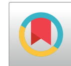
Letters

RESEARCH LETTER

Cardiac Transduction in Mini-Pigs After Low-Dose Retrograde Coronary Sinus Infusion of AAV9-*BAG3*



A Pilot Study

The gene for BCL2-associated athanogene 3, *BAG3,* is highly expressed in heart, skeletal muscle, the central nervous system, and many cancers and serves as a chaperone for the small and large heat shock proteins, inhibits apoptosis, enhances excitation-contraction coupling, and activates autophagy.[1] Mutations in *BAG3* have been found in multiple major genetics studies of patients with dilated cardiomyopathy, the majority of which are truncating or deletion variants. Intravenous delivery of adeno-associated virus 9 (AAV9)-*BAG3* in Bag3 haplo-insufficient mice suggests a beneficial effect.[2] Although the optimal route of delivery of potentially therapeutic transgenes to the heart remains controversial, studies in a porcine model of left ventricular dysfunction after occlusion of the circumflex coronary artery have shown beneficial effects on subsequent cardiac function after retrograde coronary sinus infusion (RCSI) with simultaneous occlusion of the left anterior descending coronary artery: an example being the G protein receptor kinase inhibitor mini-gene bARK (or GRK_{2ct}).[3] However, a recent report found that high-dose intravenous gene therapy administration with an AAV9 variant, led to severe neural and hepatic toxicity.[4] We hypothesized that transgene delivery via RCSI without coronary arterial occlusion might provide effective transduction of the myocardium with relatively low vector genome doses.

To test this hypothesis, healthy male Yucatan minipigs (30-40 kg) were sedated and randomized to receive either vehicle (n = 1) or 1 of 3 doses of AAV9-*BAG3* in total vector genomes (vg) per animal: group A: 5×10^{13} vg (average of 1.46×10^{12} vg/kg; n = 4); group B: 1×10^{14} vg (average of 3.45×10^{12} vg/kg; n = 2), group C:2.5×10^{14} vg (7.58×10^{12} vg/kg; n = 1) of AAV9-*BAG3*. Experiments were performed at Charles Rivers Laboratories with appropriate animal-use approvals. Following induction of anesthesia, the internal jugular vein and artery were identified and cannulated and a guide wire was advanced into the coronary sinus. An Advance 35LP COOK balloon catheter was introduced into the coronary sinus: dye was injected to ensure proper placement. After balloon inflation, dye was passed to exclude backflow and AAV9-*BAG3* was infused in 40-80 mL of vehicle over 20 minutes between 2 and 5 mL/min. Cardiac rhythm was continuously monitored and injury to myocardium or coronary sinus was assessed at time of sacrifice.

Eight weeks post dosing, 18 tissue sections from 3 short-axis circumferential segments of left ventricle tissue at 6 points along the long axis and right ventricular free wall were obtained as depicted in **Figure 1**. The presence of vg was assessed by quantitative polymerase chain reaction and a standard curve of known copy numbers of transgene plasmid. Vector messenger RNA was assessed using quantitative polymerase chain reaction targeting vector complementary DNA and expressed as relative quantities to the 18S housekeeping gene. Bag3 expression was assessed using immunohistochemistry. The results are expressed as the mean ± SEM by group of relative quantities per animal, vg/μg of DNA, or vg/cardiomyocyte where possible (eg, $n > 1$). Measurements for vg or transcript more than 3 SDs from the mean for that animal were excluded. To express vg/cardiomyocyte, each porcine cardiomyocyte was assumed to have 8 nuclei.

All animals tolerated the infusion procedure without incident. Genomic DNA (in vg/μg) were present in varying levels in all tissue samples and transduction at 8 weeks was consistent with a "U" shaped curve. (Group A: 7,043 ± 3,579 vg/μg; group B: 24,832 ± 3,307 vg/μg; group C: 15,744 vg/μg, n = 1.) The mean quantity of vg was ≥1 per porcine cardiomyocyte at doses given in groups B and C (group A: 0.70 ± 0.2; group B: 2.0 ± 0.8; group C: 1.3) (**Figure 1**).

