Adeno-Associated Virus Serotype 9–Driven Expression of BAG3 Improves Left Ventricular Function in Murine Hearts With Left Ventricular Dysfunction Secondary to a Myocardial Infarction

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HIGHLIGHTS

- BAG3 is a highly conserved protein having pleiotropic effects that is expressed at high levels in the heart, skeletal muscles, and many cancers.
- BAG3 levels are reduced in many forms of LV dysfunction including mice after ligation of the left coronary artery.
- Retro-orbital injection of mice with an adeno-associated virus coupled to murine BAG3 under the control of a CMV promoter (rAAV9-BAG3) increased myocardial levels of BAG3 by 7 days post-injection.
- Retro-orbital injection of rAAV9-BAG3 in mice post-myocardial infarction improved LV function, whereas rAAV9-BAG3 had no effect on LV function in the absence of an MI.
- BAG3 may prove to be a new therapeutic target in the treatment of heart failure.
Mutations in Bcl-2-associated athanogene 3 (BAG3) were associated with skeletal muscle dysfunction and dilated cardiomyopathy. Retro-orbital injection of an adeno-associated virus serotype 9 expressing BAG3 (rAAV9-BAG3) significantly (p < 0.0001) improved left ventricular ejection fraction, fractional shortening, and stroke volume 9 days post-injection in mice with cardiac dysfunction secondary to a myocardial infarction. Furthermore, myocytes isolated from mice 3 weeks after injection showed improved cell shortening, enhanced systolic [Ca^{2+}], and increased [Ca^{2+}] transient amplitudes, and increased maximal L-type Ca^{2+} current amplitude. These results suggest that BAG3 gene therapy may provide a novel therapeutic option for the treatment of heart failure. (J Am Coll Cardiol Basic Trans Science 2016;1:647-56) © 2016 The Authors.

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mice with LV dysfunction secondary to a myocardial infarction (MI). These studies suggest that BAG3 could be a novel target for therapeutic intervention in patients with HF with reduced ejection fraction.

**METHODS**

**ANIMAL PROTOCOLS.** Eight-week old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine) were randomly assigned to undergo an induction of an MI by left coronary artery ligation using a protocol described previously by our group that led to a significant reduction in LV function with generally acceptable levels of long-term survival (10,11). Consistent with earlier studies, nearly one-half of all infarcted mice expired within 1 week of surgery, but only 1 mouse died after week 1. Therefore, mice in which the artery was ligated were randomized to receive either gene therapy with BAG3 (rAAV9-BAG3; MI-BAG3, n = 13) or control (rAAV9-GFP; MI-GFP, n = 12) 1 week after surgery and prior to the first echocardiogram to obviate an effect of the early mortality in the model affecting outcomes. The mouse that died after week 1 had been randomized to MI-GFP and was not included in the analysis. Sham-operated control animals were treated in an identical manner except that the left anterior descending artery was not ligated. Sham mice were also randomized at the time of the week-1 echocardiogram to receive either BAG3 (rAAV9-BAG3; Sham-BAG3, n = 12) or a GFP control (rAAV9-GFP; Sham-GFP, n = 14). All experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Temple University Institutional Animal Care and Use Committee (ACUP#4360).

**ECHOCARDIOGRAPHY.** Global LV function was evaluated in all mice after light sedation (2% isoflurane) using a Vevo 770 imaging system and a 707 scan head (VisualSonics, Miami, Florida) as described previously by our group that led to a significant reduction in LV function with generally acceptable levels of long-term survival (10,11). The first echocardiogram was obtained 1 week after surgery. The left ventricular ejection fraction (LVEF) was calculated using the formula:

\[
\text{EF}\% = \frac{(\text{LV end-diastolic volume} - \text{LV end-systolic volume})}{\text{LV end-diastolic volume}} \times 100.
\]

Fractional shortening (FS) was calculated as:

\[
\text{FS}\% = \frac{(\text{LV end-diastolic dimension} - \text{LV end-systolic dimension})}{\text{LV end-diastolic dimension}} \times 100.
\]

