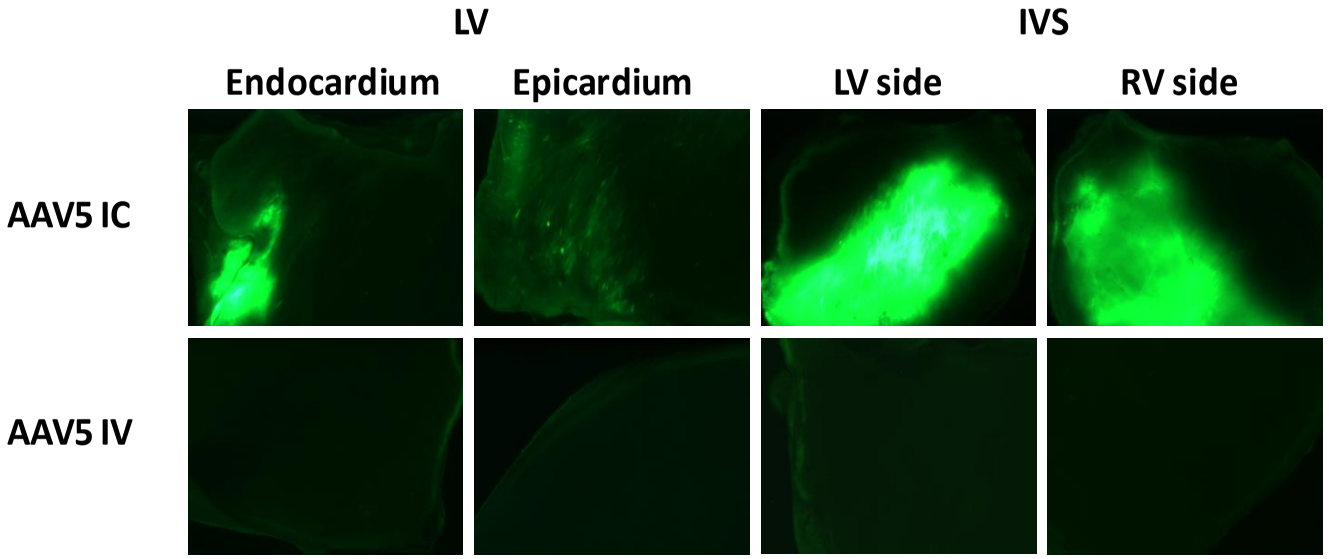

Figure 2: AAV5-mediated cardiac gene transfer using indirect intracoronary (IC) or intravenous (IV) delivery of AAV5.EGFP (5×10^{11} gc). Images were obtained three weeks after delivery. Fluorescence was detected in LV free wall and interventricular septum (IVS), and showed greater intensity with IC vs. IV delivery.

