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Cardiac Gene Transfer of Short Hairpin RNA Directed Against Phospholamban Effectively Knocks Down Gene Expression but Causes Cellular Toxicity in Canines

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Abstract

Derangements in calcium cycling have been described in failing hearts, and preclinical studies have suggested that therapies aimed at correcting this defect can lead to improvements in cardiac function and survival. One strategy to improve calcium cycling would be to inhibit phospholamban (PLB), the negative regulator of SERCA2a that is upregulated in failing hearts. The goal of this study was to evaluate the safety and efficacy of using adeno-associated virus (AAV)-mediated cardiac gene transfer of short hairpin RNA (shRNA) to knock down expression of PLB. Six dogs were treated with self-complementary AAV serotype 6 (scAAV6) expressing shRNA against PLB. Three control dogs were treated with empty AAV6 capsid, and two control dogs were treated with scAAV6 expressing dominant negative PLB. Vector was delivered via a percutaneously inserted cardiac injection catheter. PLB mRNA and protein expression were analyzed in three of six shRNA dogs between days 16 and 26. The other three shRNA dogs and five control dogs were monitored long-term to assess cardiac safety. PLB mRNA was reduced 16-fold, and PLB protein was reduced 5-fold, with treatment. Serum troponin elevation and depressed cardiac function were observed in the shRNA group only at 4 weeks. An enzyme-linked immunospot assay failed to detect any T cells reactive to AAV6 capsid in peripheral blood mononuclear cells, heart, or spleen. Microarray analysis revealed alterations in cardiac expression of several microRNAs with shRNA treatment. AAV6-mediated cardiac gene transfer of shRNA effectively knocks down PLB expression but is associated with severe cardiac toxicity. Toxicity may result from dysregulation of endogenous microRNA pathways.

Introduction

Heart failure (HF) is a major cause of morbidity and mortality, and transplantation is the only definitive cure for end-stage disease. Because the demand for donor organs is much higher than the supply, novel therapies are needed to augment current treatment modalities. It has been reported that defects in calcium handling secondary to dysregulation of the sarcoplasmic reticulum-associated proteins sarcoplasmic reticulum calcium ATPase 2a (SERCA2a) and phospholamban (PLB) are present in the failing hearts of humans and animals (Kaye *et al.*, 2008; Vinge *et al.*, 2008). Therefore, targeting these proteins in the failing heart may be a novel therapeutic option. Indeed, adeno-associated virus (AAV)-mediated over-expression of SERCA2a has been successfully used to restore mechanical and energetic function in failing rat hearts (Sakata *et al.*, 2007) and to reverse cardiac dysfunction in a porcine model of HF (Kawase *et al.*, 2008), and a clinical trial of

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AAV-mediated overexpression of SERCA2a in patients with New York Heart Association stage III/IV HF is in progress (Hajjar *et al.*, 2008; Jaski *et al.*, 2009). Other investigators have used virus-mediated expression of a dominant negative PLB (dnPLB) mutant to suppress HF progression in both small and large animal models of ischemic and nonischemic cardiomyopathy (Hoshijima *et al.*, 2002; Iwanaga *et al.*, 2004; Kaye *et al.*, 2007). No clinical trials using dnPLB have been initiated yet.

Another strategy to inhibit PLB would be to employ AAV-mediated cardiac delivery of short hairpin RNA (shRNA) directed against PLB. Studies using virus-mediated transfer of shRNA directed against PLB in cardiomyocyte culture have demonstrated that it is an effective method for both knocking down PLB expression and modulating calcium handling (Fechner *et al.*, 2007; Andino *et al.*, 2008), and one study in a rat model of HF demonstrated that AAV-mediated transfer of shRNA is effective in restoring cardiac function and geometry (Suckau *et al.*, 2009). In addition, because the gene product in this case would be a functional RNA and not a mutant protein (dnPLB), this strategy would avoid the potential of evoking a T cell response directed against cells expressing the transgene, as has been reported after AAV-mediated cardiac gene transfer of a modified, soluble TNF receptor in baboons (McTiernan *et al.*, 2007). This is an important consideration when considering large animal and clinical trials.

Therefore, in this preclinical study, our goal was to assess the safety and efficacy of using self-complementary AAV6 (scAAV6)-mediated delivery of shRNA directed against PLB in the canine heart before evaluating it in a canine model of HF. We found that AAV-mediated expression of shRNA directed against PLB is effective at knocking down PLB expression in the canine heart. However, our safety assessment revealed that healthy canines treated with shRNA, but not empty AAV6 capsid or dnPLB, experienced serum cardiac troponin I elevation, cardiac dysfunction, and alteration of cardiac microRNA expression, suggesting that this approach may not be a feasible therapeutic strategy.

