Background—We tested the hypothesis that prostacyclin synthase (PGIS) Transfer by heart specific Adeno-associated virus-8 (AAV-8) reduce heart infarct volume by augmenting synthesis of protective prostaglandins.

Method and Result-- In this study, we infused into lateral ventricle of a mice stroke model by recombinant AAV-8 containing PGIS (AAV-8- PGIS), or AAV-GFP control vector, and we determined PGIS protein and eicosanoid levels and infarct volume of the heart. PGIS proteins were increased in a time-dependent manner. AAV-8-PGIS infusion selectively augmented prostacyclin levels, with reduction of other eicosanoids in ischemic heart and a significant reduction of infarct volume. Infusion of AAV-8-PGIS also increased prostacyclin, suppressed leukotriene levels, and achieved a similar degree of heart protection. Its cardio-protection was abrogated by treatment with a selective PGIS inhibitor.

Conclusions—AAV-8-PGIS gene transfer reduce heart infarct volume by augmenting prostacyclin and suppressing leukotriene productions.

Introduction:

Ischemic myocardium results in an abrupt aggravation of cardiomyocyte injury, demonstrated experimentally by the re-introduction of oxygen into hypoxic myocardium(Hearse et al., 1975). This injury is due in part to the generation of reactive oxygen species (Bolli et al., 1988), and the injury is limited by antioxidants and free radical scavengers (Sarvazyan et al. 1995). Exposure of cardiac myocytes in culture to hypoxia is a convenient experimental system to examine some aspects of what occurs during myocardial ischemia. Evidence from studies of several in vitro systems and animals models has shown that cardiac myocyte apoptosis can be induced by stimuli sjch as hypoxia(Tanaka et al., 1994) and ischemia-reperfusion(Gottleib et al., 1994).

Medicine currently offer no effective treatment for muscular dystrophies, which afflict a significant population of children and adults worldwide. Genetherapy has great potential for the treatment of genetic muscle disease. A major challenge is how to deliver the therapeutic genes into most, if not all, of the deseased musceles. Delivery of gene vectors to local muscle of heart tissue has been achived by direct intramuscular (i.m) injection or by local blood vessel perfusion with both nonviral and viral vectors, including plasmid DNA, adenovirus and adeno-associated (AAV) vectors. Derived from a non-pathogenic human parvovirus, recombinant adeno-associated viral (rAAV) vectors are an alternative to rAd. Their small size and physical stability are advantageous for *in vivo* use, and transgene expression can persist in a wide range of tissues (Kaplitt et al., 1994). Moreover, there is no evidence of cell damage from inflammation after rAAV administration to the liver, skeletal

muscle, brain, and heart (Svensson et al., 1999;Xiao et al., 2000). rAAV vectors are being recognized as vectors for systemic and local long term delivery of gene therapy for clinical diseases (Flotte et al., 1996), yet their promiscuous tropism may lead to the undesirable expression of therapeutic genes in non-targeted cells. This limitation may be circumvented by the use of tissue-specific promoters. Li*et al.* (1999) used the muscle creatine kinase (MCK) promoter to specifically express human α-sarcoglycan in skeletal muscle using rAAV. In addition, liver-, brain-, cancer-, and rod-specific expression has been accomplished using the tissue-specific albumin, enolase, calcitonin, and rod opsin promoters, respectively. It has also been suggested that maintenance of endogenous source of PGI_2 is important in the limitation of coronary artery disease and infarct size (Luscher et al., 1993). PGI_2 is synthesized primarily in vascular endothelial and smooth muscle cells after appropriate stimulation by specific agents. Its biosynthesis is catalyzed by a series of enzymes: cytosolic phospholipase A2 cleaves arachidonic acid (AA) from the sn-2 position of phospholipids, cyclooxygenase (COX) converts AA to prostaglandin (PG) H_2, and PGI_2 synthase (PGIS) converts PGH_2 to PGI_2 (Wu et al., 1992). PGH_2 is a precursor of several biologically active prostanoids, including PGE_2, PGD_2, PGF_2, and thromboxane A2 (TXA2). PGI_2 possess cardioprotective effects in animal models of myocardial infarction. Administration of PGI_2 or its stable analogue iloprost limited infarct size in rabbits (Chiariello et al., 1988), rat (Muller et al., 1987) and dogs (Simpson et al., 1988).