**CONSTRUCTION AND ADMINISTRATION OF rAAV9-BAG3.** A sequence encoding the murine myc-tagged BAG3 (NCBI accession #BC145765) was inserted into a pAAV vector that contained a cytomegalo virus (CMV) promoter (Vector Biolabs, Malvern, Pennsylvania). The construct was then packaged into AAV-9 by transfection of HEK293 cells, and viral particles were purified by CsCl2 centrifugation (Vector Biolabs). Recombinant AAV9-BAG3 also expressed green fluorescent protein (GFP); however, GFP was not in sequence with BAG3. Fidelity of the clone and the final vector were confirmed by sequencing. Both MI mice and Sham mice were randomly assigned to receive either 60 to 80 µl rAAV9-BAG3 (5.0 to 6.5 × 1013 genome copies (GC)/ml) or rAAV9-GFP control (2.1 × 1012 GC/ml) in sterile phosphate buffered saline (PBS) at 37°C by injection into the retro-orbital venous plexus as described previously (12).

**IMMUNOBLOTS.** Hearts were rapidly frozen in liquid nitrogen and stored at −80°C. Tissue was lysed in buffer (Cell Signaling Technologies, Beverly, Massachusetts) and homogenized with beads in a Bullet Blender (Next Advance, Averill Park, New York). After centrifugation at 13,000 g for 5 min at 4°C, the supernatant was collected and protein concentration was determined by Bradford assay (Bio-Rad, Philadelphia, Pennsylvania). Equal amounts of protein were mixed with 10 µl of 5x loading buffer (350 mmol/l Tris pH 6.8, 25% β-mercaptoethanol, 30% glycerol, 10% sodium dodecyl sulfate, and 0.01% bromophenol blue), boiled, separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to Odyssey nitrocellulose membranes (LiCor, Lincoln, Nebraska) by wet transfer (Bio-Rad). Membranes were blocked in Odyssey blocking buffer (LiCor) for 1 h at room temperature (rt) before incubation with primary antibodies for 2 h at rt. The membranes were then washed 1x PBST (0.1% Tween 20 in PBS) and incubated with the secondary antibody for 1 h at rt. The signal was detected with an Odyssey scanner. Primary antibodies were Myc (Cell Signaling Technologies), BAG3 (ProteinTech Group Inc., Rosemont, Illinois), GFP (Clonetech, Mountain View, California), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Dallas, Texas) and calsequestrin (1:5,000) (Swant, Bellinzona, Switzerland). Calsequestrin was used to determine protein loading in quantitative Western blots, whereas GAPDH was used for nonquantitative blots. Secondary antibodies were goat antimouse IRDye 800 (LiCor) and IRDye 680 goat antirabbit (Rockland, Gilbertsville, Pennsylvania).

**ISOLATION OF ADULT MURINE CARDIAC MYOCYTES.** In a subset of the study cohort, cardiac myocytes were isolated from the septum and LV free wall according to the protocol of Zhou et al. (14) and plated on laminin-coated glass coverslips (15). Coverslips containing myocytes were mounted in a Dvorak-Stotler chamber, and bathed in fresh media before measurements.
MEASUREMENT OF \([\text{Ca}^{2+}]\) AND CONTRACTION IN CARDIAC MYOCYTES. GFP-expressing myocytes were fura-2 loaded (0.67 \(\mu\)mol/l fura-2 AM, 15 min), incubated in 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid–buffered (20 mmol/l, pH 7.4) medium 199 (1.8 mmol/l \([\text{Ca}^{2+}]\)o), and field stimulated to contract (2 Hz; 37°C), as described previously (9). In brief, myocytes were exposed to excitation light (360 and 380 nm) only during data acquisition. Epi-fluorescence (510 nm) was measured in steady-state twitches both before and after the addition of isoproterenol (1 \(\mu\)mol/l) (15–20). For contraction measurements, images of myocytes (not loaded with fura-2) were captured by a charge-coupled device video camera, and myocyte motion was analyzed offline with an edge detection algorithm (15–20).