Materials and Methods

Vector design and production

Self-complementary AAV6 vectors were produced according to the previously described pseudotyping protocol by the Vector Core of the University of Pennsylvania (Philadelphia, PA) (Gao *et al.*, 2002). Briefly, recombinant AAV genomes containing AAV2 inverted terminal repeats (ITRs) were packaged by triple transfection of 293 cells with a *cis* plasmid carrying the enhanced green fluorescent protein (EGFP) transgene, an adenovirus helper plasmid, and a chimeric *trans* plasmid carrying the AAV2 *rep* gene fused to the capsid gene of AAV6. Self-complementary vectors contained a mutation in the termination sequence of the 5′ ITR to allow synthesis and encapsidation of a dimeric inverted repeat of the transgene cassette (McCarty *et al.*, 2001). The empty capsid used in this study was AAV6 capsid devoid of ITR-containing DNA. It was purified through a CsCl step gradient as described in detail previously (Grimm *et al.*, 2003) and was quantified by silver stain analysis.

The scAAV6-dnPLB construct was designed to express canine PLB with an S16E mutation under the control of the constitutive chicken β-actin promoter with cytomegalovirus enhancer (CB) (Kaye *et al.*, 2007). The scAAV6-shRNA-PLB construct was designed to express shRNA directed against canine PLB under the control of the U6 promoter (Suckau *et al.*, 2009).

Animal use and vector delivery protocol

All animals were handled in compliance with National Institutes of Health (Bethesda, MD) and institutional guidelines that were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Eleven mongrel canines (5–10 kg, 3–6 months old) were used in this study. They were randomized to receive either scAAV6-U6-shRNA-PLB ($n=6$), scAAV6-CB-dnPLB ($n=2$), or AAV6 capsid only ($n=3$) at a dose of 5×10^{11} genome copies/kg.

The vector delivery procedure was performed under general anesthesia, and dogs were placed in left lateral recumbency. Heart rate, respiratory rate, systolic blood pressure, electrocardiogram, and oxygen saturation were monitored throughout the anesthetic period. A right carotid arteriotomy was performed, and a 7-French introducer was placed in the vessel, followed by insertion of the injection catheter. This catheter was a steerable injection catheter with an adjustable-length core needle (MyoCath; Bioheart, Sunrise, FL), which has previously been employed to deliver AAV vector in our laboratory (Bish *et al.*, 2008, 2010). The catheter was flushed with heparinized blood before vector infusion to prevent inactivation of the virus (Marshall *et al.*, 2000). Next, under fluoroscopic guidance, the catheter was advanced into the left ventricular cavity, and by steering the needle tip and adjusting the needle length, approximately 60 transendocardial injections of 250 μl each were performed to target the left ventricular free wall and interventricular septum from base to apex and from endocardium to epicardium with AAV vector. Contrast medium was added to the vector solution so that injection sites could be visualized. This allowed us to differentiate between injected and uninjected regions of the heart and helped to ensure that the vector solution was distributed globally throughout the myocardium.

For each procedure vector was mixed with 2 mL of sterile contrast solution (Omnipaque; GE Healthcare, Piscataway, NJ) and diluted with sterile saline to produce 15 mL for injection. Lidocaine (2 mg/kg as a bolus followed by a constant rate infusion at 50 μg/kg/min) was initiated if ventricular tachycardia developed during the procedure. After recovery, dogs were treated with carprofen for 2 days and with amoxicillin–clavulanic acid for 5 days.

Echocardiography

Echocardiography was performed with a Sonos 7500 (Philips Healthcare, Bothell, WA) with standard two-dimensional, M-mode, and Doppler capability (color and spectral imaging at each time point). Evaluation was performed with the animal in right and left lateral recumbency by using a cut-out table and imaging from below. The same sonographer performed all assessments (M.M.S.).

Serum cardiac troponin I quantification

Serum cardiac troponin I (cTnI) was assessed with a Stratus analyzer (Dade-Behring, Newark, DE). This analyzer uses a two-site sandwich immunoassay to detect free and complexed cTnI. Canine normal ranges have previously been established (Sleeper *et al.*, 2001).

RT-PCR analysis

Total RNA was isolated from cardiac samples snap-frozen in liquid nitrogen (TRIzol; Invitrogen, Carlsbad, CA) and further purified with RNeasy columns (Qiagen, Valencia, CA) as previously described (Evans *et al.*, 2008). Two hundred and fifty nanograms of total RNA from each sample were subjected to single-strand reverse transcription (Applied Biosystems, Foster City, CA). The resultant cDNA was used for quantitative RT-PCR (qRT-PCR) with oligonucleotides specific for canine PLB and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control, using the LightCycler system and reagents (LightCycler FastStart DNA MasterPLUS SYBR Green I; Roche Applied Science, Indianapolis, IN). Expression levels were assessed by calculating the crossing point (Cp), where measured fluorescence rises above background, as the second derivative maximum of the reaction curve. Each sample was analyzed in duplicate and the resulting data were averaged. The relative change in expression between groups was based on the assumption that a difference of 1 Cp results from a 2-fold change in expression. Melting point analysis of experimental samples confirmed that all primers were specific for their respective transcripts, when there was only one melting point observed for each primer pair. Controls included RNA not subjected to reverse transcription, and water only. Primer sequences were as follows:

Canine PLB Forward: atggataaagtccaatacctca
Canine PLB Reverse: tcagagaagcatcacaatgat
Canine GAPDH Forward: atggtgaaggtcggagtcaacggat
Canine GAPDH Reverse: gaagatggagatggacttcccgttg