In this study, we evaluated the effects of AAV-8-PGIS versus a control vector (AAV-8-GFP) on eicosanoid levels in ischemic heart and on infarct volume in a rat ischemic stroke model.

Materials & Methods:

Construction of plasmid and recombinant AAV

The linear, single-stranded AAV-derived vector can be adapted for several genes and promoters between the inverted terminal repeats (ITRs) at each end. We inserted a reporter gene, green fluorescent protein (GFP), and a rat 1.7 kb HPGK promoter. Methods to prepare recombinant AAV (rAAV) have been described previously(Phillips et al., 1996). The HPGK-GFP was packaged into AAV-8 (AAV8--GFP). The construct of PGIS use human phosphoglycerate kinase (PGK) promoters to drive PGIS, (AAV8-PGIS). Titers of virus stocks are determined by plaque titration on the 293 cell line, and are expressed in plaque-forming units (PFU). Virus stocks are aliquoted in small volumes and stored in phosphate-buffered saline (PBS) with 10% glycerol at -80°C until use. All experiments are carried out using aliquots of the same virus stocks.

Myocardial ischemia injury

Mice are anesthetized and placed on a ventilator. The left front chest is opened through thoractomy between the second and third intercostal spaces. An 5-0 prolene suture is passed and tied to produce coronary occlusion with tapered needle under the left anterior descending coronary artery 2–3 mm from the tip of the left auricle for 2 weeks.

Measurements of heart Tissue Eicosanoids by Enzyme Immunoassay

Heart was homogenized gently in 1 mL ice-cold buffer (0.05 mol/L Tris at pH 7.0, 0.1 mol/L NaCl, 0.02 mol/L EDTA) and centrifuged at 55 000*g* for 1 hour. The supernatant was acidified and passedn through a Sep-Pak C18 cartridge. Eicosanoids were eluted with 100% methanol, dried under nitrogen gas, redissolved in a small amount of buffer, and analyzed using enzyme immunoassay kits: PGE2, 6-keto-PGF1α, TXB2, and LTB4 kits from R&D System Ins and PGD2 and LTC4 kits from Cayman Chemical Co.

Determination of Infarct size

At the end of the ischemia period, 5 ml of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; 5% in phosphate buffered saline; 37 °C) is injected into the heart via a sidearm in the aortic cannula. The ischemic area (area at risk) of the myocardium is dilineated by the absence of blue staining. The heart is then weighed and frozen at –20°C overnight prior to the assessment of infarct size. While the hearts are still frozen, the ventricles are sliced into six to eight transverse sections, each approximately 2-3 mm thick, from apex to base. The right ventricle is then dissected from each slice and the left ventricular slices weighed. These slices are then incubated in triphenyltetrazolium chloride solution (2% in phosphate buffer; pH 7.4) in a water bath at 37°C for 15 min to identify infarcted tissue. Viable tissue within the area at risk is stained red (as a result of the dehydrogenase conversion of TTC to a red formazan), and infarcted tissue remained pale. Slices are rinsed and fixed in 10 % formalin for 24 h before being photographed on to slide film. Area at risk and infarct size are quantified by image analysis and corrected for weight, with area at risk expressed as a percentage of the left ventricle and infarct size as a percentage of the area at risk.

