Gene delivery of medium chain acyl-coenzyme A dehydrogenase (MCAD) induces physiological cardiac hypertrophy and protects against pathological remodelling

Bianca C. Bernardo1,2,*, Kate L. Weeks1,*, Thawin Pongsukwechkul1,3, Xiaoming Gao1, Helen Kiriazis1, Nelly Cemerlang1, J.H. Boey1, Yow Keat Tham1,4, Chad J. Johnson5, Hongwei Qian1, Xiao-Jun Du1,4, Paul Gregorevic1,6,7,8 and Julie R. McMullen1,4,9*

1 Baker Heart and Diabetes Institute, Melbourne, 3004, Australia.
2 Department of Paediatrics, University of Melbourne, Melbourne, 3010, Australia.
3 Department of Biochemistry and Molecular Biology, University of Melbourne, Melbourne, 3010, Australia.
4 Department of Medicine, Monash University, Clayton, 3800, Australia.
5 Monash Micro Imaging, Burnet Institute, Melbourne, 3004, Australia.
6 Department of Physiology, University of Melbourne, Melbourne, 3010, Australia.
7 Department of Biochemistry and Molecular Biology, Monash University, Clayton, 3800, Australia.
8 Department of Neurology, The University of Washington School of Medicine, Seattle, WA 98195, USA.
9 Department of Physiology, Monash University, Clayton, 3800, Australia.

*Authors contributed equally to the work

*Correspondence should be addressed to J.R.M, B.C.B or K.L.W

Julie R. McMullen: Email: julie.mcmullen@baker.edu.au Tel: +61 3 8532 1194
Bianca C. Bernardo: Email: bianca.bernardo@baker.edu.au Tel: +61 3 8532 1167
Kate L. Weeks: Email: kate.weeks@baker.edu.au Tel: +61 3 8532 1205
PO Box 6492
Melbourne 3004, Australia
Fax: +61 3 8532 1100
**ABSTRACT**

We previously showed that medium chain acyl-coenzyme A dehydrogenase (MCAD, key regulator of fatty acid oxidation) is positively modulated in the heart by the cardioprotective kinase, phosphoinositide 3-kinase [PI3K(p110α)]. Disturbances in cardiac metabolism are a feature of heart failure patients, and targeting metabolic defects is considered a potential therapeutic approach. The specific role of MCAD in the adult heart is unknown. To examine the role of MCAD in the heart and to assess the therapeutic potential of increasing MCAD in the failing heart, we developed a gene therapy tool using recombinant adeno-associated viral vectors (rAAV) encoding MCAD. We hypothesised that increasing MCAD expression may recapitulate the cardioprotective properties of PI3K(p110α). rAAV6:MCAD or rAAV6:control was delivered to healthy adult mice and to mice with pre-existing pathological hypertrophy and cardiac dysfunction due to transverse aortic constriction (TAC). In healthy mice, rAAV6:MCAD induced physiological hypertrophy (increase in heart size, normal systolic function and increased capillary density). In response to TAC (~15 weeks), heart weight/tibia length increased ~60% in control mice and ~45% in rAAV6:MCAD mice compared to sham. This was associated with an increase in cardiomyocyte cross-sectional area in both TAC groups which was similar. However, hypertrophy in TAC rAAV6:MCAD mice was associated with less fibrosis, a trend for increased capillary density, and a more favourable molecular profile compared with TAC rAAV6:control mice. In summary, MCAD induced physiological cardiac hypertrophy in healthy adult mice, and attenuated features of pathological remodelling in a cardiac disease model.

**ABBREVIATIONS**

AAV, adeno-associated virus; AF, atrial fibrillation; Akt, protein kinase B; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; caPI3K, constitutively active PI3K(p110α); DCM, dilated cardiomyopathy; dnPI3K, dominant negative PI3K (p110α); FS, fractional shortening; HF, heart failure; IGF1R, insulin-like growth factor 1 receptor; LV, left ventricular; LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension; LVPW, LV posterior wall; MCAD, medium chain acyl-coenzyme A dehydrogenase; MI, myocardial infarction; Ntg, non-transgenic; PI3K, phosphoinositide 3-kinase; rAAV, recombinant adeno-associated viral vectors; SERCA2a, sarcoplasmic reticulum Ca2+ ATPase; TAC, transverse aortic constriction; Tg, transgenic.
INTRODUCTION
Heart failure (HF) is a debilitating condition that affects 1-3% of people in Western society. The incidence of HF is likely to rise over the coming decades due to the aging population, rising rates of obesity and diabetes, and the availability of interventions that prolong survival following cardiac insults such as myocardial infarction (MI) (1). Consequently, an increasing number of patients will contribute significantly to the economic burden caused by HF (2). There are very limited therapies or procedures to treat or cure HF, and long term survival following HF remains poor, with one third of patients dying within a year of diagnosis (3). Thus, there is a clear need for new therapeutic strategies to treat HF.

In general, research and therapeutics have focused on inhibiting pathological processes that contribute to HF. As an alternative approach, we previously identified a proactive intervention for protecting the failing heart, based on activation of the phosphoinositide 3-kinase (PI3K\(p_{110}^D\)) pathway (4-10). Increasing PI3K\(p_{110}^\beta\) activity via cardiac-specific transgene expression or gene therapy (recombinant adeno-associated viral vectors [rAAV]) preserved or improved cardiac function, and attenuated pathological remodelling in multiple mouse models of cardiac dysfunction and heart failure (4-10). While these studies demonstrate the benefits of activating the PI3K\(p_{110}^D\) pathway in the stressed heart, there are challenges associated with targeting PI3K\(p_{110}^D\) directly. There are numerous drugs or agents which activate PI3K signalling in the heart (11). However, many of these drugs also activate other signalling cascades, and/or do not specifically target the \(p_{110}^\alpha\) isoform of PI3K. PI3K has multiple isoforms with both protective (e.g. \(p_{110}^D\)) and adverse effects (e.g. \(p_{110}^\gamma\)) in the heart. For example, mice with reduced PI3K\(p_{110}^\alpha\) activity (due to cardiac-specific expression of a dominant negative PI3K\(p_{110}^\alpha\) construct; dnPI3K-Tg mice) were more susceptible to pathological remodelling and HF when subjected to MI (10) or pressure overload (5, 9), demonstrating that PI3K\(p_{110}^\alpha\) is required for protecting the heart in these disease settings. In contrast, mice deficient for PI3K\(p_{110}^\gamma\) were protected from developing HF in response to chronic isoproterenol infusion, suggesting that PI3K\(p_{110}^\gamma\) contributes to pathological remodelling downstream of \(\beta\)-adrenergic receptor activation (12). Further, PI3K has numerous actions in various cell types and has widespread tissue distribution (13, 14). In non-cardiac tissues, enhanced PI3K\(p_{110}^\alpha\) activity due to mutations within *PIK3CA*, increased gene copy
number or abnormal phosphatase and tensin homolog (PTEN) activity has been linked with
tumorigenesis (15). Consistent with this observation, inhibiting PI3K signalling is a current
therapeutic strategy for the treatment of many cancers (16). Seeking to emulate the therapeutic
potential of PI3K(p110α) while addressing potential concerns, we have identified novel mRNAs
regulated by PI3K(p110α) that may represent alternative drug targets (10).