Western blot analysis

Cardiac biopsies obtained for Western blotting were snap-frozen in liquid nitrogen. Specimens were pulverized, homogenized in 10 vol of triple-detergent lysis buffer (50 m*M* Tris [pH 8.0], 0.1% sodium dodecyl sulfate [SDS], 1.0% Triton X-100, 0.5% deoxycholate [DOC], 5 m*M* EDTA, 50 m*M* dithiothreitol [DTT], Complete protease inhibitor [0.4 tablet/10 ml; Roche, Indianapolis, IN]), and centrifuged at 13,000 rpm for 5 min. The protein concentration of the supernatant was then determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Fifty micrograms of each sample were electrophoresed on a 4–20% SDS–polyacrylamide gel (Lonza, Rockland, ME) after the addition of 2 × sample loading buffer (130 m*M* Tris [pH 8.0], 20% glycerol, 4.6% SDS, 2% DTT, 0.02% bromophenol blue) and 5 min of denaturation at 100°C. Proteins were then transferred to Immobilon-P (Millipore, Bedford, MA), using the iBlot transfer apparatus (Invitrogen). The membrane was subsequently blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20. Immunoblotting was performed to detect phospholamban (1:500 dilution; Millipore) and actin (1:2000 dilution; Sigma-Aldrich, St. Louis, MO) as a loading control. Detection was performed with a SuperSignal West Pico chemiluminescent substrate kit (Pierce Protein Research Products, Rockford, IL).

Enzyme-linked immunospot assay for T cell responses

Peripheral blood mononuclear cells (PBMCs) were isolated as per standard protocol and cryopreserved until assayed as previously described (Bish *et al.*, 2008). Briefly, tissues were obtained fresh and homogenized; red blood cells were lysed; lymphocytes were then collected and cryopreserved until assayed. For the enzyme-linked immunospot (ELISpot) assay, a peptide library consisting of 15-mers overlapping by 10 amino acids with the adjacent peptide was synthesized for the capsid sequence of the AAV6 VP1 protein (736 amino acids, 146 peptides; Mimotopes, Clayton, Victoria, Australia). Peptides were resuspended in 50% acetonitrile–0.1% acetic acid at a concentration of 5 mg/ml. Peptides were arranged into a 24-pool matrix, with each pool consisting of 12 or 13 peptides and each peptide contained in 2 orthogonal pools. To test for T cell responses, 2×10^5 cells were incubated for either 24 hr (interferon-γ) or 48 hr (interleukin-10) at 37°C, 10% CO_2, in the presence of AAV6 peptide pools (individual peptide final concentration, 10 μg/ml) or AAV6 empty capsid (10 μg/ml; Research Vector Core, Children's Hospital of Philadelphia, Philadelphia, PA). Phorbol 12-myristate 13-acetate (PMA, 50 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) were used as positive controls. Medium alone (DMEM; Gibco/Invitrogen) served as a negative control. All assays were performed in triplicate. Canine interferon-γ and interleukin-10 were assayed with canine development module kits (R&D Systems, Minneapolis, MN). Assays were scored with an immunospot reader (CTL, Cleveland, OH), and positive T cell responses were scored as 3-fold above medium control. Results are displayed as spot-forming units/10^6 cells.

MicroRNA microarray analysis

Myocardial biopsies were collected from dogs treated with scAAV6-U6-PLB-shRNA 16–26 days previously ($n = 3$) and from untreated, age-matched controls ($n = 3$) and snap-frozen in liquid nitrogen. Two biopsies were collected per animal. MicroRNA (miRNA) was extracted with an miRNA Easy mini kit (Qiagen). miRNA expression was analyzed with miRCURY LNA version 9.2 (Exiqon, Woburn, MA). GenePix (GPR) files were imported into Partek Genomics Suite version 6.4 (Partek, St. Louis, MO), where foreground median minus background median were extracted for each channel on each array. Data were transformed such that any extracted intensity that was less than 1 was assigned a value of 1. Data were then LOESS (locally weighted scatterplot smoothing) normalized using $\log_2$ ratios for treated dogs over control dogs for each array. Dye-swap pairs were averaged, yielding three biological replicates for treated over control. Statistical analysis for significantly dysregulated miRNAs was conducted for those probes, which had $\log_2$ ratios for at least two of the three pairs.

Statistical analysis

Mean values from each experimental group were compared by two-tailed Student t test. Analysis of microarray data is described in the previous section.

Results

Study design

shRNA-mediated knockdown of cardiac PLB expression has potential as a novel therapeutic approach in the treatment of HF. Before evaluating the long-term therapeutic

potential of this approach in a canine model of HF, we chose to assess the safety of shRNA-mediated knockdown of PLB in healthy canines. Six dogs were treated with scAAV6-U6-shRNA-PLB. Three control dogs were treated with AAV6 empty capsid to establish a virus control group. An additional two canines were treated with scAAV6-CB-PLB-S16E (dnPLB) (Kaye *et al.*, 2007) to establish a control group for PLB inhibition. PLB mRNA and protein levels as well as microRNA expression were analyzed between days 16 and 26 in three of the dogs treated with PLB shRNA. The remaining dogs (three treated with PLB shRNA, three control dogs treated with AAV6 capsid only, and two dogs treated with dnPLB) were monitored long term with monitoring of serum cardiac troponin I (cTnI) and cardiac function to assess the safety of AAV-mediated PLB knockdown with shRNA.