Western Blot Analysis

A total of 15 μg of cardiac homogenate lysate proteins are applied to each lane and analyzed by Western blots. antibodies are each diluted to 1:2000. Peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:2000 dilution) is used as the second antibody to detect PGIS, COX-1, COX-2, eNOS, iNOS and nNOS bands, respectively, by enhanced chemiluminescence (Amersham)

Measurement of Infarct Volume

The infarct volume in the left ventricle was measured as described.17 Mice were euthanized, and the heart was carefully removed, cooled in ice-cold saline for 5 minutes, and dissected coronally into 2-mm slices using a Jacobowitz brain slicer (Zivic- Miller), which were incubated in PBS (pH 7.4) containing 2% 2,3,5-triphenyltetrazolium chloride at 37°C for 30 minutes and then stored in 10% neutral-buffered formalin. The cross-sectional area of infarction in the left ventricle territory for each heart slice was measured with a Zeiss IBAS image analyzer.

Statistical Analysis

ANOVA was used to compare the temporal expression of infarct volume and levels of eicosanoid. The level of differences among groups was analyzed by Fisher's protected *t* tests (GB-STAT 5.0.4, Dynamic Microsystem Inc). *P*_0.05 was considered statistically significant.

Results:

Systemic gene transfer intravenously in adult mice

We want to investigated whether intravenous injection of AAV8 vectors in adult mice could achieve systemic gene delivery to heart. Two-month-old mice were injected with the AAV8 vector containing GFP via the tail vein at a dose2×10^{12}v.g.(6×1010v.g./g body weight). One month after i.v. injection, adult mice also showed strong GFP expression in the heart and to a lesser degree in skeletal muscles **(Fig.1)**

Intravenous Infusion of AAV8-PGIS Increased PGIS Protein and Prostanoid Levels in mice heart

To evaluate the efficiency of gene expression of AAV8-PGIS administration via intravenous route , we infused total 2×10^{12}v.g.(6×1010v.g./g body weight)of AAV8-PGIS into the tail vein of normal mices and determined PGIS protein levels 1 to 4 months after administration. Compared with AAV-GFP control, AAV8-PGIS augmented PGIS protein levels in a time-dependent manner **(Figure 2)**. The PGIS protein augmentation was noted at 0.5 months after administration and last to 4 months. Thus, intravenous infusion of AAV8-PGIS was efficacious in over-expression of PGIS.

AAV8-PGIS Gene Transfer Selectively Augmented Heart Prostacyclin Levels

We have recently shown in brain infusion experiments that concurrent COX-1 and PGIS expressions by using a bicistronic COX-1/PGIS vector selectively augmented PGI2 synthesis following brain ischemia/reperfusion (lin et al., 2002).To determine whether the AAV8-PGIS will selective prostacyclin increase occurred in heart of mice, we intravenously injected with AAV8-PGIS and measured eicosanoid levels in no ischemic or 24 hours after ischemia/reperfusion of heart. Compared with normal

heart intravenously injected with AAV-GFP control vectors, the AAV8-GFP transduced ischemic heart had a significantly higher level of PGE2 ($P<0.01$), PGD2 ($P<0.01$), TXB2 ($P<0.01$), LTB4 ($P<0.01$), and LTC4 ($P<0.01$) **(Figure 3 versus Figure 4)**. The eicosanoid levels in AAV transduced ischemic the 6-keto-PGF1α level in AAV8-PGIS–transduced ischemic brain was increased by 3-fold over that of AAV8-GFP control (Figure 4). In contrast, PGE2, PGD2, TXB2, LTB4, and LTC4 levels were all significantly reduced in AAV8-PGIS–transduced ischemic heart tissues **(Figure 4)**. These results are consistent with selective augmentation of PGI2 productions by shunting PGH2 into the PGIS pathway.