Transcription of the *BAG3* transgene was also measured in groups A, B, and C. Relative quantities of vector transcript (± SEM) normalized to 18S ribosomal RNA were vehicle group: 0.99; group A: 4.1 ± 1.0; group B: 9.0 ± 4.5, and group C: 8.5. Bag3 protein levels measured by immunohistochemistry remained unchanged.

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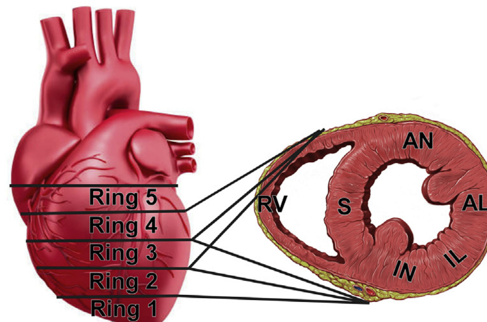
FIGURE 1 **Distribution of vg Across Myocardium in CS After AAV9-BAG3 Infusion**



Location	Vehicle			5e13vg/animal			1e14vg/animal			2.5e14vg/animal		
	Vg/ myocyte	*SEM*	*N*	*Vg/ myocyte*	*SEM*	*N*	*Vg/ myocyte*	*SEM*	*N*	*Vg/ myocyte*	*SEM*	*N*
Ring 2												
AN	0.0	0	1	0.3	0.2	4	0.9	0.3	2	1.4	0	1
AL	0.0	0	1	0.1	0.0	4	0.1	0.0	2	0.6	0	1
IL	0.0	0	1	0.5	0.3	4	1.8	1.4	2	0.7	0	1
IN	0.0	0	1	0.3	0.1	4	0.4	0.1	2	6.6	0	1
S	0.0	0	1	0.1	0.1	3	1.4	1.1	2	1.5	0	1
RV	0.0	0	1	0.5	0.3	4	0.8	0.5	2	0.7	0	1
Ring 3												
AN	0	0	1	0.2	0.1	4	12.8	12.0	2	0.9	0	1
AL	0.0	0	1	0.1	0.0	4	1.4	0.8	2	1.3	0	1
IL	0.0	0	1	0.3	0.2	4	4.0	3.6	2	1.4	0	1
IN	0.0	0	1	4.1	2.4	4	0.6	0.2	2	1.5	0	1
S	0.0	0	1	0.2	0.1	4	0.6	0.2	2	0.9	0	1
RV	0.0	0	1	1.0	0.7	4	0.3	0.2	2	-	0	0
Ring 4												
AN	0.3	0	1	0.2	0.1	4	1.0	0.1	2	1.0	0	1
AL	0.0	0	1	0.3	0.1	4	0.4	0.2	2	0.4	0	1
IL	0.1	0	1	3.7	3.2	4	10.8	10.0	2	1.0	0	1
IN	0.0	0	1	0.5	0.3	3	0.8	0.0	1	0.7	0	1
S	0.0	0	1	1.6	1.4	4	1.1	0.0	2	0.9	0	1
RV	0.0	0	1	0.2	0.1	4	0.5	0.1	2	1.3	0	1

AN=Anterior, AL=Anterolateral, IL=Inferolateral, IN=Inferior, S=Septum, RV=Right Ventricle

Distribution of vector genomes (vg) across the myocardium in the coronary sinus (CS) of the pig after retrograde infusion of adeno-associated virus 9 (AAV9)-BAG3. Mean ± SEM vg per myocyte. Measurements from tissues obtained from 5 left ventricular regions. RV = right ventricle.

Using RCSI, we were able to transduce the myocardium safely and efficiently with AAV9-*BAG3* in this pilot study such that, on average, each cardiomyocyte had at least 1 copy of the introduced gene using total vg doses of 1×10^{14} vg (3.45×10^{12} vg/kg) and 2.5×10^{14} vg (7.58×10^{12} vg/kg). We used the fewest animals possible to test our hypothesis because of the high cost of mini-pigs and the difficulty and expense in finding animals free of preformed AAV antibodies. However, this pilot study has provided strong evidence that AAV vectors can deliver DNA to the heart. As expected, Bag3 protein levels were not altered presumably because Bag3 is autoregulated.