PLB knockdown

The spatial distribution of cardiac gene transfer after injection via the percutaneously inserted catheter described in Materials and Methods has been previously published (Bish *et al.*, 2008, 2010). RT-PCR results revealed that PLB mRNA expression was reduced approximately 16-fold (Fig. 1A), and Western blot results revealed that PLB protein was reduced approximately 5-fold (Fig. 1B) after treatment with scAAV6-U6-shRNA-PLB. Thus, AAV-mediated cardiac delivery of shRNA directed against PLB is effective at knocking down cardiac PLB expression in the canine.

Long-term cardiac safety assessment

We next monitored the three remaining dogs treated with PLB shRNA long-term to assess the safety of this procedure. The three dogs treated with AAV6 capsid only and two dogs treated with dnPLB were monitored as controls. Serum cardiac troponin I elevations were noted in three of three dogs treated with PLB shRNA, zero of three dogs treated with AAV6 capsid only, and zero of two dogs treated with dnPLB. The elevation was apparent by 2 to 3 weeks postinjection, with a peak at 4–5 weeks to approximately 100-fold baseline levels (Fig. 2A). This was associated with a decrease in fractional shortening as measured via echocardiography (Fig. 2B). At this time, one of three dogs treated with shRNA was killed, and tissue was collected for analysis as detailed below. In the remaining two of three dogs treated with shRNA, serum cTnI levels and fractional shortening gradually improved over the next 6–8 weeks. No abnormalities were observed in any of the three dogs treated with AAV6 capsid only with one of three monitored for 6 months and two of three monitored for 2 years, or in either of the dogs treated with dnPLB, both of which were monitored for greater than 18 months. Expression of the dnPLB construct was confirmed both in canines before its use in this study and in one of the long-term dnPLB canines used in this study at 19 months (see Supplementary Fig. S1; supplementary data are available online at www.liebertonline.com/hum). The remaining dnPLB canine used in this study continues to be monitored for long-term safety assessment and is now over 2 years posttreatment.

ELISpot assay

Next, we sought to determine the etiology of the cardiac toxicity observed after treatment with scAAV6-U6-shRNA-PLB. Because no toxicity was observed in any of the canines treated with either AAV6 capsid only or scAAV6-dnPLB, it seemed unlikely that it was secondary to a T cell response directed against AAV6 capsid. However, because a capsid-directed T cell response was observed in a human trial of AAV-mediated hepatic gene transfer (Manno *et al.*, 2006) and a canine study of AAV-mediated skeletal muscle gene transfer (Wang *et al.*, 2007) at a similar time frame postinjection, we screened peripheral blood mononuclear cells (PBMCs) from dogs experiencing toxicity via ELISpot for T cells reactive to AAV6 capsid. No T cells reactive to AAV6 capsid were identified in the PBMCs isolated from these dogs at 4 and 7 weeks postinjection, or from dogs treated with AAV6 capsid only (Fig. 3A and B and data not shown). In addition, no T cells reactive to AAV6 capsid were identified in the heart or spleen of the shRNA-treated dog that was killed (Fig. 3C, D).