AAV8 Mediated Intravenous Infusion of PGIS Provides Long-Term Protection From I/R Injury and Reduces Infarct Size

The ischemia induced infarct volume was significantly lower in mices receiving AAV8-PGIS 2 months before ischemia than in mices receiving AAV-GFP under an identical experimental protocol **(Figure 5).**

Figure lagend

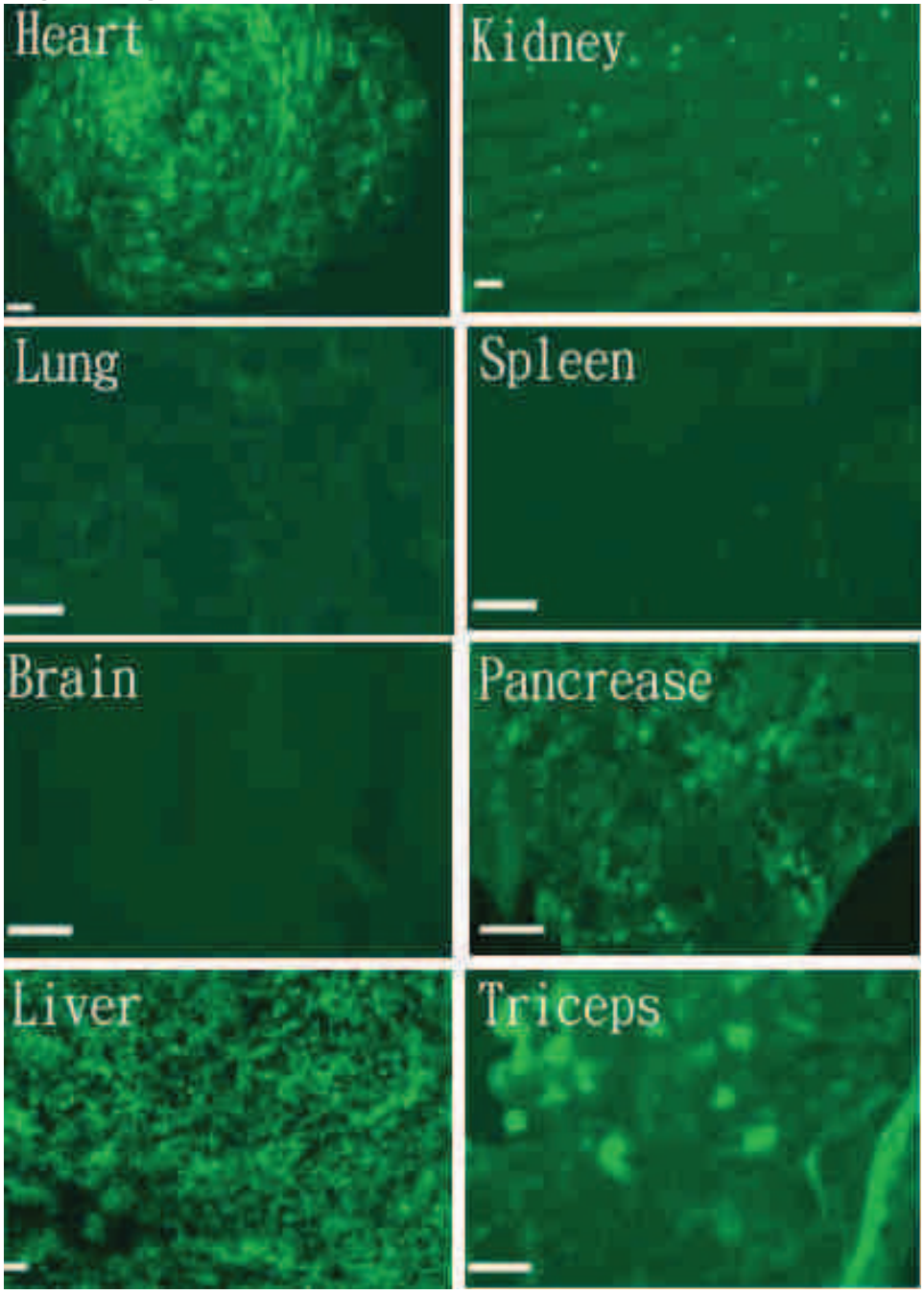

Figure 1. Systemic gene delivery to variable organ by intravenous(i.v.) injection in adult mice. Fluorescent microscopy of cryosection of different organ for GFP expression two months after tail vein injection of $2x10^{12}$v.g. of dsAAV8-GFP in 10-week-old adult mice.