One such target is medium chain acyl-coenzyme A dehydrogenase (MCAD; encoded by Acadm).
Oxidation of fatty acids by MCAD (and by other acyl-CoA dehydrogenases) is a key step in
mitochondrial fatty acid β-oxidation (17, 18). In mice, cardiac MCAD gene expression correlated
with PI3K(p110α) activity as well as with cardiac function. Specifically, MCAD gene
expression was i) elevated in hearts with increased PI3K activity, ii) reduced in hearts with
decreased PI3K activity, iii) reduced in a setting of MI, and iv) positively correlated with cardiac
function (10). MCAD was of particular interest, as reduced MCAD levels have also been
observed in the failing human heart (19) and cardiac abnormalities have been documented in
infants with MCAD deficiency (20, 21). Furthermore, global deletion of MCAD caused
cardiomyopathy in mice (22). The aims of this study were i) to assess the effect of selectively
increasing MCAD expression in cardiac myocytes of adult mice, and ii) to examine whether
increasing MCAD expression in a mouse model of cardiac stress with pre-existing cardiac
dysfunction could attenuate pathological cardiac remodelling. Here, we report that increasing
MCAD expression in the murine heart using a gene therapy approach (rAAV delivery of MCAD
expression cassette) induced physiological cardiac hypertrophy in healthy adult mice, and
partially attenuated the disease phenotype of mice subjected to pressure overload.

MATERIALS AND METHODS
Experimental animals and groups
Animal care and experimentation were conducted in accordance with the Australian code for the
care and use of animals for scientific purposes (National Health & Medical Research Council of
Australia, 8th Edition, 2013), and approved by the Alfred Medical Research and Education
Precinct Animal Ethics Committee.
Male 10–12-week old mice on a C57BL/6 background were used in this study. Nine animals were used for the study in normal healthy mice (rAAV6:CON n=4; rAAV6:MCAD n=5). Seventeen mice underwent transverse aortic constriction (TAC) surgery (TAC rAAV6:CON n=9; TAC rAAV6:MCAD n=8). Four mice underwent sham surgery and received rAAV6:CON as a control for the TAC study.

**Left ventricular (LV) structure and function**

Transthoracic echocardiography (M-mode two-dimensional echocardiography) was performed on anaesthetised mice (1.8% isoflurane, inhalation) using a Philips iE33 ultrasound machine with a 15M Hz linear array transducer. Echocardiography was performed 8 weeks post rAAV6:control or rAAV6:MCAD delivery in normal wildtype mice. For the TAC surgical study, echocardiography was performed prior to surgery (baseline), ~4 weeks post-TAC (prior to treatment: rAAV6:MCAD), and post-treatment (i.e. ~15 weeks post-TAC, endpoint, Figure S1). LV chamber dimensions (LV end-diastolic dimension, LVEDD; LV end-systolic dimension, LVESD), LV wall thickness (LV posterior wall, LVPW), heart rate (HR), and fractional shortening (FS) were analysed offline using dedicated software (ProSolv Cardiovascular Analyser version 3.5; ProSolv, Indianapolis, IN, USA) obtained from M-mode traces.

**Pressure overload**

Male C57BL/6 mice were subjected to TAC (n=17) or a sham (n=4) operation as previously described (23, 24). The TAC model induces a chronic pressure load on the heart and is associated with progressive pathological hypertrophy and cardiac dysfunction within four weeks of surgery (25). Mice were anesthetised with ketamine, xylazine and atropine (KXA) (100, 20 and 1.2 mg/kg respectively) via intraperitoneal injection and administered carprofen (5mg/kg) as an anti-inflammatory and analgesic subcutaneously. Mice were intubated to assist ventilation during surgery. Mice were then administered a local anaesthetic subcutaneously (lignocaine, 7 mg/kg) at the site of incision. A sternotomy was performed to access the aorta and a non-absorbable 5-0 braided silk suture was tied around the aorta between the right innominate and left carotid arteries, causing a constriction of approximately 65% using a 0.46 mm probe as a guide. For sham operations to serve as controls, mice received the same surgical procedure except that the aorta was not banded.
AAV generation

Complementary DNA of the MCAD gene (Homo sapiens cDNA sequence for Acadm AK312629.1) was modified by adding a NotI restriction site to the 5’ end, and a FLAG tag sequence and HindIII restriction site to the 3’ end. The construct was made by GenScript (Jiangsu Province, People’s Republic of China) and cloned into an adeno-associated viral vector plasmid using standard cloning techniques. The plasmid (hereon referred to as pAAV6:MCAD) contained a cytomegalovirus (CMV) promoter and a synthetic poly(A) (9). The rAAV6:MCAD vector was produced by co-transfecting HEK 293T cells with pAAV6:MCAD and pDGM6, a packaging plasmid which provides the additional genes required for AAV vector production. Purification of vectors from transfected cells was achieved by standard methods, as reported previously (26). The titre of purified vector stocks was determined by RT-qPCR using primers for the CMV promoter: forward 5’-gcggtaggctgtacgggtgg-3’, reverse 5-cggtgatatggtctccaggc-3’ (9).

Cell culture of HEK293T cells

HEK 293T (Australia Cell Bank) were seeded at 300,000 cells/well (passages 2-4). HEK 293T (DMEM, 25 mM, 10% FBS, 1% PS) were transfected with pAAV6:MCAD or a control plasmid at a total concentration of 4 μg per well in 6-well plates using lipofectamine 2000 (Life Technologies) for 24 hours. To extract protein, HEK 293T cells were washed in 1X PBS, then lysed in cell lysis buffer (300 mM Hepes, 2% Triton X, 150 mM NaCl, 50 mM NaF, 1 mM PMSF, 4 μg/mL pepstatin) at 4°C for 30 minutes. Total protein concentration was determined using the Bradford Assay (BioRad, Hercules, CA, USA).

AAV administration

rAAV6:empty vector (referred to as rAAV6:CON) or rAAV6:MCAD was administered to 1) healthy adult mice or 2) mice subjected to TAC after pathological hypertrophy and cardiac dysfunction were confirmed by echocardiography at ~4 weeks (Figure S1). For the TAC study, four sham-operated animals were injected with rAAV6:CON as a control. 2 x 10¹¹ vector genomes in a total volume of 150 μL saline was injected into the tail vein using a 29 G syringe. Prior to injection, mice were placed under a heat lamp for approximately 10 minutes to dilate
their veins, and the tail was wiped with an alcohol swab. Mice were returned to their cages and checked daily for 3 days for any symptoms such as redness, scabbing, tail necrosis and abnormal behaviour. Uptake of rAAV6:MCAD in the heart was confirmed by Western blotting for the FLAG tag.

**Microarray data**

Microarray data was mined from an earlier study (10). The array dataset is deposited at Gene Expression Omnibus (GEO), GSE14267.

**RNA and protein isolation**

Total RNA was isolated from frozen mouse tissues using TRI Reagent (Sigma-Aldrich, St Louis, MO, USA) and quantitated on a Nanodrop™ Spectrometer (Thermo Scientific, Waltham, MA, USA). For protein lysates, frozen mouse ventricles were homogenised in a lysis buffer (10% glycerol, 137 mM NaCl, 20 mM Tris-HCl (pH 7.4), 20 mM NaF, 10 mM EDTA, 1 mM EGTA, 1 mM sodium pyrophosphate, 1 mM vanadate, 1 mM PMSF, 4 μg/mL pepstatin, 4 μg/mL aprotinin, 4 μg/mL leupeptin) and total protein concentration was determined using the Bradford Assay. RNA samples from previously characterised genetic mouse models with dilated cardiomyopathy (DCM) and atrial fibrillation (AF), or with increased or decreased PI3K activity and Ntg littermates (Ntg, caPI3K, dnPI3K) were also included in this study (7, 27-29).