CONFOCAL MICROSCOPY. Confocal microscopy was used to detect BAG3 localization in adult cardiomyocytes as described previously (9). Briefly, adult mouse LV cardiomyocytes were isolated and plated on laminin-coated 4-well chamber slides (Lab-Tek, Rochester, New York). BAG3 was identified using a primary rabbit antibody (1:200; ProteinTech Group Inc.). After incubation for 12 h at 20°C, myocytes were rinsed with PBS and then incubated with secondary Alexafluor 594-labeled goat anti-rabbit antibodies (1:500 Invitrogen, Eugene, Oregon) at rt in the dark.
for 60 min. A Carl Zeiss 710 confocal microscope (63× oil objective) with ZEN software was used for imaging. Total laser intensity and photomultiplier gain were set constant for all groups and settings, and data were verified by 2 independent observers who were blinded to the experimental group. Three coverslips were used for each experimental group and at least 3 cell images were acquired from each coverslip.

REAL-TIME POLYMERASE CHAIN REACTION MEASUREMENT OF BRAIN NATRIURETIC PEPTIDE

Total messenger ribonucleic acid (mRNA) was isolated from a randomly selected group of sham or MI mice as described previously, with the exception of the use of a MirVana miRNA isolation kit (Thermo Fisher Scientific, Waltham, Massachusetts). Reverse-transcribed cDNA from 1 μg mRNA was used to determine the expression of B-type natriuretic peptide (BNP) from MI-GFP (6) and -BAG3 (5) mice as described previously (16). BNP primers were 5′-CTG AAG GTG TCC CAG AT 3′ and 5′-CCT TGG TCC TTC AAG AGG TG 3′, and GAPDH primers were 5′-AAC GAC CCC TTC ATT GAC 3′ and 5′-TCC ACG ACA TAC TCA GCA C 3′. GAPDH was used as a reference for normalization of BNP measurements. The ΔCT method was used to quantify the results. BNP expression was presented as a relative to the GAPDH gene.

STATISTICAL ANALYSIS. Data was analyzed using GraphPad Prism 6 (La Jolla, California) or JMP version 12 (SAS Institute, Cary, North Carolina). Data are presented as means ± SEM for continuous variables. Our experience with echocardiographic measurements in test animals and measures of Ca2+ homeostasis and contractility in cells has shown that the underlying distribution is normal. Furthermore, analysis of variance (ANOVA) is robust to moderate variations from normalcy. Therefore, we used 2-way ANOVA for repeated measures to assess the significance of the different groups, time, and the first-order interaction between group and time for echocardiographic measures. For analysis of the single-cell data in which cells were exposed to isoproterenol, we first analyzed the data for all 3 groups (Sham-GFP, MI-GFP, and MI-BAG3) together to make sure that statistical significance existed amongst the 3 groups and then considered group, isoproterenol, and first-order interaction between group and isoproterenol using 2-way ANOVA with Bonferroni multiple comparisons adjustments. A p value < 0.05 was considered statistically significant.