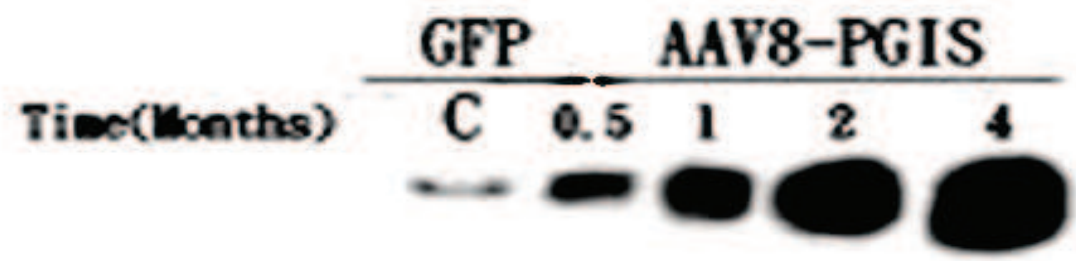

Figure 2. Western blot analysis of PGIS proteins in nonischemic mice heart tissues transduced with AAV vectors.

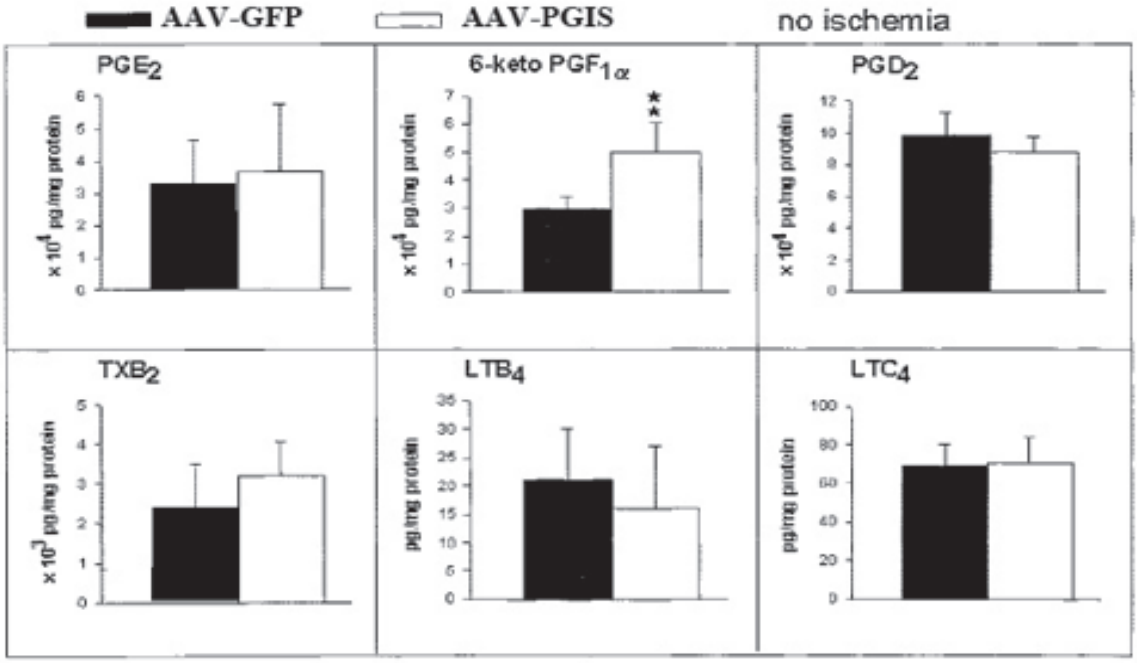

Figure 3. Eicosanoid levels in nonischemic heart tissues transduced by AAV8-PGI and AAV8-GFP as control. Bar is mean_SD of 3 experiments. *$P < 0.05$; **$P < 0.01$.