**Northern blotting**

For mRNA analysis, 10 μg of total RNA was separated on a formaldehyde denaturing agarose gel and transferred to a Hybond-N membrane (GE Healthcare, Pittsburgh, PA, USA) in 20X SSC by upward capillary transfer, and probed for MCAD (Acadm), atrial natriuretic peptide (Anp), B-type natriuretic peptide (Bnp) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as previously described (29).

**qRT-PCR**

2 μg of total RNA was reverse transcribed using the High Capacity RNA-to-cDNA kit (Life Technologies, Carlsbad, CA, USA) according to manufacturer’s recommendations. qPCR was performed using TaqMan chemistry on an Applied Biosystems 7500 or Quant Studio 7 Flex real-
time PCR instrument. Hypoxanthine phosphoribosyltransferase 1 (Hprt1) was used to standardise for cDNA concentration and data was analysed using the $2^{-\Delta\Delta Ct}$ method of quantification.

**Western blotting**

100 μg of protein lysates were separated by SDS-PAGE, blotted onto PVDF membrane (Merck, Frankfurt, Germany), incubated with antibody overnight and detected by chemiluminescence. Signals were quantitated using ImageJ 1.44p pixel analysis (NIH Image Software, Bethesda, MD, USA) and data normalised to a control value of 1. Blots were probed with pAkt (Cell Signaling, 1:1000, #9271), total Akt (Cell Signaling, 1:2500, #9272), FLAG (Cell Signaling, 1:1000, #2368S), α-tubulin (Cell Signaling, 1:2500, #2144S), MCAD (Santa Cruz, 1:500, sc-49046) or GAPDH (Cell Signaling, 1:5000, #14C10). The Kaleidoscope™ Prestained SDS-PAGE Standards, broad range (BioRad, #1610324) or the Precision Plus Protein™ All Blue Prestained Protein Standards (BioRad, #1610373) were used as the molecular weight marker.

**Histological analyses**

Tissue samples were fixed in 4% paraformaldehyde and paraffin embedded for histological analysis. Cardiac collagen deposition/interstitial fibrosis (Masson’s trichrome stain, 6 μm cross-sections) was assessed as previously described (23, 24, 30). For assessment of angiogenesis and cardiomyocyte cross-sectional area, 4 μm cross-sections were deparaffinised in xylene and graded ethanol series, blocked in PBA (Thermo Shandon 407210) and co-stained with Alexa Fluor 568-conjugated isolectin B4 (Invitrogen I21412, 1:10 dilution) and FITC-conjugated wheat germ agglutinin (WGA; Vector Labs FL1021, 1:50 dilution). Sections were mounted with ProLong® Gold antifade reagent (Life Technologies) and visualised under an Olympus BX61 fluorescence microscope using a 60x 1.42NA objective lens. For each heart, capillary density was calculated by dividing the number of capillaries by the number of cardiomyocytes from at least 4 fields of view, and averaged. To determine cardiomyocyte cross-sectional area, a custom script was written in the FIJI distribution of Image J (31). A background subtraction was first applied to improve contrast. Cardiomyocytes were then segmented in their respective channel using their intensity maxima to watershed the objects and create regions of interest (ROIs) from which the area was measured. Erroneous or missing ROIs were edited manually and partial cells
at the image edges were automatically excluded. All transverse cardiomyocytes from at least 4 fields of view per heart were measured (i.e. >100 cardiomyocytes per heart) and the average area determined. Image acquisition and analysis was performed blinded to treatment group.

**Statistical Analyses**

Statistical analyses were performed using GraphPad Prism (Version 7, La Jolla, CA, USA). Results are presented as mean ± SEM. Differences between groups were identified using one-way analysis of variance (ANOVA) followed by Fisher’s post-hoc tests, unless otherwise indicated. Unpaired t-tests were used when comparing two groups for a single measure. A value of P≤0.05 was considered significant. All relative units are expressed as a fold change with the relevant control group normalised to 1.

**RESULTS**

**Differential expression of MCAD in physiological and pathological cardiac hypertrophy**

We previously identified MCAD as a potential therapeutic target from our microarray screen in mouse models of cardiac stress and protection (10). This screen utilised RNA extracted from hearts of cardiomyocyte-specific transgenic mice with increased or decreased PI3K(p110α) activity (caPI3K-Tg and dnPI3K-Tg, respectively). caPI3K-Tg mice develop physiological hypertrophy, whereas dnPI3K-Tg mice have smaller hearts and are more susceptible to cardiac remodelling and HF in settings of cardiac stress (4, 5, 7-10). Compared to the hearts of non-transgenic (Ntg) mice, MCAD gene expression was increased in the hearts of caPI3K-Tg mice and reduced in the hearts of dnPI3K-Tg mice (Figure 1A). The microarray results were validated by Northern blot analysis using independent Ntg, caPI3K and dnPI3K hearts (Figure 1B). MCAD gene expression was also decreased in the mouse heart in settings of MI (10) (Figure 1C), DCM (27) (Figure 1D) and AF (7) (Figure 1D). The development of a therapeutic tool which could increase MCAD expression was also of interest because of the high abundance of MCAD in the heart in comparison to other tissues (Figure 1E).

**rAAV6:MCAD induced physiological heart growth in healthy adult mice**

To investigate the effect of increasing MCAD expression in the adult heart, we developed a new gene therapy tool, i.e. recombinant AAV encoding FLAG-tagged MCAD (rAAV6:MCAD)
(Figure 2A). We have previously used this AAV subtype (AAV6) in combination with a CMV promoter to achieve cardiac-selective expression of the caPI3K transgene in mice (6, 9). Transfection of HEK 293 cells with the vector plasmid encoding FLAG-MCAD led to expression of a protein product of the correct molecular weight (~47 kDa; Figure 2B), and the recombinant protein was detectable in heart lysates from mice following systemic administration of 2×10^{11} vector genomes (vg) rAAV6:MCAD (Figure 2C). Consistent with previous studies utilising this vector (9, 26), FLAG-MCAD was highly expressed in the heart and to a lesser extent in skeletal muscle, but not in lung, kidney or spleen (Figure 2D). FLAG-MCAD was detectable at very low levels in the liver (Figure 2D).

First, we investigated the effects of rAAV6:MCAD administration on heart morphology and function in healthy mice. Male mice examined 8 weeks after administration of rAAV6:MCAD had larger hearts than mice that received a control vector (rAAV6:CON; Figure 3A, Table 1). This was accompanied by a comparable increase in cardiomyocyte cross-sectional area, although this did not reach statistical significance (Figure 3B). Atrial weight was also modestly increased in mice administered rAAV6:MCAD (Figure 3C, Table 1), consistent with what is observed with exercise-induced physiological hypertrophy. Echocardiography revealed that mice administered rAAV6:MCAD had thicker left ventricular walls and preserved systolic function (Figure 3D, Table 2). Capillary density was significantly increased in MCAD-treated mice (Figure 3E) and there was no evidence of left ventricular fibrosis (Figure 3F), lung congestion (Figure 3G) or expression of the pathological hypertrophy markers ANP and BNP (foetal genes, Figure 3H) in mice administered rAAV6:MCAD. The expression of key metabolic genes also did not differ between groups (Figure S2). Collectively, these data indicate that increasing MCAD expression induces physiological cardiac hypertrophy in healthy mice. Physiological hypertrophy is often associated with increased PI3K-Akt signalling. In the current study, MCAD-induced hypertrophy was not associated with increased phosphorylation of Akt (Figure S3).