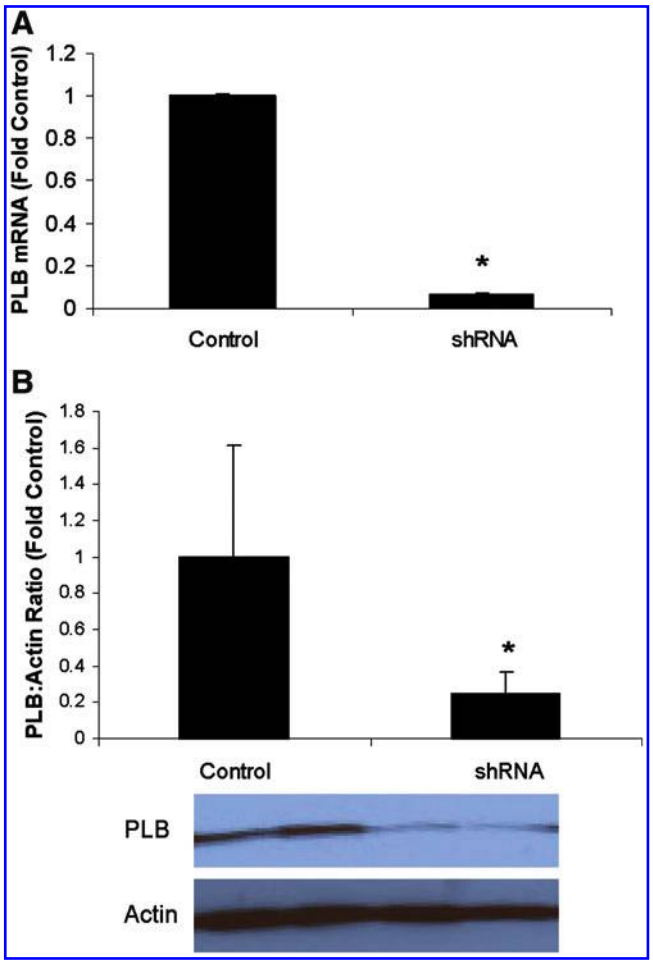

FIG. 1. Analysis of phospholamban (PLB) mRNA and protein levels reveals efficacy of scAAV6-U6-shRNA-PLB in knocking down PLB expression. **(A)** Quantitative RT-PCR analysis demonstrates approximately 16-fold decreased PLB mRNA with short hairpin RNA (shRNA) overexpression compared with control. **(B)** Western blot demonstrates approximately 5-fold decreased PLB protein with shRNA overexpression compared with control. $*p < 0.05$, $n = 3$ per group. Tissue was collected 16–26 days postinjection. Color images available online at www.liebertonline.com/hum

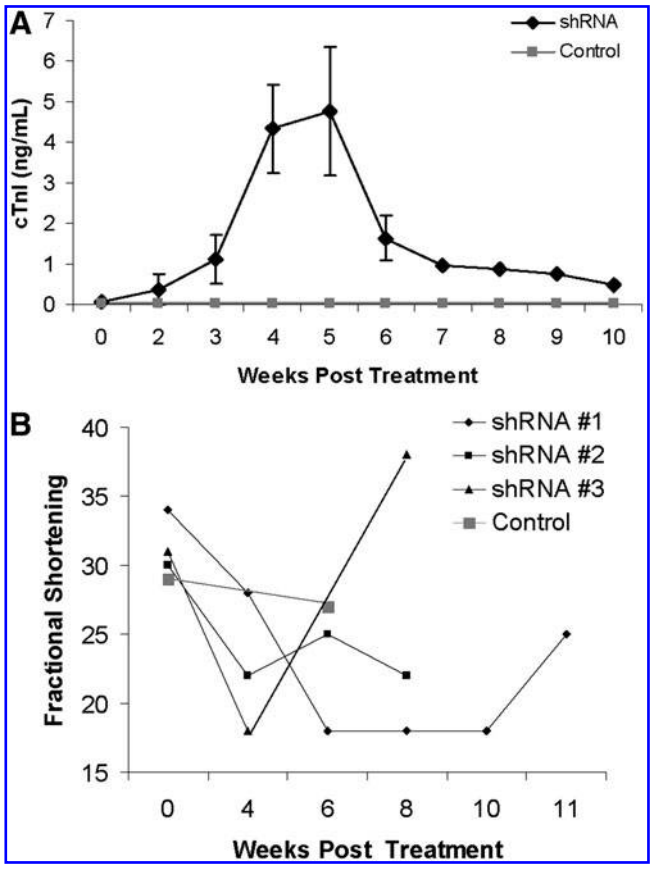

FIG. 2. Long-term safety assessment demonstrates cardiac toxicity in canines treated with scAAV6-U6-shRNA- phospholamban (PLB). **(A)** Serum cardiac troponin I (cTnI) measurements were performed at baseline and up to 10 weeks after treatment with scAAV6-shRNA-PLB ($n=3$), AAV6 capsid only ($n=3$), or scAAV6-dnPLB ($n=2$). Serum cTnI levels peaked at 4–5 weeks in canines treated with scAAV6-shRNA-PLB. Because no elevation in serum cTnI was observed in either the AAV6 capsid group or the scAAV6-dnPLB group, both groups are represented here as Control. **(B)** Cardiac function as assessed by echocardiography. Fractional shortening (FS), a measure of cardiac contractility, is reported for canines treated with scAAV6-shRNA-PLB vs. control. Echocardiography was performed at multiple time points from baseline to 11 weeks as depicted. Canines treated with scAAV6-shRNA-PLB experienced a decline in FS beginning at 4 weeks. dnPLB, dominant negative PLB.

MicroRNA microarray

Because a T cell response directed against AAV6 capsid was not responsible for the cardiac toxicity and because PLB inhibition via dnPLB did not induce toxicity in this study, we continued to investigate its etiology. It was reported that oversaturation of endogenous microRNA pathways after AAV-mediated shRNA overexpression in mouse liver was responsible for hepatotoxicity and death (Grimm *et al.*, 2006). Therefore, we next analyzed cardiac microRNA expression by means of microarray technology. We found that cardiac miRNA-124 expression was decreased approximately 2-fold, and miRNA-302d expression was increased approximately 3-fold, after treatment with scAAV6-U6-shRNA-PLB (Fig. 4). No significant changes in expression were noted for the following microRNAs with previously reported roles in cardiovascular physiology and pathophysiology: miRNA-1 (Yang *et al.*, 2007; Zhao *et al.*, 2007), miRNA-21 (Yin *et al.*, 2008), miRNA-133 (Care *et al.*, 2007), miRNA-195 (van Rooij *et al.*, 2006), miRNA-199a (Rane *et al.*, 2009), miRNA-208a (van Rooij *et al.*, 2007; Callis *et al.*, 2009), miRNA-320 (Ren *et al.*, 2009) (Fig. 4).