Given the presence and transcription of low doses of AAV9-*BAG3* vg in the pig heart, we hypothesize that RCSI can effectively increase expression of functional Bag3 protein, potentially exerting therapeutic effects in the setting of Bag3 haploinsufficiency. The variability in biodistribution seen across the myocardium supports the hypothesis that gene distribution may be influenced by inherent unevenness in myocardial strain. Our finding of a dosing curve trending to a “U” shape is consistent with previous studies with AAV vectors; however, ongoing studies will also clarify this possibility.

In conclusion, we report that we were able to safely deliver low doses of AAV9-*BAG3* vg using catheter-based RCSI in healthy mini-pigs, which resulted in diffuse transduction of the myocardium. Using lower total vg doses may translate to important safety advantages in the clinic, although the optimal route and dose of delivery in humans is not yet known.

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Drs Myers and Semigran are employees of Renovacor, Inc and have either equity or options in the company. Mr Landsberg is a consultant with Renovacor, Inc. Mrs Bologna is a consultant to the company and has equity in Renovacor, Inc., Dr Feldman is the founder of Renovacor, Inc; has equity in the company; is a consultant to the company; and has pending U.S. patents that have been optioned by Temple University to Renovacor, Inc. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

Barry Greenberg, MD, served as Guest Editor for this paper.

The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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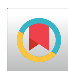
RESEARCH LETTER

LncRNAs in Inflammation



Lessons From a Preclinical Investigation of *Mexis* Therapy in Atherosclerosis

Work in the last decade established long noncoding RNAs (lncRNAs) as critical regulators of many biologic processes required for life including X-inactivation.[1] However, the significance of lncRNAs in cardiovascular disease is still in question. Hundreds of lncRNA genes have been proposed to be critical for cardiovascular health but in vivo genetic perturbations have either been missing or showing subtle effects.[2] In addition, development of lncRNA-based therapies remains an aspirational goal. Our group discovered that conserved lncRNA *Mexis* acts as a key modulator of cholesterol efflux and atherosclerosis development in mice and humans.[3] *Mexis* orchestrates macrophage responses to sterol overload by boosting Abca1 expression and in part explains spatial variations in Abca1 across tissues. *Mexis*-based therapies may be highly attractive because they would enhance cholesterol efflux. Conversely, recent evidence suggests that sustained activation of DDX17 (the binding partner of Mexis) by RNAs triggers noncanonical inflammasome signaling.[4] Thus, we aimed to decipher how enhancing *Mexis* in macrophages would impact multiple nonredundant pathways causality linked with risk of atherothrombotic disease.

Our previous work showed that deletion of *Mexis* reduces Abca1, enhances inflammation and foam cell formation, as well as promotes atherosclerosis development.[3] To test the therapeutic effects of lncRNAs within lesions, we used a novel genetic model that allows spatial control of *Mexis* expression from the endogenous locus (**Figure 1A**). Our study was approved by the UCLA Institutional Animal Care and Research Advisory Committee. We crossed conditional *Mexis* knockin mice with Cre^{LysM} to generate macrophage-specific enhanced *Mexis* expression (**Figure 1A**). Isolation of peritoneal macrophages from Cre+ mice confirmed a marked increase in *Mexis* compared with Cre- controls (**Figure 1B**). Consistent with the notion that *Mexis* acts in *trans* to boost *Abca1* levels, we observed an increase in *Abca1* mRNA and protein (**Figure 1B**). *Mexis* overexpression was associated with enhanced cholesterol efflux to an ApoA1 acceptor (**Figure 1B**). In addition, enhancing *Mexis* reduced foam cell formation in vitro and in vivo (**Figure 1C**). To test the efficacy of *Mexis*-based therapy on atherosclerosis we performed a bone marrow transplant in $LDLR^{-/-}$ mice with Cre+ and Cre- marrow (**Figure 1D**). We confirmed bone marrow reconstitution and found that *Cre+* bone marrow transplantation did not impact serum cholesterol, triglycerides, or fasting glucose (not shown). Analysis of atherosclerosis lesions by en face and aortic root section did not show differences in plaque burden between groups (**Figure 1D**). Surprisingly, and in contrast to the in vitro results, enhanced *Mexis* expression in the atherosclerosis model led to significant elevation in systemic inflammatory markers including serum amyloid A (**Figures 1E to 1F**).