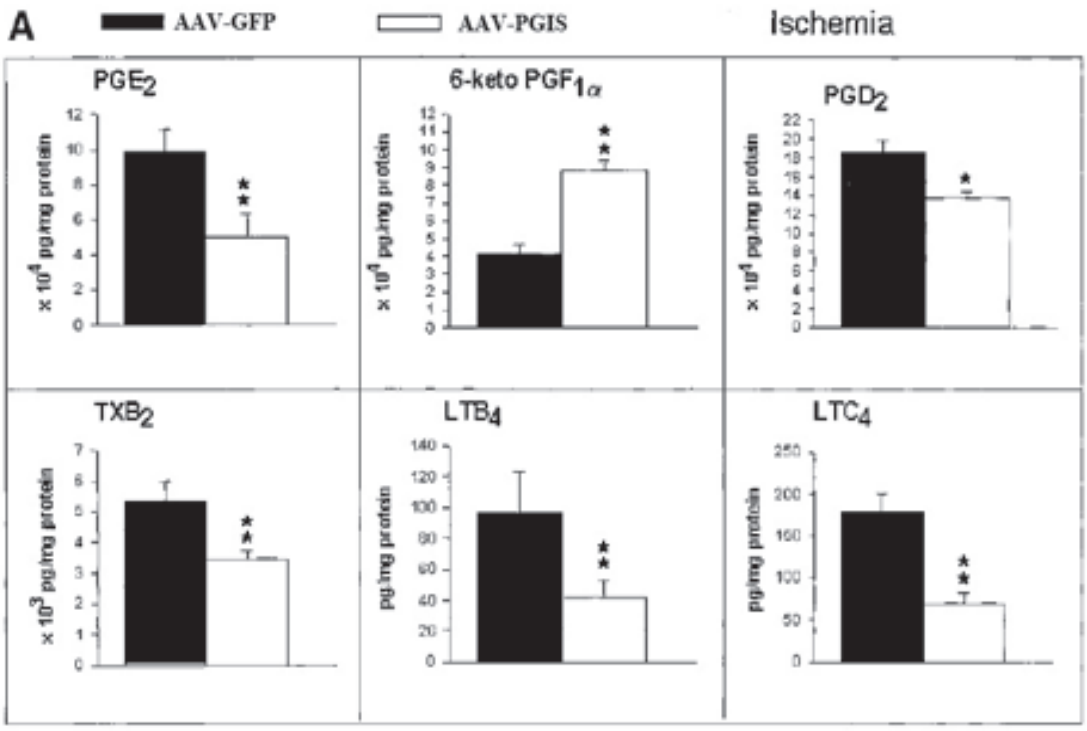

Figure 4. A, Eicosanoid levels in ischemic heart transduced with AAV8-PGIS or AAV8-GFP as control. AAV vectors were infused through tail vein of mices 2 months before ischemia, and eicosanoid levels in whole heart were measured by 24 hours after ischemia/reperfusion. Bar is mean_SD of 3 experiments. $^{**}P < 0.01$.