Discussion

In this study, we demonstrated that percutaneous delivery of scAAV6-U6-shRNA-PLB is effective at knocking down expression of PLB in the canine heart. Our study was the first to assess the efficacy of AAV-mediated transfer of shRNA directed against PLB in a large animal model. A previous report described the effective use of scAAV9-U6-shRNA-PLB to knock down expression of PLB in a rat model of HF induced by transaortic banding (Suckau *et al.*, 2009). In this model, decreased PLB expression was associated with restoration of cardiac function and geometry over a 3-month period without any adverse effects. We designed our vector and shRNA sequence on the basis of this report and on another in the literature describing virus-mediated transfer of shRNA directed against PLB in rat neonatal cardiomyocyte culture, with some modifications for use in a canine model (Fechner *et al.*, 2007). Namely, our shRNA sequence was directed against the same region of PLB as previously described in the rat but modified to be canine specific. In addition, we used scAAV6 instead of scAAV9 because it has been previously reported that scAAV6 is superior to scAAV9 in the canine heart (Bish *et al.*, 2008).

After demonstrating the efficacy of this approach, we next assessed its long-term safety in healthy canines as a final step before the design of a preclinical study in a canine model of HF. Surprisingly, we found that treatment with AAV-shRNA-PLB was associated with elevation of serum cTnI, with a peak at 4 to 5 weeks postinjection, and depressed cardiac function. Cardiac function gradually improved with normalization of serum cTnI levels by approximately 3 months postinjection. After observing this severe toxicity, we directed our attention toward identifying its etiology.

The canines that experienced toxicity were treated with AAV6 expressing shRNA directed against PLB; therefore, the toxicity could have been induced by the AAV6 vector, by inhibiting PLB, or by the shRNA itself. Because the gene product in this case was a functional RNA and not a protein, we did not need to consider a T cell response directed against the expressed transgene as the etiology, as has been previously reported (McTiernan *et al.*, 2007). However, a T cell response directed against the AAV capsid could still be responsible for the observed toxicity. Indeed, robust cellular immune responses directed against AAV capsid have been described approximately 4 weeks postinjection in human liver (Manno *et al.*, 2006) and canine skeletal muscle (Wang *et al.*, 2007). To rule this out in our study, we performed two separate experiments. First, we injected three dogs with AAV6 empty capsid and monitored these animals up to 2 years. These animals did not experience elevation of serum cTnI or depression of cardiac function, suggesting that AAV capsid was not the cause of the toxicity. To be sure, we next screened PBMCs, spleen, and heart from dogs that received shRNA-PLB for T cells reactive to AAV6 capsid, using the