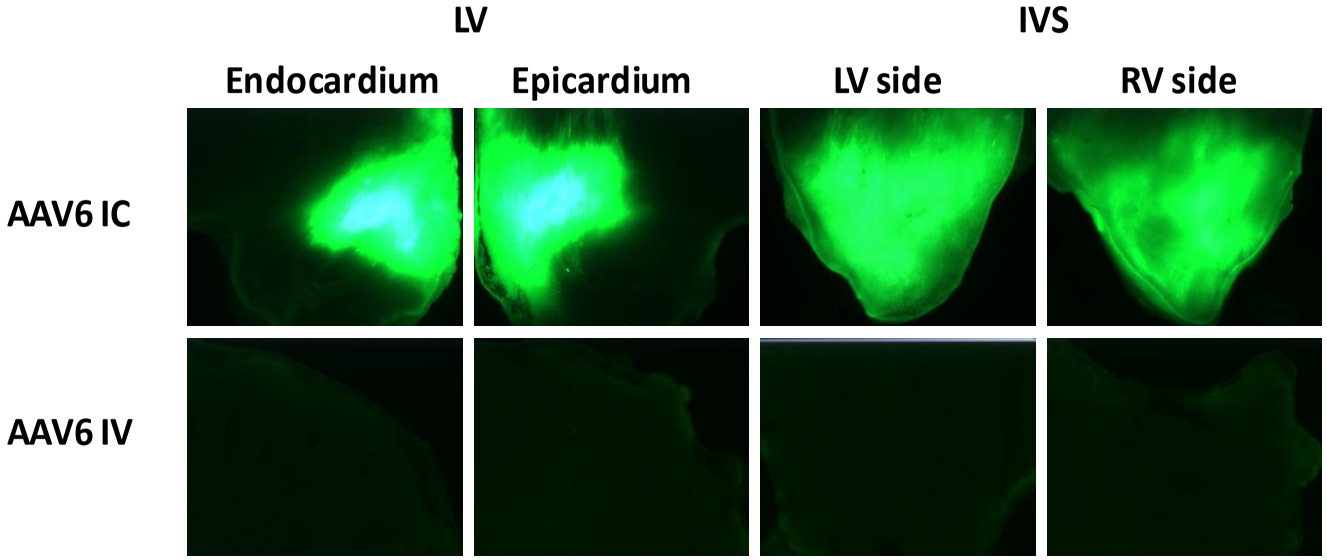

Figure 3: AAV6-mediated cardiac gene transfer using indirect intracoronary (IC) or intravenous (IV) delivery of AAV6.EGFP (5×10^{11} gc). Images were obtained three weeks after delivery. Fluorescence was detected in LV free wall and interventricular septum (IVS), and showed greater intensity with IC vs. IV delivery.

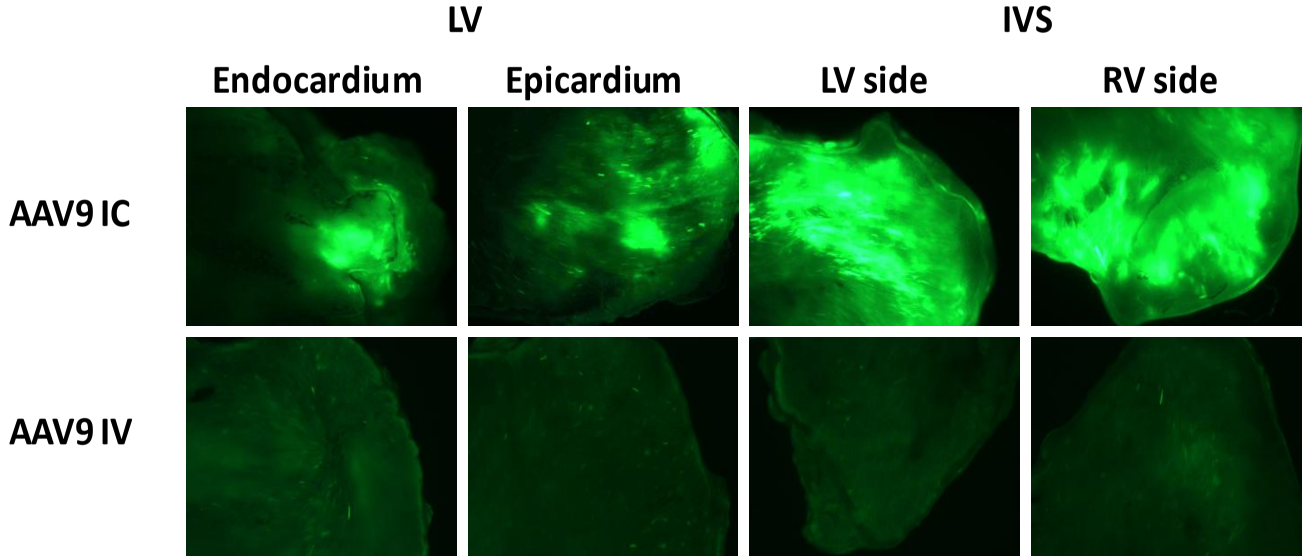

Figure 4: AAV9-mediated cardiac gene transfer using indirect intracoronary (IC) or intravenous (IV) delivery of AAV9.EGFP (5×10^{11} gc). Images were obtained three weeks after delivery. Fluorescence was detected in LV free wall and interventricular septum (IVS), and showed greater intensity with IC vs. IV delivery.