Administration of rAAV6:MCAD was associated with favourable effects on features of pressure overload-induced pathological remodelling including fibrosis, capillary density, SERCA2a and metabolic gene expression
Having established that administration of rAAV:MCAD is associated with beneficial adaptations in the hearts of healthy mice (i.e. physiological cardiac hypertrophy), we investigated whether increasing cardiac MCAD levels could improve the cardiac phenotype of mice with established pathological remodelling and cardiac dysfunction induced by pressure overload. Mice were subjected to TAC surgery, which places a chronic pressure load on the heart. Following 4 weeks of TAC, LV remodelling (i.e. increased LVPW thickness) and systolic dysfunction (i.e. reduced fractional shortening) was confirmed by echocardiography (Table 3), and mice were then randomised to receive a single intravenous injection of $2 \times 10^{11}$ vg rAAV6:CON or rAAV6:MCAD (Figure S1). A subset of mice underwent a sham operation and received rAAV6:CON. Cardiac function was assessed by echocardiography ~15 weeks post-surgery, and tissue collected for assessment of morphological, histological and biochemical parameters (Figure S1).

Cardiac expression of FLAG-MCAD at the end of the treatment period was confirmed by Western blot (Figure 4A). Two mice from the TAC rAAV6:MCAD group were excluded due to absent expression of FLAG by Western blot (Figure S4). Fifteen weeks of TAC induced significant increases in heart weight. Heart weight/tibial length increased by ~60% in TAC rAAV6:CON vs. sham rAAV6:CON, and ~45% in TAC rAAV6:MCAD vs. sham rAAV6:CON (Figure 4B, Table 4). However, cardiomyocyte cross-sectional area increased to a similar degree in both TAC groups (Figure 4C). Consistent with the development of pathological cardiac hypertrophy, TAC rAAV6:CON mice displayed left ventricular fibrosis (Figure 4D), the expression of pathological stress genes (ANP, BNP and α-skeletal actin; Figure 4E) and downregulation of the gene encoding sarcoplasmic reticulum Ca$^{2+}$ ATPase (SERCA2a; Figure 4F). rAAV6:MCAD administration post-TAC was associated with reduced amounts of left ventricular fibrosis (Figure 4D) and preserved SERCA2a gene expression levels (Figure 4F). There was also a trend for increased capillary density with rAAV6:MCAD treatment (Figure 4G). Finally, rAAV6:MCAD treatment was also associated with increased expression of genes that are positively correlated with mitochondrial biogenesis (PGC1α), intracellular transport of long chain fatty acids (CPT1B) and glucose transport (GLUT4) (Figure 5A-C). However, the expression of other genes associated with fatty acid oxidation (PPARα) and glucose metabolism (PDK4) were unchanged (Figure 5D-E).
Despite favourable effects of MCAD gene therapy on cardiac fibrosis and gene expression in TAC mice, there was no significant improvement in cardiac function at the end of the treatment period (Table 3). However, it was noteworthy that 3 out of the 6 MCAD-treated mice displayed a higher fractional shortening by the end of the treatment period. By contrast, 7 out of 9 mice that received the control vector displayed a lower or no change in fractional shortening at study endpoint. Given MCAD was initially identified as an interesting candidate to study because expression was positively correlated with cardiac function (10), it was of interest to ascertain whether transduction efficiency of rAAV in individual mice was correlated with fractional shortening. Transduction of rAAV (as assessed by measuring FLAG expression) was more efficient in some TAC mice than others (Figure 6A). Regression analysis of fractional shortening with the expression of FLAG in the TAC rAAV6:MCAD group showed a trend (P=0.06) for a positive correlation (Figure 6B).

**DISCUSSION**

The major goal of this study was to generate a cardiac-selective MCAD gene therapy tool to determine the effect of increasing MCAD expression in the adult murine heart, basally and in a setting of cardiac stress. Rationale for these experiments was based on earlier observations that MCAD expression was positively regulated by the cardioprotective kinase PI3K in the adult heart, and depressed in the failing mouse heart in a number of cardiac disease models (MI, AF and DCM). To this end, we cloned the MCAD construct into rAAV6 with a CMV promoter to attain cardiac selectivity, as previously described (6, 9). The most important findings to arise from this study were that MCAD gene therapy (rAAV6:MCAD): 1) induced physiological cardiac hypertrophy in healthy adult mice, and 2) provided a degree of cardiac protection in a model of pressure overload with established cardiac dysfunction.

MCAD has relatively high abundance in the heart and is a key fatty acid metabolism gene. In healthy adult mice, MCAD gene therapy was associated with an increase in heart mass which was characteristic of physiological cardiac hypertrophy, as systolic function was preserved, capillary density was increased, and there were no signs of left ventricular fibrosis or foetal gene expression (hallmarks of pathological, but not physiological, cardiac hypertrophy (32-35)). The
induction of physiological hypertrophy with rAAV6:MCAD was reminiscent of what we had previously observed with rAAV6:caPI3K (also 8 weeks post-AAV administration) (9). Physiological hypertrophy in mice administered caPI3K gene therapy has been attributed to an increase in Akt phosphorylation (9), as Akt is a downstream target of PI3K, and a key regulator of physiological heart growth (36). The mechanism by which the increase in MCAD expression led to an increase in myocardial mass in the current study is unclear, but may involve crosstalk with other hypertrophic signalling pathways (pAkt was not elevated). In a seminal study investigating postprandial cardiac growth in the Burmese python, Riquelme and colleagues (37) demonstrated that circulating fatty acids were responsible for triggering myocardial growth in the python following a meal, and infusion of a cocktail of fatty acids that mimicked fed python plasma induced physiological cardiac hypertrophy in mice (37). Fatty acid-induced hypertrophy of the python heart was associated with activation of three signalling pathways that have been associated with physiological cardiac hypertrophy in mammals: FAK, NRF2 and RhoA (38). Thus, it appears that metabolic and hypertrophic pathways in the heart are linked. Furthermore, while it is well known that cells undergoing growth display changes in metabolism, there is increasing evidence to show that alterations in metabolism are also sufficient for promoting cell growth. The ability of cells to sense changes in metabolism to control cell growth has been reported in the most primitive of organisms. Additional layers of growth control, such as protein kinase networks are reported to have evolved later in the development of multicellular organisms (39). The precise role of MCAD in promoting cardiac growth will require further investigation.