RESULTS

rAAV9-BAG3 IMPROVES LV DYSFUNCTION IN MICE POST-MI. Preliminary studies demonstrated that myc-tagged rAAV9-BAG3 was appreciated by day 7 after retro-orbital injection and reached maximal levels of expression by day 9. (Figure 1A) Therefore, to assess the effects of increased expression of BAG3 on LV function post-MI, mice in both the Sham and MI groups were randomized within group just prior to the week-1 echocardiogram to receive either myc−tagged rAAV9-BAG3 (Sham- and MI-BAG3) or rAAV9-GFP (Sham- and MI-GFP) followed by sacrifice 3 weeks later. As shown in Figure 1B, compared with Sham-GFP mice, MI-GFP mice demonstrated a significant decrease in LVEF at 1 week post-MI with a progressive diminution in contractility over time. By contrast, compared with MI-GFP mice, MI-BAG3 mice demonstrated significantly (p < 0.0001) higher LVEF at 9 days post-rAAV9-BAG3 injection, and LVEF continued to improve at 3 weeks post-injection (p < 0.0001) (Table 1). Similarly, at 3 weeks post-injection, rAAV9-BAG3 resulted in a significant increase in stroke volume (p < 0.0001), FS (p < 0.0001), and LV posterior wall thickness in diastole (p < 0.025) in MI-BAG3 mice when compared with MI-GFP mice. However, injection of rAAV9-BAG3 did not affect LV mass, LV volume, or LV internal diameter in MI-BAG3 mice when compared with measurements in MI-GFP mice (Table 1). Importantly, injection of rAAV9-BAG3 had no effect on LVEF in Sham mice when compared with Sham mice that received rAAV9-GFP. It is also noteworthy that there was no statistical difference in LVEF between MI-BAG3 and -GFP mice at weeks 1, 3, 5, or 7—all of which were prior to the injection of rAAV9-BAG3 or -GFP at week 8. Preliminary studies had demonstrated that the fall in ejection fraction post-MI in our model plateaued.
between weeks 5 and 7. Therefore, we chose to inject AAV9-BAG3 at week 8, a time when the EF was relatively stable, to optimize our ability to assess the effects of rAAV9-BAG3. Also, it should be noted that there were no deaths in mice randomized to any of the 4 treatment groups after injection of either rAAV9-BAG3 or -GFP.

As we have previously observed in other models of HF (1), levels of BAG3 were significantly diminished in MI-GFP mice at 11 weeks post-MI compared with Sham-GFP mice (p < 0.04); however, MI-BAG3 mice had significantly elevated levels of BAG3 compared with MI-GFP mice (p < 0.04) (Figure 1C). Consistent with earlier findings in our laboratory (9), BAG3 was found predominantly in the sarcolemma and t-tubules of Sham-GFP myocytes. BAG3 levels were reduced in MI-GFP myocytes. Importantly, not only did rAAV9-BAG3 injection reconstitute BAG3 levels, but the exogenous BAG3 was also correctly targeted to the sarcolemma and t-tubules of MI-BAG3 myocytes (Figures 1D to 1F).

We did not measure BNP levels as part of our original protocol; however, we were able to assess levels of BNP in a subgroup of MI-BAG3 and -GFP hearts. There was a trend toward a decrease in BNP mRNA levels in the hearts of MI mice that received rAAV9-BAG3 (4.87 ± 0.52; n = 5; presented as relative to GAPDH expression) as compared with those that received rAAV9-GFP (2.96 ± 0.71; n = 6); however, the difference did not meet statistical significance (p = 0.670).

**EFFECTS OF AAV9-BAG3 ON INDIVIDUAL MYOCYTES POST-MI.** To confirm the salutary in vivo effects of rAAV9-BAG3 on myocardial contractility in mice that had undergone an MI and to dissect the physiological effects of gene therapy, we assessed the effect of rAAV9-BAG3 expression on individual myocytes isolated from a randomly selected cohort of MI-BAG3,
MI-GFP, and Sham-GFP hearts 11 weeks post-MI (3 weeks post-AAV9-BAG3 injection). Compared with Sham-GFP myocytes, contraction amplitudes in MI-GFP myocytes were significantly lower at baseline, and the differences were amplified in the presence of isoproterenol (Figures 2A to 2C) (Sham-vs. MI-GFP; p = 0.0004; group × iso interaction effect). BAG3 overexpression improved contractility in post-MI myocytes toward normal: the beneficial effect was more prominent in the presence of isoproterenol (MI-GFP vs. -BAG3; p = 0.054; group × iso interaction effect). Compared with Sham-GFP myocytes, maximal shortening (Figure 2D) (p = 0.0162) and relengthening (MI-BAG3 vs. -GFP; p = 0.012) velocities post-MI.