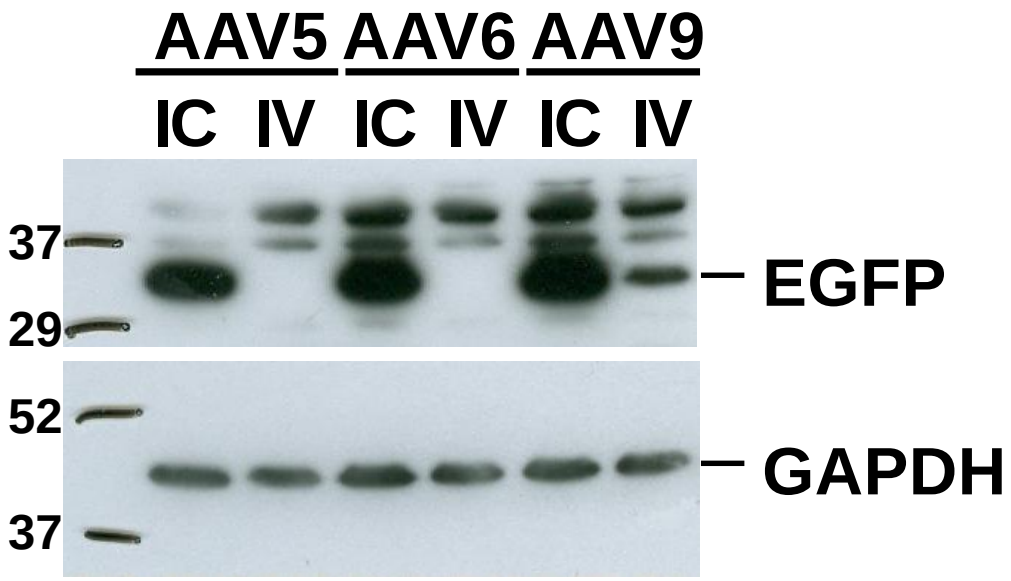

Figure 5: Immunoblotting to assess EGFP content in LV homogenates from mice three weeks after indirect intracoronary (IC) or intravenous (IV) delivery of AAV.EGFP (5×10^{11} gc). GAPDH was used to normalize data for protein loading. LV EGFP content was greater with IC vs. IV delivery in all serotypes.