Having established that MCAD gene therapy induced physiological cardiac hypertrophy, we next assessed the therapeutic potential of increasing MCAD in the TAC model of pathological hypertrophy. Previous studies in caPI3K and insulin-like growth factor 1 receptor (IGF1R) transgenic mice provided rationale for promoting physiological signalling in a setting of pathological hypertrophy. Both caPI3K and IGF1R transgenic mice with physiological hypertrophy were protected in settings of pressure overload-induced hypertrophy (4, 40). Instead of mice displaying an exaggerated hypertrophic response as a consequence of hearts being subjected to a physiological stimulus (enhanced IGF1-PI3K signalling due to transgene expression) in addition to a pathological stimulus (pressure overload), mice displayed a comparable or reduced hypertrophic response to pressure overload which was associated with a
more favourable phenotype (e.g. lower fibrosis, more favourable gene expression, better cardiac function) (4, 40). The blunted pathological response appears to be related to physiological signalling inhibiting signalling cascades activated by pathological stimuli (4). In the current study, there was a ~60% increase in normalised heart weight in TAC rAAV6:CON mice relative to sham, compared with a ~45% increase in TAC rAAV6:MCAD mice. However, cardiomyocyte cross-sectional area increased with TAC compared to sham to a similar degree in both the rAAV6:CON and rAAV6:MCAD groups. Despite no significant differences in normalised heart weight or cardiomyocyte cross-sectional area between the TAC groups, TAC rAAV6:MCAD mice displayed a more favourable phenotype than TAC rAAV6:CON mice. The most striking difference was less left ventricular fibrosis in TAC mice treated with rAAV6:MCAD compared with the TAC control mice. In addition, TAC rAAV6:MCAD mice had higher levels of SERCA2a gene expression, a more favourable metabolic gene expression profile, and tended to have a higher capillary density. Collectively, this is consistent with the phenotypes observed in caPI3K and IGF1R transgenic mice subjected to pressure overload (4, 40). Notwithstanding these salutary characteristics of the hearts from TAC rAAV6:MCAD mice, this was not accompanied by a significant improvement in systolic function. Though, in assessing AAV transduction and cardiac function in individual mice, it was apparent that i) AAV transduction was more efficient in some hearts than other hearts, ii) within the TAC rAAV6:MCAD cohort, a greater percentage of mice had a higher fractional shortening post-treatment than the TAC rAAV6:CON mice, and iii) there was a trend for a positive correlation between fractional shortening and AAV transduction. The reason for differential AAV transduction is not entirely clear but may be related to the degree of cardiac pathology prior to AAV delivery (fibrosis deposition, reduced angiogenesis). It is possible that delivering the AAV at an earlier time point, extending the treatment period, or increasing the dose of AAV to increase MCAD levels even further, may provide additional benefit.

Metabolic remodelling is a key feature of both pathological and physiological cardiac hypertrophy. In the healthy adult heart, fatty acid oxidation is the primary metabolic pathway responsible for generating energy, accounting for 60-70% of ATP production (41). Pathological cardiac remodelling is associated with a substrate switch, with increased reliance on glucose as an energy substrate (42). In contrast, physiological cardiac hypertrophy is associated with
enhanced fatty acid and glucose oxidation (42). PGC1α is a transcriptional coactivator that regulates transcription of PPARα, which in turn regulates the transcription of fatty acid oxidation genes (including MCAD). Administration of rAAV6:MCAD in normal healthy adult mice had no significant effect on the expression of metabolic genes including PGC1α, CPT1b, GLUT4, PPARα and PDK4. However, in the TAC model, MCAD gene delivery was associated with higher expression of PGC1α, CPT1b and GLUT4 compared with TAC control mice. As noted, PGC1α is a critical regulator of fatty acid oxidation genes, and CPT1 is a rate-limiting enzyme of mitochondrial fatty acid uptake. Deficiency of CPT1b in a setting of pressure overload or conditional deletion in muscle under basal conditions was associated with accelerated cardiac pathology (43, 44). GLUT4 is one of the major glucose transporters in the adult heart. A number of studies from GLUT4 genetic mouse models suggest that GLUT4-mediated glucose transport is an important mechanism for regulating cardiac energy metabolism and growth, and protecting the heart in settings of stress (45). Consistent with the current study, the TAC model has previously been shown to be associated with significantly lower, or trend for lower, expression of metabolism related genes including CPT1b, PGC1α, and PPARα (46, 47). Furthermore, TAC was reported to lead to accelerated HF in PGC1α knock out mice, and MCAD was one of the most down-regulated metabolic genes (47).

The current study is consistent with prior studies which have suggested that restoring TAC-induced defects in metabolism (e.g. via increasing PGC1α (47), inhibiting acetyl CoA carboxylase 2 (48)) may have therapeutic potential in the failing heart. Given limitations regarding the number of procedures which can be performed on any one mouse, the size of the mouse heart, and access to specialised equipment, it was not possible to perform some analyses e.g. comprehensive metabolic studies (such as isolated perfused hearts with labelled substrates to examine substrate utilisation), global metabolite and gene expression profiling, mitochondrial analyses, and analysis of blood pressure. Another limitation is the smaller than anticipated number of animals in the rAAV6:MCAD TAC group due to the unexpected absence of AAV transduction in some mice, which had to be removed. Given the phenotypes identified in this study, further studies and investigation are warranted but were beyond the scope of the current study. Finally, blood pressure was not assessed after aortic banding or after gene therapy. All mice were subjected to a similar degree of aortic constriction by using the same gauge needle at
the time of the TAC surgery. Whether the cardiac-selective gene therapy had any impact on hemodynamic conditions could be assessed in future studies. However, in the TAC model, in which the degree of cardiac pressure overload is largely dependent on the degree of aortic constriction, any downstream impact of the gene therapy on blood pressure is unlikely to alter the extent of the pressure overload.

Gene therapy using AAVs has been considered a potential therapeutic strategy to selectively target the heart. However, despite many animal studies demonstrating benefit of gene transduction in preclinical models of heart failure, few have translated to the clinic. In addition, the most recent clinical trials have not met primary efficacy endpoints (49-51), most likely because of the difficulties associated with efficiently transducing the human heart (52). Approaches to improve cardiac gene delivery are actively being pursued (e.g. optimal dosing, more efficient gene delivery methods, re-engineered vectors with higher cardiac tropism), and it is anticipated that some of these approaches will be incorporated into future clinical trials. Despite these hurdles, a major finding from these clinical trials was the favourable safety profile of gene delivery vectors targeting the heart in patients, supporting further investigations in this field of gene transfer (53).

In summary, we have developed a tool for interrogating the therapeutic potential of MCAD in the heart. The current study reveals a novel role for MCAD as an inducer of physiological heart growth, and protection against pathological remodelling. Further investigation will be required to identify the critical molecular mechanisms, and the direct effects of MCAD on cardiac metabolism.

**CLINICAL PERSPECTIVES**

- HF is a significant global health problem which is becoming worse as the population ages. As a multifactorial clinical syndrome, HF still represents an epidemic threat, highlighting the need for deeper insights into disease mechanisms and the development of innovative therapeutic strategies.
- Physiological heart growth is considered protective in animals and humans. The two major findings from this study were that MCAD gene therapy 1) induced physiological
cardiac hypertrophy in healthy adult mice, and 2) provided a degree of cardiac protection in a model of pressure overload with established cardiac dysfunction (e.g. attenuation of cardiac fibrosis, maintained Serca2a gene expression and a favourable metabolic gene signature).

- MCAD gene delivery may present a potential therapeutic approach for correcting metabolic disturbances in the failing heart and attenuating cardiac pathology including fibrosis.