**EFFECTS OF BAG3 OVEREXPRESSION ON \([Ca^{2+}]\), TRANSIENTS POST-MI.** \(Ca^{2+}\) occupies a central role in excitation-contraction coupling, and alterations in \([Ca^{2+}]\) dynamics may account for the contractility differences observed among the 3 groups of myocytes. Compared to Sham-GFP myocytes, systolic \([Ca^{2+}]\), was significantly lower in MI-GFP myocytes (p = 0.009; group × iso interaction effect) (Figures 3A to 3C). BAG3 overexpression restored systolic \([Ca^{2+}]\), in post-MI myocytes toward that observed in Sham-GFP myocytes (Figure 3C) (Sham-GFP vs. MI-BAG3; p = 0.504; group × iso interaction effect). Diastolic \([Ca^{2+}]\), was not different among the 3 groups (p = 0.722; group × iso interaction effect) (Figure 3C). Compared with
Sham-GFP myocytes, \([\text{Ca}^{2+}]_i\) transient amplitudes were significantly reduced in MI-GFP myocytes (Figure 3D) \((p = 0.026; \text{group \times iso interaction effect})\). In addition, rAAV9-BAG3 increased \([\text{Ca}^{2+}]_i\) transient amplitudes in post-MI myocytes toward levels observed in Sham-GFP myocytes (Sham-GFP vs. MI-BAG3; \(p = 0.518, \text{group \times iso interaction effect}\)) (Figure 3D).

**EFFECTS OF BAG3 OVEREXPRESSION IN L-TYPE \text{Ca}^{2+} \text{CURRENT POST-MI.}** L-type \text{Ca}^{2+} current (I_{Ca}), the physiological trigger for sarcoplasmic reticulum (SR) \text{Ca}^{2+} release, is a major determinant of \([\text{Ca}^{2+}]_i\) transient amplitude. Our observation that \([\text{Ca}^{2+}]_i\) transient amplitudes differed among the 3 groups of myocytes prompted us to measure I_{Ca}. At baseline, maximal I_{Ca} amplitudes and voltage at which I_{Ca} peaked were similar among Sham-GFP, MI-GFP, and MI-BAG3 myocytes \((p = 0.042)\) (Figure 4A). In the presence of isoproterenol, maximal I_{Ca} amplitude tended to be lower \((p = 0.081)\) in MI-GFP when compared with Sham-GFP myocytes (Figure 4B). Treatment with rAAV9-BAG3 increased maximal I_{Ca} amplitude post-MI to levels that were even higher \((p = 0.042)\) than those measured in Sham-GFP myocytes (Figure 4B).

**DISCUSSION**

In this proof-of-concept study, we demonstrate for the first time that delivery of BAG3 to the heart using rAAV9 and murine BAG3 under the control of the CMV promoter significantly increased LV function in mice with substantially diminished LV function secondary to an MI. BAG3 has pleiotropic effects in cells due to the presence of multiple protein binding motifs. For example, it binds to the ATPase domain of heat shock protein-70 to effect protein quality control \((21)\), interacts with Bcl-2 to impart an antiapoptosis function, and regulates a diverse portfolio of cellular processes including development, cytoskeletal arrangement, and mitophagy \((2)\). Our observation that BAG3 couples the \(\beta_1\)AR and the L-type \text{Ca}^{2+} channel within the sarcolemma of adult cardiac myocytes and the finding that rAAV9-BAG3 increases LV performance in hearts with diminished LV function after an MI are consistent with this pleiotropism \((9)\).