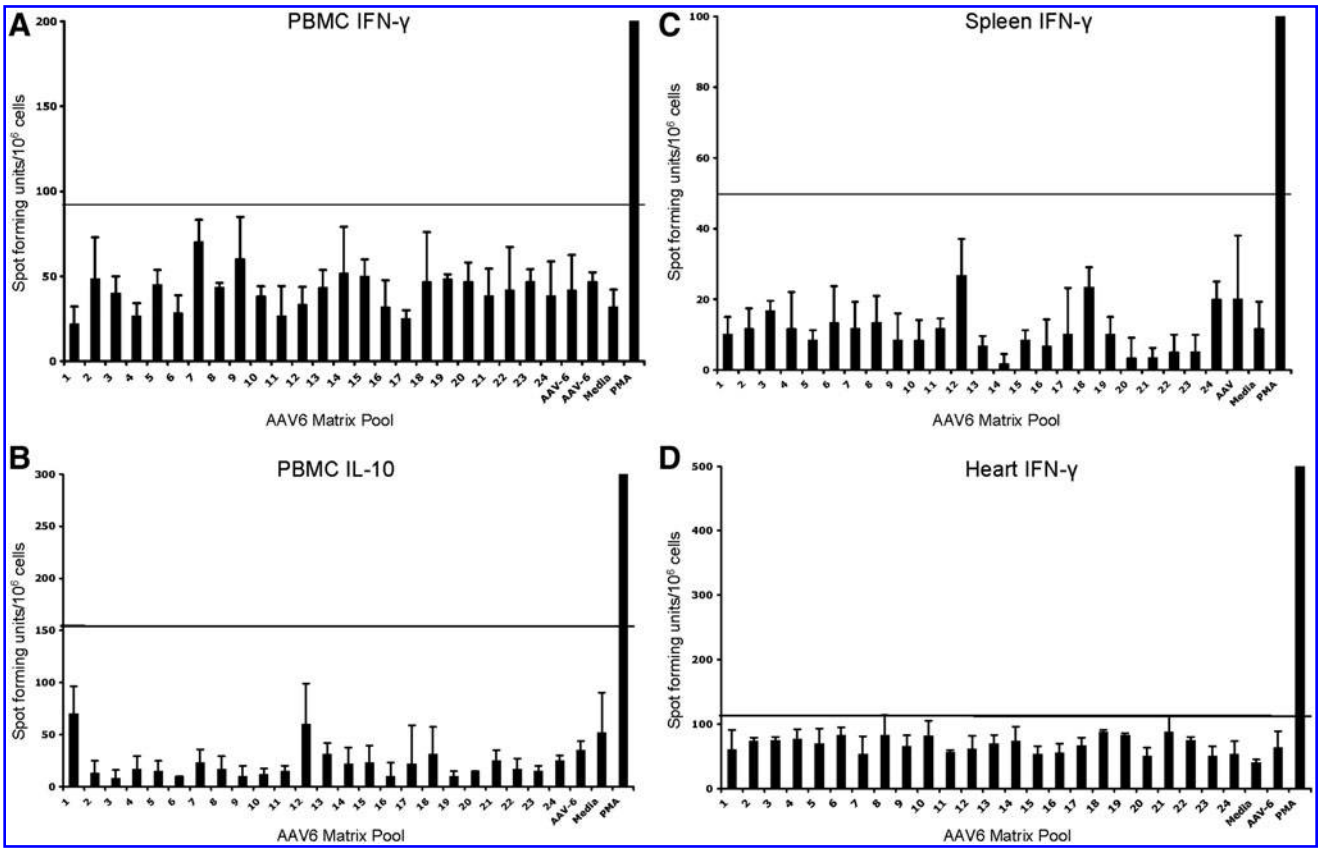

FIG. 3. AAV6 capsid does not induce a T cell-specific response in canines treated with scAAV6-U6-shRNA-PLB. Representative ELISpot data from PBMCs obtained 4 weeks postinjection and assayed for (**A**) interferon-γ and (**B**) interleukin-10 production are shown. Representative ELISpot data from lymphocytes isolated from (**C**) spleen and (**D**) heart 7 weeks postinjection and assayed for interferon-γ are shown. For all assays, cells were tested against 24 pools (5 μg/peptide, each peptide) comprising an AAV6 capsid peptide matrix, 10 μg/ml AAV6 empty capsid, phorbol 12-myristate 13-acetate (PMA)/ionomycin (positive control) or medium (negative control). The assay was performed in triplicate. Data are shown as spot-forming units/10^6 cells. The solid line indicates 3-fold over medium background control, used to determine positive T cell responses.

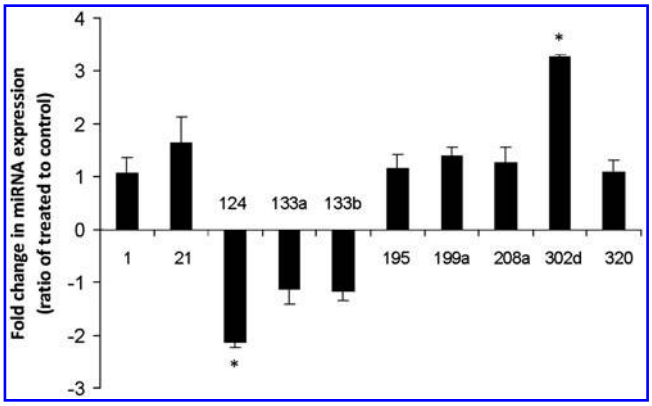

FIG. 4. Alterations in cardiac microRNA expression 16–26 days after percutaneous cardiac delivery of scAAV6-U6-shRNA-PLB ($n=3$) compared with control ($n=3$). Microarray analysis revealed significant alterations in expression of two miRNAs after overexpression of shRNA. No significant changes were noted in several other miRNAs with previously defined roles in the heart. *Significant, as defined by $p<0.05$ and >1.5-fold expression change (Thum *et al.*, 2007; Evans *et al.*, 2008).

ELISpot assay. ELISpot is the most widely accepted and most sensitive assay for detecting T cell responses in this case. ELISpot was used to determine the T cell response against AAV in a published clinical trial for hemophilia B (Manno *et al.*, 2006), and this same rigorous ELISpot analysis continues to be used as the primary assay for detection of T cell responses in current clinical and nonhuman primate trials of AAV-mediated gene transfer for hemophilia (Mingozzi and High, 2011; Nathwani *et al.* 2011). No capsid-reactive T cells were identified, further suggesting that AAV capsid was not responsible for the toxicity.

Another possibility is that inhibition of PLB induced cardiomyocyte death, as there is controversy in the literature concerning the safety of cardiac PLB inhibition. Although PLB knockout mice exhibit enhanced contractility without evidence of cardiomyopathy (Slack *et al.*, 2001), and PLB inhibition in hamsters (Hoshijima *et al.*, 2002) and rats (Iwanaga *et al.*, 2004; Sakata *et al.*, 2007; Zhao *et al.*, 2008; Suckau *et al.*, 2009) is effective in suppressing the progression of HF, humans homozygous and heterozygous for a null PLB L39Stop mutation develop left ventricular hypertrophy, premature dilated cardiomyopathy, and HF (Haghighi *et al.*, 2003). This discrepancy in phenotype may exist because

humans have a large cardiac reserve that can be regulated by altering sarcoplasmic reticulum calcium content via PLB, whereas small animals such as mice with resting heart rates over 600 beats/min have relatively little cardiac reserve and minimal ability to increase sarcoplasmic reticulum calcium stores above their near-maximal baseline (Haghighi *et al.*, 2003; MacLennan and Kranias, 2003). As a result, loss of PLB in humans, where it functions as a gatekeeper of the large cardiac reserve, may lead to significantly increased cardiac work and eventual cardiomyopathy and HF over years (Haghighi *et al.*, 2003), whereas loss of PLB in mice, where it regulates only a minimal cardiac reserve, may lead to a relatively smaller increase in cardiac work without cardiomyopathy over the short life span of the animal (Slack *et al.*, 2001).