ACKNOWLEDGEMENTS

We acknowledge Iśka Carmichael and the Monash Micro Imaging Facility for provision of instrumentation and training. This study was funded by National Health and Medical Research Council Project Grant (APP1009025 to J.R.M), and also supported in part by the Victorian Government’s Operational Infrastructure Support Program. X.J.D., P.G. and J.R.M are National Health and Medical Research Council Senior Research Fellows (IDs 1043026, 1117835, 586604, 1078985). J.R.M was also supported by an Australian Research Council Future Fellowship (FT0001657). B.C.B is supported by an Alice Baker and Eleanor Shaw Fellowship (The Baker Foundation, Melbourne, Australia). K.L.W was supported by an Australian Postgraduate Award and a National Heart Foundation Overseas Training Fellowship (O12M6802). The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

J.R.M. conceived the study; B.C.B coordinated the study; K.L.W. designed the Acadm construct; B.C.B., K.L.W., T.P., X.M.G., H.K., N.C., Y.K.T., E.J.H.B., X.J.D. and J.R.M. performed experiments; H.Q. made the AAV; P.G. provided advice regarding the Acadm construct and contributed analytical tools; B.C.B., K.L.W. and J.R.M. analysed data, wrote the paper and prepared the figures; B.C.B., K.L.W., H.K., P.G. and J.R.M critically reviewed and edited the paper. All authors reviewed the manuscript and approved the final version.
REFERENCES

51. Chung ES, Miller L, Patel AN, Anderson RD, Mendelsohn FO, Traverse J, et al. Changes in ventricular remodelling and clinical status during the year following a single administration of
FIGURE LEGENDS

Figure 1: Acadm expression is elevated in a setting of physiological hypertrophy and reduced in settings of cardiac stress. A) Microarray analysis of Acadm expression in hearts from non-transgenic (Ntg), caPI3K and dnPI3K transgenic mice (control/sham model). B) Validation of microarray by Northern blot analysis in a separate cohort of Ntg, caPI3K and dnPI3K mice (control un-operated adult mice). Representative Northern blots and quantification of Acadm relative to Gapdh. C) Microarray analysis of Acadm expression in non-transgenic mice subjected to sham or myocardial infarction (MI) surgery. D) Northern blot analysis of Acadm expression in mouse models of dilated cardiomyopathy (DCM) and atrial fibrillation (AF). Representative Northern blots and quantification of Acadm relative to Gapdh (from the same Northern blot). All data are expressed as mean ± SEM. One-way ANOVA followed by Fisher’s post-hoc tests (A, B, D); unpaired t-test (C). N=3-4/group. *P<0.05 vs. Ntg/Sham. †P<0.05. E) Gene expression of Acadm in different mouse tissues by qPCR normalised to liver. N=5/group. Data expressed as mean ± SEM. *P<0.05 vs. kidney, liver, lung, spleen and skeletal muscle. One-way ANOVA followed by Fisher’s post-hoc tests

Figure 2: rAAV6:MCAD transduces heart muscle and leads to MCAD expression in vivo. A) Design of new MCAD gene therapy tool. ITR = inverted terminal repeat; SpA = synthetic polyadenylation signal. AAV components (ITR regions, rep and cap genes) are shown in red; adenoviral components (VA, E2A and E4 regions) are shown in green. B) FLAG-tagged MCAD is detectable in HEK 293 cells transfected with the plasmid used to generate rAAV6:MCAD (MCAD). CON = control plasmid. C) FLAG-tagged MCAD is detectable in heart lysates from mice administered 2x10^{11} vg rAAV6:MCAD (M). Representative Western blots probed with anti-FLAG and anti-MCAD antibodies. C = control vector, rAAV6:CON. D) Mice administered 2x10^{11} vg rAAV6:MCAD (M) express FLAG-tagged MCAD in heart (He) and skeletal muscle (Sk), and at very low levels in the liver (Li), but not in the kidney (Kid), spleen (Spl) or lung (Lu). Representative Western blot. C = control vector, rAAV6:CON.

Figure 3: Delivery of rAAV6:MCAD in healthy adult mice induces heart growth reminiscent of physiological hypertrophy. Mice were assessed 8 weeks after a single
intravenous injection of $2 \times 10^{11}$ vg rAAV6:MCAD or a control vector (rAAV6:CON).  

**A)** Representative hearts at dissection and heart weight (HW) normalised to tibial length (TL). Scale bar = 0.2 cm.  

**B)** Representative cross-sections of the left ventricle, stained with wheat germ agglutinin (WGA), and quantification of cardiomyocyte cross-sectional area. Scale bar = 25 μm. For each heart, 300-500 cardiomyocytes were measured and the area averaged.  

**C)** Atria weight (AW) normalised to TL.  

**D)** Representative M-mode echocardiograms and quantification of left ventricular posterior wall (LVPW) thickness and fractional shortening.  

**E)** Representative cross-sections of the left ventricle, stained with WGA and isolectin B4, and quantification of capillary density. Scale bar = 25 μm.  

**F)** Representative cross-sections of the left ventricle stained with Masson’s trichrome and quantification of left ventricular (LV) fibrosis. Scale bar = 200 μm.  

**G)** Lung weight (LW) normalised to TL.  

**H)** Representative Northern blots of *Anp* and *Bnp* relative to *Gapdh* in hearts of mice that received rAAV6:CON (C) and rAAV6:MCAD (M). Expression of *Anp* and *Bnp* in a non-transgenic (Ntg) mouse and a mouse with heart failure (HF, due to overexpression of Mst1) is shown for comparison. All data are presented as mean ± SEM. N=4-5/group, except in panel F (3-4/group). Unpaired *t*-tests. *P*<0.05 vs rAAV6:CON.  

**Figure 4: Delivery of rAAV6:MCAD attenuated fibrosis and restored SERCA2a levels in a mouse model of pressure overload.** Male mice were subjected to transverse aortic constriction (TAC) or a sham operation and received $2 \times 10^{11}$ vg rAAV6:MCAD (M) or rAAV6:CON (C). Mice were dissected ~15 weeks post-surgery.  

**A)** Representative Western blots showing cardiac expression of FLAG-MCAD and endogenous MCAD in mice administered rAAV6:MCAD or rAAV6:CON.  

**B)** Representative hearts at dissection and heart weight (HW) normalised to tibial length (TL). Scale bar = 0.2 cm. Data are expressed as mean ± SEM. N=4 (sham), 6-9 (TAC) per group. One-way ANOVA followed by Fisher’s post-hoc tests. ***P*<0.001 vs. Sham, **P*<0.01 vs. Sham.  

**C)** Representative cross-sections of the left ventricle, stained with wheat germ agglutinin, and quantification of cardiomyocyte cross-sectional area. Scale bar = 25 μm. For each heart, >100 cardiomyocytes were measured and the area averaged. Data are expressed as mean ± SEM. N=3 (Sham), 6-9 (TAC) per group. One-way ANOVA followed by Fisher’s post-hoc test. *P*<0.05 vs Sham.  

**D)** Representative cross-sections of the left ventricle stained with Masson’s trichrome and quantification of fibrosis. Scale bar = 100 μm. Data are expressed as mean ± SEM. N=4 (Sham), 5-9 (TAC) per group. One-way ANOVA followed by Fisher’s post-hoc test.
qPCR quantification of *Anp*, *Bnp*, *α-skeletal actin* and *Serca2a* expression relative to *Hprt1*. Data are expressed as mean ± SEM. One-way ANOVA followed by Fisher’s post-hoc tests. N=3 (Sham), 6-9 (TAC) per group. *P<0.05 vs. Sham. †P<0.05.

**G)** Representative cross-sections of the left ventricle, stained with wheat germ agglutinin (WGA) and isolectin B4, and quantification of capillary density. Scale bar = 25 μm. Data are expressed as mean ± SEM. N=9 (TAC rAAV6:CON) and 6 (TAC rAAV6:MCAD) per group. Unpaired t-test.