Studies in mice harboring a homozygous deletion of BAG3 that develop myofibrillar degeneration and death by 4 weeks of age \((7)\), observations in children with a point mutation \((p.\text{Pro209Leu})\) in BAG3 who have a dystrophin-like phenotype and cardiac hypertrophy \((3-6, 22, 23)\), diminished levels of BAG3 in failing human hearts and in animal models of HF \((1)\), the observation that the myofibrillar structure of neonatal myocytes is disrupted after BAG3 is knocked down \((8)\), and our observation that BAG3 facilitates the ability of \(\beta\)-adrenergic signaling to augment cardiac contraction through linkage of the \(\beta_1\)AR and the L-type \text{Ca}^{2+} channel and subsequent alterations in \text{Ca}^{2+} homeostasis suggest that BAG3 plays an important role in cardiac homeostasis \((9)\). Our findings that rAAV9-BAG3 correctly targeted the exogenous BAG3 to the sarcolemma and t-tubules and improved LV function in mice after an MI is consistent with these earlier studies. Furthermore, we confirmed our in vivo findings in individual myocytes that were isolated from MI-BAG3, MI-GFP, and Sham-GFP mice.
Intuitively, an increase in contractility due to enhanced Ca\(^{2+}\) homeostasis might presage an increased risk for arrhythmogenesis. However, several lines of reasoning suggest that this is not the case: 1) diastolic Ca\(^{2+}\) was not different between MI-GFP and -BAG3 myocytes in the presence of isoproterenol, suggesting that the cells were not Ca\(^{2+}\) overloaded; 2) the lower I\(_{\text{Ca}}\) in post-MI myocytes compared with Sham myocytes in the presence of isoproterenol was attributable to βAR uncoupling and not to alterations in ion channel function, because forskolin or dibutyryl cyclic adenosine monophosphate restored I\(_{\text{Ca}}\) to normal (24); and 3) there were no deaths in any of the experimental groups after injection of rAAV9. Thus, there does not appear to be a cellular substrate for arrhythmias, although longer follow-up post-rAAV9-BAG3 will be required to completely eliminate this possibility.

**STUDY LIMITATIONS.** This proof-of-concept study was focused on evaluating the early effects of increasing BAG3 expression in the heart in vivo using an adeno-associated virus under the control of a CMV promoter. As a result, we were not able to detect the potential effects of BAG3 on cardiac remodeling. For example, the salutary effects of rAAV9-BAG3 on LV function were not associated with a change in LV mass or size. However, we did see a trend toward a decrease in BNP levels in MI-BAG3 hearts when compared with MI-GFP hearts in an evaluation of a subset of the overall study group.

**CONCLUSIONS**

The consistent observation that rAAV9-BAG3 had no untoward effect on ventricular function in Sham-BAG3 mice when compared with Sham-GFP mice has important implications for the potential use of rAAV9-BAG3 as a novel treatment strategy, because it suggests that after a finite level of cellular BAG3 is reached, subsequent protein expression does not have deleterious effects. Because inotropic responses do not necessarily guarantee clinical responses, it will be important to evaluate the long-term effects of BAG3 overexpression on contractile function and on cellular processes that play a role in cell survival, including apoptosis and autophagy. Nonetheless, the results of this proof-of-concept study suggest that BAG3 may be a novel and potentially effective therapeutic target in patients with loss of function biallelic mutations and in those with nonfamilial dilated cardiomyopathy, and that further investigations to clarify the effects of rAAV9-BAG3 and its mechanisms of action in the heart are warranted.

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**COMPETENCY IN MEDICAL KNOWLEDGE:** It is becoming increasingly evident that a substantial proportion of idiopathic dilated cardiomyopathy is due to genetic variants and that these variants are often passed down among family members in an autosomal dominant fashion. Although recognition of familial forms of heart failure has not yet led to specific treatments, identification of large families with heritable forms of the disease can lead to the discovery of new therapeutic targets, thus reinforcing the importance of the family history in the workup of a patient with new onset heart failure.

**TRANSLATIONAL OUTLOOK:** Although the present study demonstrates proof of concept that re-expression of BAG3 can improve LV function in mice after an MI, several steps will be required before these findings can be translated into man, including evaluation of the long-term effects of BAG3 expression and proof of concept in a large animal model.

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