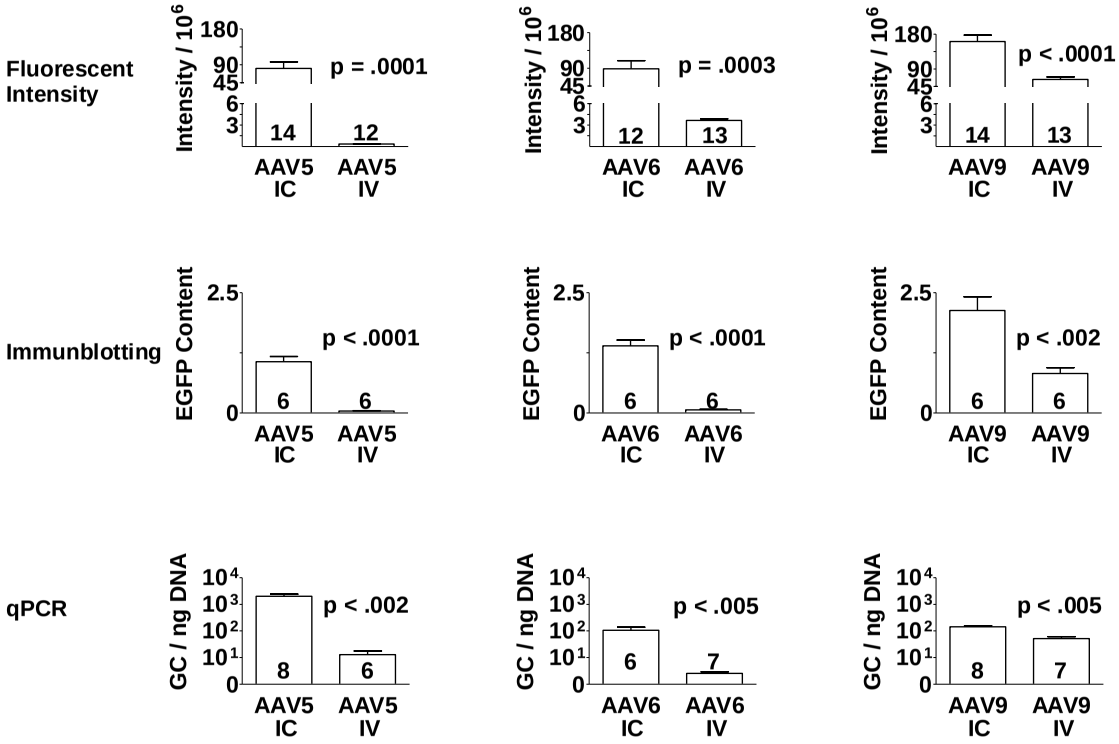

Figure 6: Analysis of AAV serotype and delivery method. Columns display results of indirect intracoronary (IC) vs. intravenous (IV) delivery (5×10^{11} gc) within a single AAV serotype. Rows display data from three methods of assessing efficiency of gene transfer. Fluorescence, EGFP content and AAV DNA copy number were higher with indirect IC vs. IV delivery in all three serotypes. Data are from entire heart, a combination of LV free wall and interventricular septum. Probability values from the Student's t-test (unpaired, 2-tailed). Error bars denote 1 SE; numbers in bars indicate group size