**Figure 5:** Delivery of rAAV6:MCAD was associated with an improved metabolic gene expression profile. Male mice were subjected to transverse aortic constriction (TAC) or a sham operation and received 2x10^{11} vg rAAV6:MCAD (M) or rAAV6:CON (C). qRT-PCR quantification of A) PGC1α (*Ppargc1a*), B) CPT1B (*Cpt1b*), C) GLUT4 (*Glut4*), D) PPARα (*Ppara*) and E) PDK4 (*Pdk4*). Data are expressed as mean ± SEM. Unpaired t-tests. N=3 (Sham), 6-9 (TAC) per group. †P<0.05.

**Figure 6:** rAAV6:MCAD transduction efficiency and correlation with cardiac function. A) Differential expression of FLAG as a marker of AAV transduction efficiency in TAC mice. rAAV6:MCAD (M) or rAAV6:CON (C). B) Linear correlation of fractional shortening and FLAG expression (marker of AAV transduction efficiency) in the TAC rAAV6:MCAD group. P=0.06, Pearson correlation.
Table 1: Morphological data from wildtype mice 8 weeks after administration of $2 \times 10^{11}$ vg rAAV6:CON or rAAV6:MCAD

<table>
<thead>
<tr>
<th></th>
<th>rAAV6:CON</th>
<th>rAAV6:MCAD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. of animals</strong></td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>34.8 ± 1.7</td>
<td>33.7 ± 1.1</td>
</tr>
<tr>
<td><strong>Tibial length (mm)</strong></td>
<td>16.2 ± 0.1</td>
<td>16.5 ± 0.1</td>
</tr>
<tr>
<td><strong>HW (mg)</strong></td>
<td>134.2 ± 9.5</td>
<td>166.9 ± 8.8*</td>
</tr>
<tr>
<td><strong>AW (mg)</strong></td>
<td>6.9 ± 0.4</td>
<td>9.9 ± 0.8*</td>
</tr>
<tr>
<td><strong>LW (mg)</strong></td>
<td>139.3 ± 2.2</td>
<td>151.4 ± 4.5</td>
</tr>
<tr>
<td><strong>KW (mg)</strong></td>
<td>390.6 ± 30.8</td>
<td>425.5 ± 30.6</td>
</tr>
<tr>
<td><strong>LivW (g)</strong></td>
<td>1.6 ± 0.1</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td><strong>HW/TL (mg/mm)</strong></td>
<td>8.3 ± 0.5</td>
<td>10.1 ± 0.5*</td>
</tr>
<tr>
<td><strong>AW/TL (mg/mm)</strong></td>
<td>0.4 ± 0.0</td>
<td>0.6 ± 0.1*</td>
</tr>
<tr>
<td><strong>LW/TL (mg/mm)</strong></td>
<td>8.6 ± 0.2</td>
<td>9.2 ± 0.3</td>
</tr>
<tr>
<td><strong>KW/TL (mg/mm)</strong></td>
<td>24.1 ± 1.7</td>
<td>25.8 ± 1.8</td>
</tr>
<tr>
<td><strong>LivW/TL (mg/mm)</strong></td>
<td>96.3 ± 7.2</td>
<td>91.1 ± 8.2</td>
</tr>
</tbody>
</table>

HW, heart weight; AW, atrial weight; LW, lung weight; KW, kidney weight; LivW, liver weight; BW, body weight; TL, tibial length.
Data are shown as mean ± SEM.
*P<0.05 vs. rAAV6:CON, unpaired t-test
Table 2: Echocardiography measurements from wildtype mice 8 weeks after administration of $2 \times 10^{11}$ vg rAAV6:CON or rAAV6:MCAD.

<table>
<thead>
<tr>
<th></th>
<th>rAAV6:CON</th>
<th>rAAV6:MCAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>BW, g</td>
<td>32.9 ± 0.6</td>
<td>34.1 ± 1.3</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>599 ± 28</td>
<td>650 ± 15</td>
</tr>
<tr>
<td>LVPW, mm</td>
<td>0.82 ± 0.03</td>
<td>1.00 ± 0.05*</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.99 ± 0.04</td>
<td>3.81 ± 0.13</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>2.34 ± 0.08</td>
<td>2.00 ± 0.13</td>
</tr>
<tr>
<td>FS, %</td>
<td>41 ± 2</td>
<td>48 ± 2</td>
</tr>
</tbody>
</table>

BW, body weight; HR, heart rate; LV, left ventricular; LVPW, LV posterior wall thickness; LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension; FS, fractional shortening.

Data are shown as mean ± SEM.

*P<0.05 vs. rAAV6:CON, unpaired t-test
Table 3: Echocardiography measurements of rAAV6:CON- and rAAV6:MCAD-treated mice at baseline, 4 weeks post-surgery and post-AAV delivery.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>4 weeks post-surgery (prior to AAV administration)</th>
<th>~15 weeks post-surgery / post AAV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>TAC (prior to surgery)</td>
<td>Sham</td>
</tr>
<tr>
<td>rAAV6:CON</td>
<td>4</td>
<td>9</td>
<td>rAAV6:MCAD</td>
</tr>
<tr>
<td>rAAV6:CON</td>
<td>4</td>
<td>9</td>
<td>rAAV6:MCAD</td>
</tr>
<tr>
<td>rAAV6:MCAD</td>
<td>6</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>rAAV6:CON</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>rAAV6:CON</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>rAAV6:MCAD</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>rAAV6:CON</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>rAAV6:CON</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>rAAV6:MCAD</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>BW, g</th>
<th>HR, bpm</th>
<th>LVPW, mm</th>
<th>LVEDD, mm</th>
<th>LVESD, mm</th>
<th>FS, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>4</td>
<td>9</td>
<td>6</td>
<td>4</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>BW, g</td>
<td>27.4 ± 0.9</td>
<td>28.7 ± 0.6</td>
<td>27.0 ± 0.7</td>
<td>27.5 ± 0.9</td>
<td>29.3 ± 0.4</td>
<td>27.9 ± 0.7</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>546 ± 16</td>
<td>539 ± 9</td>
<td>536 ± 20</td>
<td>574 ± 42</td>
<td>555 ± 16</td>
<td>543 ± 18</td>
</tr>
<tr>
<td>LVPW, mm</td>
<td>0.80 ± 0.05</td>
<td>0.77 ± 0.02</td>
<td>0.77 ± 0.02</td>
<td>0.76 ± 0.03</td>
<td>1.00 ± 0.03*†</td>
<td>0.96 ± 0.03*†</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>4.03 ± 0.09</td>
<td>4.18 ± 0.09</td>
<td>4.16 ± 0.06</td>
<td>3.95 ± 0.18</td>
<td>4.33 ± 0.05</td>
<td>4.27 ± 0.17</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>2.65 ± 0.09</td>
<td>2.75 ± 0.08</td>
<td>2.81 ± 0.06</td>
<td>2.52 ± 0.11</td>
<td>3.17 ± 0.07*†</td>
<td>3.09 ± 0.15*†</td>
</tr>
<tr>
<td>FS, %</td>
<td>34 ± 1</td>
<td>34 ± 1</td>
<td>33 ± 1</td>
<td>36 ± 2</td>
<td>27 ± 1*†</td>
<td>28 ± 1*†</td>
</tr>
</tbody>
</table>

BW, body weight; HR, heart rate; LV, left ventricular; LVPW, LV posterior wall thickness; LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension; FS, fractional shortening.

Data are shown as mean ± SEM. Two-Way Repeated Measures ANOVA followed by Fisher's post-hoc test.