However, there is also speculation that loss of PLB in humans may not be deleterious. Because the mutant L39Stop PLB peptide contains the cytoplasmic domain of PLB that interacts with and inhibits SERCA2a, it has been suggested that PLB L39Stop is not a null mutation, but rather a constitutively active inhibitor (Chien *et al.*, 2003; Haghighi *et al.*, 2004). If this is the case, then L39Stop is simply another example of a constitutively active PLB mutant responsible for human dilated cardiomyopathy, such as the R9C (Schmitt *et al.*, 2003) or R14 deletion (Haghighi *et al.*, 2006). In addition, a report describing reversal of advanced HF after delivery of adenovirus expressing dnPLB in a pacing-induced porcine model provides direct evidence for the safety of PLB inhibition in large animal hearts (Kaye *et al.*, 2007). However, animals in this study were monitored for only 2 weeks after injection of dnPLB, so data describing the long-term safety of PLB in large animal hearts are lacking. Because we wanted to rule out PLB inhibition as a potential source of toxicity in our dogs and because no other long-term studies of cardiac PLB inhibition in a large animal model have been published, we established a control group for PLB inhibition by injecting two healthy dogs with scAAV6 expressing dnPLB. We did not observe elevation of serum cTnI or depression in cardiac function in these animals, which were monitored for over 18 months, confirming the safety of long-term cardiac PLB inhibition in a large animal.

However, it should be noted that we cannot rule out a very late onset of cardiac dysfunction resulting from PLB inhibition in these canines. Although PLB knockout mice exhibit enhanced contractility (Slack *et al.*, 2001), the effects of inhibiting PLB in healthy hearts in a large animal model are unknown. It may have no effect, or the effect may take more than 2 years (the observation time in this study) to develop. Indeed, humans with PLB mutations do not typically present with cardiac disease until their late teens or early twenties at the earliest (Haghighi *et al.*, 2003).

Because we had ruled out AAV capsid and PLB inhibition as sources of toxicity, we next investigated shRNA overexpression as a source. A study described hepatotoxicity and death in mice approximately 1 month after intravenous injection of an AAV vector expressing various shRNA sequences, and it was determined that oversaturation of endogenous microRNA pathways by the exogenous shRNA was responsible (Grimm *et al.*, 2006). Because the cardiac toxicity in our study occurred at a similar time, it seemed plausible that the same mechanism may be responsible. Indeed, previous work has demonstrated the importance of microRNA in the heart (Thum *et al.*, 2008a; Small *et al.*, 2010). As in other tissues, microRNAs in the heart control gene expression by binding to target mRNAs and either promoting their degradation or inhibiting their translation. In doing so, they regulate expression of genes involved in multiple processes, such as hypertrophy (van Rooij *et al.*, 2007), conduction (Callis *et al.*, 2009), fibrosis (Thum *et al.*, 2008b), and apoptosis (Rane *et al.*, 2009). In our study, we found that treatment with shRNA was associated with alterations in expression of miRNAs 124 and 302d. Although no definitive roles for these miRNAs have been described in the heart, they have been implicated in embryonic development (Maiorano and Mallamaci, 2009; Rosa *et al.*, 2009). In addition, dysregulation of the miRNA-302 family has been reported in human HF (Thum *et al.*, 2008a). It has been suggested that reactivation of fetal miRNA programs may contribute to altered gene expression in human HF (Thum *et al.*, 2007). Future studies in experimental animal models are needed to define the mRNA targets and physiological role of miRNAs 124 and 302 in the heart. In addition, future investigation will be directed at assessing alterations in cardiac function and in the miRNA expression profile after treatment with additional shRNAs, including a second PLB-specific sequence, a nonessential gene sequence, and a nonspecific/negative control sequence, to strengthen the validity of our findings in this initial study.

In summary, we demonstrated in this study that percutaneous delivery of scAAV6-U6-shRNA-PLB is effective in knocking down PLB expression in the canine heart. However, it is associated with severe cardiac toxicity that may be secondary to oversaturation of endogenous miRNA pathways. Future studies using this approach should focus on strategies to limit shRNA expression to the minimally effective dose, perhaps by designing vectors with promoters that are weaker than the traditional polymerase III promoters (H1 and U6) used to drive shRNA expression, such as tissue-specific, polymerase II promoters (Giering *et al.*, 2008).

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Author Disclosure Statement

J.M.W. and G.P.G. are inventors on patents that have been licensed to various biopharmaceutical companies.

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