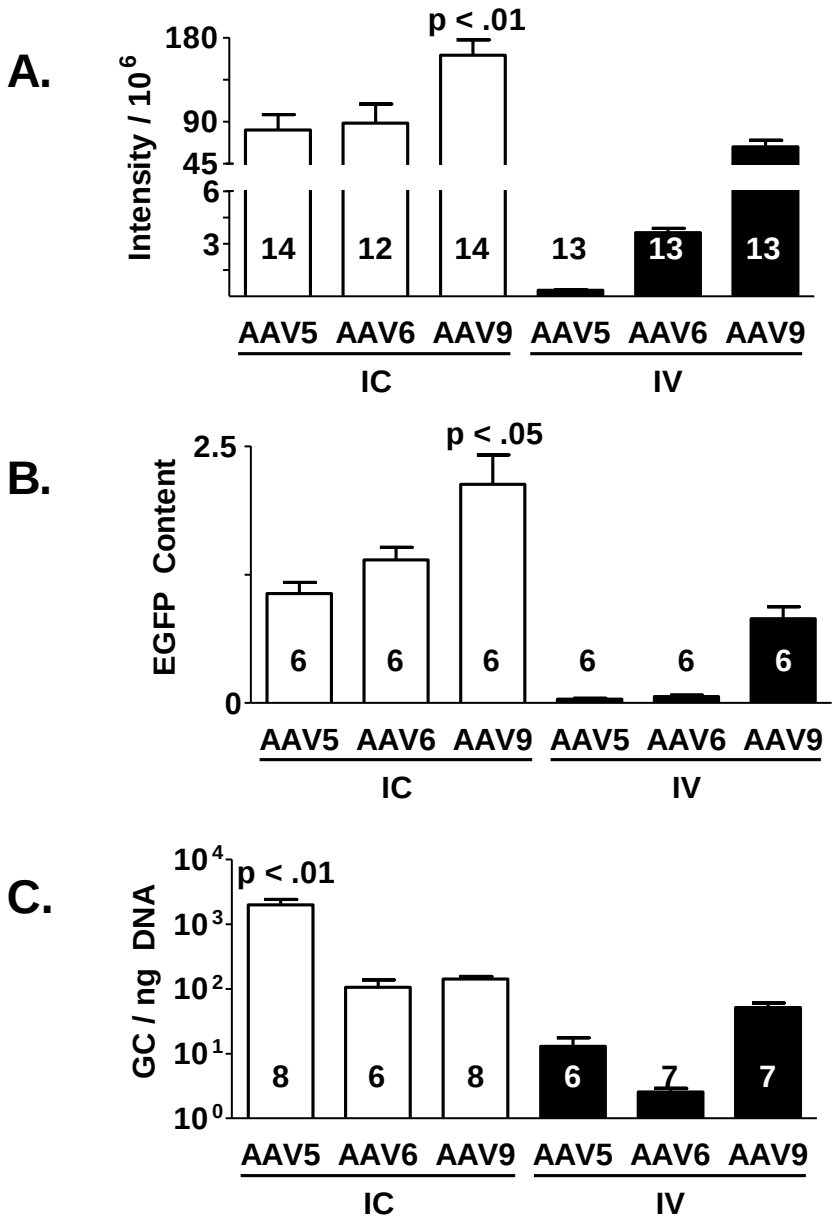

Figure 7: Optimal AAV serotype and delivery method. Analysis of cardiac gene transfer three weeks after indirect intracoronary (IC) or intravenous (IV) delivery of AAV.EGFP (5×10^{11} gc). Indirect IC delivery of AAV9 shows the highest levels of LV fluorescent intensity ($p < 0.01$; **A)** and EGFP content ($p < 0.05$; **B**). **C.** Indirect IC delivery of AAV5 shows the highest LV AAV copy number ($p < 0.01$). Probability values from ANOVA (3x2), followed by Dunn's Bonferroni Correction for post-hoc analysis. Error bars denote 1 SE; numbers in bars indicate group size.