* P<0.05 vs. baseline of same group, and sham at same timepoint.
† P<0.05 vs. sham at the same timepoint
‡ P<0.05 vs. same group at 4 weeks post-surgery.
Table 4: Morphological data from mice treated with rAAV6:MCAD or rAAV6:CON ~15 weeks after the induction of pressure overload

<table>
<thead>
<tr>
<th></th>
<th>Sham rAAV6:CON</th>
<th>TAC rAAV6:CON</th>
<th>TAC rAAV6:MCAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>4</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>32.3 ± 0.6</td>
<td>33.3 ± 0.3</td>
<td>32.9 ± 1.1</td>
</tr>
<tr>
<td>Tibial length (mm)</td>
<td>16.7 ± 0.1</td>
<td>16.7 ± 0.1</td>
<td>16.6 ± 0.1</td>
</tr>
<tr>
<td>HW (mg)</td>
<td>134.5 ± 6.4</td>
<td>214.6 ± 11.9***</td>
<td>194.5 ± 8.0**</td>
</tr>
<tr>
<td>AW (mg)</td>
<td>7.4 ± 0.9</td>
<td>11.6 ± 1.4</td>
<td>10.2 ± 1.0</td>
</tr>
<tr>
<td>LW (mg)</td>
<td>146.0 ± 1.6</td>
<td>173.0 ± 8.0*</td>
<td>165.0 ± 5.3</td>
</tr>
<tr>
<td>KW (mg)</td>
<td>405.8 ± 23.6</td>
<td>403.9 ± 14.0</td>
<td>399.0 ± 14.9</td>
</tr>
<tr>
<td>LivW (g)</td>
<td>1.4 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>HW/TL (mg/mm)</td>
<td>8.1 ± 0.4</td>
<td>12.9 ± 0.7***</td>
<td>11.7 ± 0.5**</td>
</tr>
<tr>
<td>AW/TL (mg/mm)</td>
<td>0.4 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>LW/TL (mg/mm)</td>
<td>8.8 ± 0.1</td>
<td>10.4 ± 0.5*</td>
<td>10.0 ± 0.3*</td>
</tr>
<tr>
<td>KW/TL (mg/mm)</td>
<td>24.3 ± 1.4</td>
<td>24.2 ± 0.8</td>
<td>24.1 ± 0.9</td>
</tr>
<tr>
<td>LivW/TL (mg/mm)</td>
<td>82.3 ± 7.8</td>
<td>76.0 ± 4.3</td>
<td>68.1 ± 4.2</td>
</tr>
</tbody>
</table>

AW, atria weight; HW, heart weight; KW, kidney weight; LW, lung weight; LivW, liver weight; TL, tibial length.
Data are shown as mean ± SEM. One-way ANOVA with Fisher's post hoc test [except LW, LW/TL: Krustal Wallis with Dunn post hoc test].
***P<0.001 vs sham, **P<0.01 vs sham, *P<0.05 vs sham.
Figure 2

A. Schematic diagram of the vector plasmid and AAV6 helper packaging plasmid. The vector plasmid encodes the FLAG-MCAD construct, while the AAV6 helper packaging plasmid contains the rep and cap genes.

B. Western blot analysis of HEK 293 cells. Comparison of CON and MCAD samples for FLAG, α-tubulin, MCAD, and GAPDH.

C. Western blot analysis of mouse hearts. Comparison of CON and MCAD samples for FLAG, α-tubulin, MCAD, and GAPDH.

D. Western blot analysis of mouse tissues. Comparison of He, Kid, Sk, Spl, Lu, and Li samples for FLAG and α-tubulin.
Figure 3

A. Heart weight

B. Cardiomyocyte cross-sectional area

C. Atria weight

D. LV wall thickness and systolic function

E. WGA, Isolectin B4, Composite, Capillary density

F. LV fibrosis

G. Lung weight

H. Stress gene expression

Legend:
- rAAV6: CON
- rAAV6: MCAD

Graphs and images comparing heart, atria, LV wall, capillary density, and fibrosis between CON and MCAD groups, with statistical significance indicated by asterisks.
SUPPLEMENTARY INFORMATION

Gene delivery of medium chain acyl-coenzyme A dehydrogenase (MCAD) induces physiological cardiac hypertrophy and protects against pathological remodelling

Bianca C. Bernardo1,2*, Kate L. Weeks1,2*, Thawin Pongsukwechkul1,2, Xiaoming Gao1, Helen Kiriazis3, Nelly Cemerlang1, Esther J.H. Boey1, Yow Keat Tham1,4, Chad J. Johnson5, Hongwei Qian1, Xiao-Jun Du1,4, Paul Gregorevic1,6,7,8 and Julie R. McMullen1,4,9*

1 Baker Heart and Diabetes Institute, Melbourne, 3004, Australia.
2Department of Paediatrics, University of Melbourne, Melbourne, 3010, Australia.
3Department of Biochemistry and Molecular Biology, University of Melbourne, Melbourne, 3010, Australia.
4Department of Medicine, Monash University, Clayton, 3800, Australia.
5Monash Micro Imaging, Burnet Institute, Melbourne, 3004, Australia
6Department of Physiology, University of Melbourne, Melbourne, 3010, Australia.
7Department of Biochemistry and Molecular Biology, Monash University, Clayton, 3800, Australia.
8Department of Neurology, The University of Washington School of Medicine, Seattle, WA 98195, USA.
9Department of Physiology, Monash University, Clayton, 3800, Australia.

#Authors contributed equally to the work

*Correspondence should be addressed to J.R.M, B.C.B or K.L.W

Julie R. McMullen: Email: julie.mcmullen@baker.edu.au Tel: +61 3 8532 1194
Bianca C. Bernardo: Email: bianca.bernardo@baker.edu.au Tel: +61 3 8532 1167
Kate L. Weeks: Email: kate.weeks@baker.edu.au Tel: +61 3 8532 1205
PO Box 6492
Melbourne 3004, Australia
Fax: +61 3 8532 1100
Figure S1: Schematic showing experimental timeline. Prior to surgery, ~10-week old male mice underwent baseline echocardiography (echo) for assessment of heart function. Mice were then subjected to transverse aortic constriction (TAC) or a sham surgery. Following ~4 weeks of TAC, left ventricular remodelling and systolic dysfunction was confirmed by echocardiography, and mice were randomised to receive a single intravenous injection of 2x10^{11} vg rAAV6:CON or rAAV6:MCAD. Cardiac function was assessed by echocardiography ~15 weeks post-surgery (9-10 weeks post-AAV injection), and tissue collected for analysis.
Figure S2: Metabolic gene expression in healthy mice treated with rAAV6:MCAD. qRT-PCR quantification of PGC1α, CPT1B, GLUT4, PPARα and PDK4. Data are expressed as mean ± SEM. Unpaired t-test. N=4-5/group. No significant differences by unpaired t-test.
Figure S3: Phosphorylation of Akt was unaltered in healthy mice treated with rAAV6:MCAD. Top: Representative Western blots probed with phospho-Akt and total-Akt in healthy mice treated with rAAV6:CON (C) or rAAV6:MCAD (M). Bottom: Quantification of pAkt relative to tAkt. Data are expressed as mean ± SEM. Unpaired t-test. N=4-5/group.
Figure S4: FLAG expression in TAC mice treated with rAAV6:MCAD. Representative Western blots probed with anti-FLAG and anti-α tubulin antibodies in TAC mice administered 2x10^{11} vg rAAV6:MCAD. No detectable expression of FLAG in heart lysates from mice run in lanes 3 and 4.