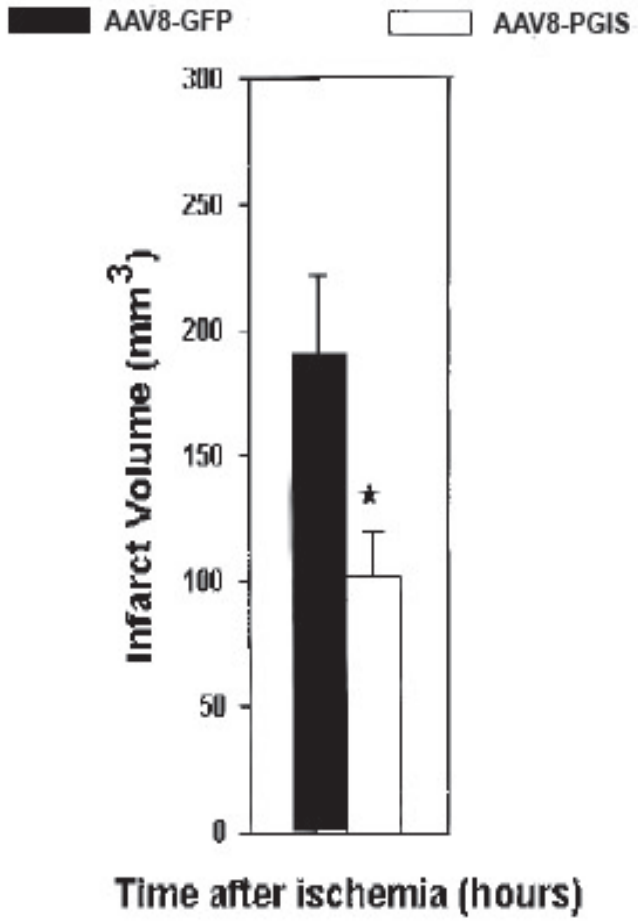

Figure 5. Heart infarct volume in mices treated with AAV8-PGIS or AAV8-GFP at 72 before ischemia/reperfusion. Bar is mean ±SD of 3 experiments. *$P < 0.05$ and **$P < 0.01$.

References

- Bolli R, Patel BS, Jeroudi MO, Lai EK, McCay PB (1988). J Clin Invest 82, 476-485.
- Chiariello M, Golino P, Cappelli-Bigazzi M, Ambrosio G, Tritto I, Salvatore M (1988). Am Heart J 115, 499-504.
- Flotte T, Carter B, Conrad C,Guggino W, Reynolds T, Rosenstein B, Taylor G, Walden S, Wetzel R.(1996). Hum Gene Ther. 1996 Jun 10;7(9):1145-59
- Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL (1994). J Clin Invest 94, 1621-1628.
- Hearse DJ, Humphrey SM, Nayler WG, Slade A, Border D (1975) J. Mol. Cell. Cardiol. 7, 315-324.

- Kaplitt MG, Xiao X, Samulski RJ, Li J, Ojamaa K, Klein IL, Makimura H, Kaplitt MJ, Strumpf PK, Diethrich EB (1996) Ann Thorac Surg 62, 1669-1676.
- Luscher TF, Tanner FC, Tschudi MR, Noll G (1993) e. Annu Rev Med 44, 395-418.
- Muller B, Schmidtke M.(1987). Adv Prostaglandin Thromboxane Leukot Res. 1987;17A:455-8
- Philip R, Clary B, Brunette E, Kilinski L, Murugesh D, Sorich M, Yau J, Lebkowski J, Lyerly HK, Philip M. (1996). Clin Cancer Res. an;2(1):59-68
- Sarvazyan NA, Askari A, Huang WH (1995) Life Sci. 1995;57(10):1003-10.
- Simpson PJ, Fantone JC, Mickelson JK, Gallagher KP, Lucchesi BR (1988). Circ Res 63, 1070-1079.
- Svensson EC, Marshall DJ, Woodard K, Lin H, Jiang F, Chu L, Leiden JM (1999). Circulation 99, 201-205
- Tanaka M, Ito H, Adachi S, Akimoto H, Nishikawa T, Kasajima T, Marumo F, Hiroe M.(1994). Circ Res. 1994 Sep;75(3):426-33.
- Wu KK, Kulmacz RJ, Wang L-H, et al. (1992). Amsterdam, the Netherlands: Elsevier Science Publishers BV:11–23.
- Xiao X, Li J, Tsao YP, Dressman D,Hoffman EP, Watchko JF.(2000). J Virol. 2000 eb;74(3):1436-42