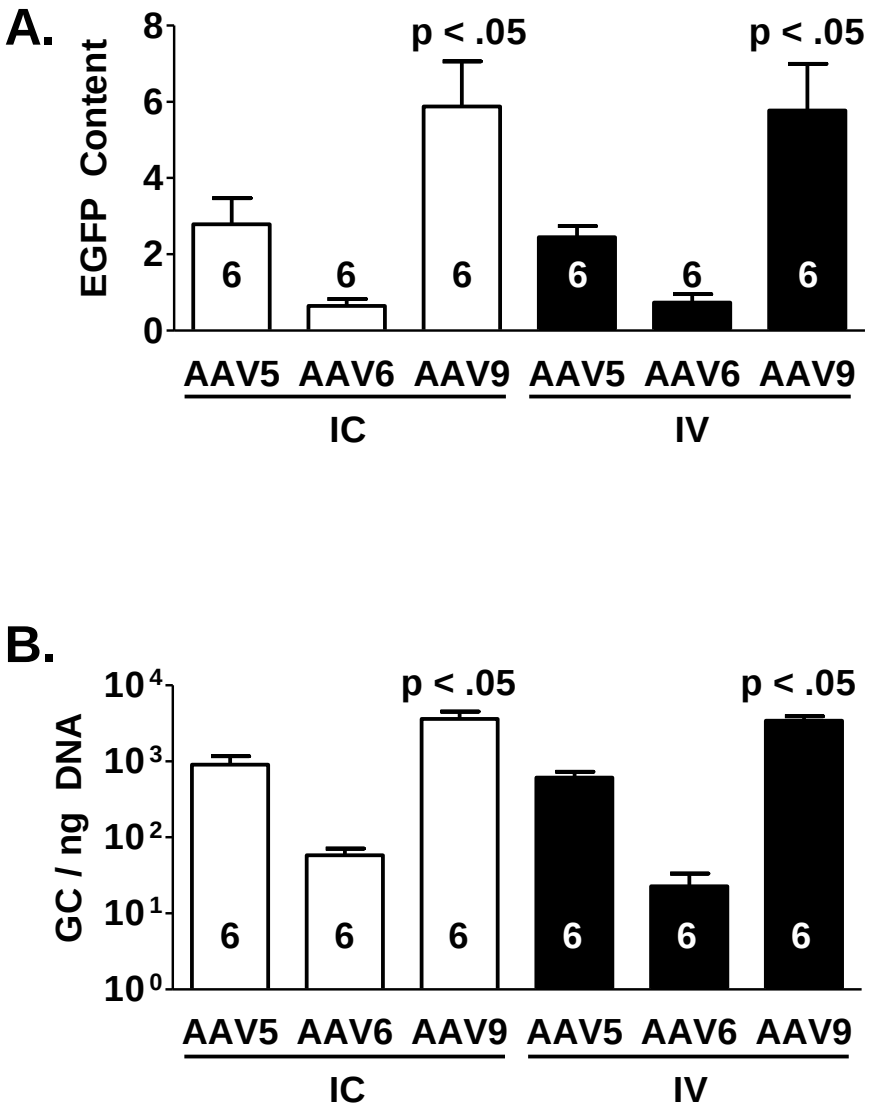

Figure 8: Analysis of liver gene transfer three weeks after indirect intracoronary (IC) or intravenous (IV) delivery of AAV.EGFP (5×10^{11} gc). **A**. Immunoblotting showed that AAV9 provided the highest levels of EGFP expression, with no difference between delivery methods for any serotype. **B.** Quantitative PCR showed that AAV9 provided the highest levels of AAV genome copies, with no difference between delivery methods for any serotype. Probability values from ANOVA (3x2), followed by Dunn's Bonferroni Correction for post-hoc analysis. Error bars denote 1 SE; numbers in bars indicate group size

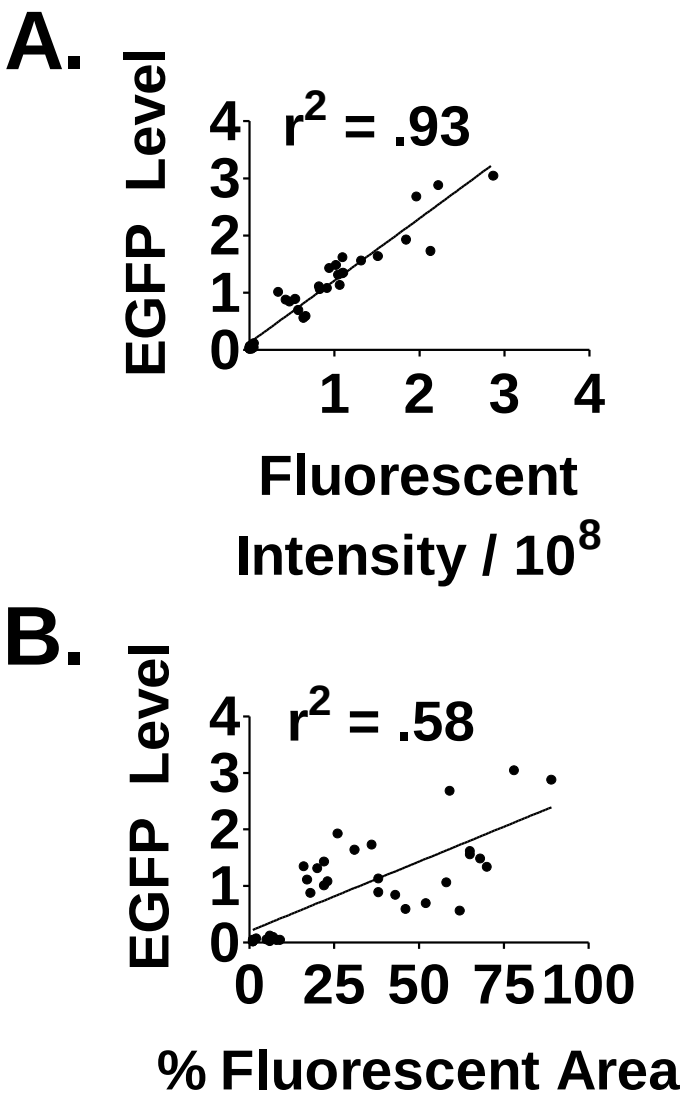

Figure 9: A. Correlation between fluorescent intensity-area product and EGFP content by immunoblotting. **B**. Correlation between percent of LV showing fluorescence and EGFP content by immunoblotting. A statistical test (Meng et al., 1992) confirmed that fluorescent intensity better correlates with EGFP content ($p<0.0001$) than does fluorescent area.

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