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Phosphoinositide 3-Kinase (p110 α) Gene Delivery Limits Diabetes-induced Cardiac NADPH Oxidase and Cardiomyopathy in a Mouse Model with Established Diastolic Dysfunction

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ABSTRACT

Phosphoinositide 3-kinase [PI3K (p110 α)] is able to negatively regulate the diabetes-induced increase in NADPH oxidase in the heart. Patients affected by diabetes exhibit significant cardiovascular morbidity and mortality, at least in part due to a cardiomyopathy characterised by oxidative stress and left ventricular (LV) dysfunction. Thus, PI3K (p110 α) may represent a novel approach to protect the heart from diabetes-induced cardiac oxidative stress and dysfunction. In the current study, we investigated the therapeutic potential of a delayed intervention with cardiac-targeted PI3K gene therapy, administered to mice with established diabetes-induced LV diastolic dysfunction. Diabetes was induced in 6-week-old male mice by streptozotocin. After 8 weeks of untreated diabetes, LV diastolic dysfunction was confirmed by a reduction in echocardiography-derived transmitral E/A ratio. Diabetic and non-diabetic mice were randomly allocated to receive either recombinant adeno-associated viral vector-6 carrying a constitutively-active PI3K construct (rAAV6-caPI3K, single i.v. injection, 2×10^{11} vector genomes) or null vector, and were followed for a further 6 or 8 weeks. At study endpoint, diabetes-induced LV dysfunction was significantly attenuated by a single administration of rAAV6-caPI3K, administered 8 weeks after the induction of diabetes. Diabetes-induced impairments in each of LV NADPH oxidase, endoplasmic reticulum stress, apoptosis, cardiac fibrosis, and cardiomyocyte hypertrophy, in addition to LV systolic dysfunction, were attenuated by delayed intervention with rAAV6-caPI3K. Hence, our demonstration that cardiac-targeted PI3K(p110 α) gene therapy limits diabetes-induced upregulation of NADPH oxidase and cardiac remodelling suggests new insights into promising approaches for the treatment of diabetic cardiomyopathy, at a clinically-relevant time point (after diastolic dysfunction is manifest).

Keywords: Diabetic cardiomyopathy, reactive oxygen species, myocardial remodelling, superoxide, gene therapy, phosphoinositide 3-kinase

Abbreviations

AAV	Adeno-associated virus
Akt.....	Protein kinase B
ANP.....	Atrial natriuretic peptide
Bax	Bcl-2-associated X protein
Bcl-2.....	B-cell lymphoma 2
caPI3K.....	Constitutively active PI3K (p110 α)
CMV	Cytomegalovirus
CTGF	Connective tissue growth factor
dnPI3K	Dominant-negative PI3K (p110 α)
ER	Endoplasmic reticulum
Nox2.....	NADPH oxidase 2
PI3K	Phosphoinositide 3-kinase
PI3K (p110 α).....	Phosphoinositide 3-kinase, p110 α isoform
PKC β 2.....	Protein kinase C, β 2 isoform
rAAV6-caPI3K	Recombinant adeno-associated-virus 6- caPI3K
STZ	Streptozotocin

Introduction

The alarming global diabetes pandemic is predicted to affect almost 600 million adults by 2035 (1). In addition to substantial healthcare costs, diabetic patients suffer significantly poorer health outcomes, posing a significant heart failure (HF) burden (2,3). Diabetes not only escalates the risk of developing myocardial abnormalities (often initially asymptomatic) as a prelude to HF (4,5), but also increases HF incidence 2.5-fold, independent of age, concomitant hypertension, obesity, dyslipidaemia or coronary heart disease (6). Left ventricular (LV) diastolic dysfunction in particular is common in diabetic patients; 50–75% of asymptomatic, normotensive patients with well-controlled diabetes exhibit diastolic dysfunction (yet are free of concomitant coronary disease or impairments in LV ejection fraction) (4–6). Despite the epidemiological links between diabetes and increased risk of HF, first revealed almost 50-years ago (5,7), there are still no therapies targeting specifically the diabetic heart (8).

Progression of diabetic cardiomyopathy in preclinical models is closely linked to hyperglycaemia-induced generation of reactive oxygen species (ROS), triggering early LV diastolic dysfunction. This often precedes LV systolic impairment and is accompanied by concomitant cardiomyocyte abnormalities (apoptosis, hypertrophy) and cardiac fibrosis (9,10). Phosphoinositide-3-kinases (PI3Ks) are a family of lipid kinases that regulate several physiological functions, including membrane-trafficking, adhesion, cell growth and survival (11–13). The p110 α subunit of PI3K exhibits cardioprotection, at least in part via anti-apoptotic and ROS-limiting properties (12,14,15). Indeed our research has previously identified PI3K as a negative regulator of NADPH oxidase in the heart (14). Cardiac-specific transgenic (Tg) expression of constitutively-active (ca) PI3K reduces susceptibility to cardiac pathologies including pressure-overload-induced hypertrophy (12,16). Further, we previously demonstrated that caPI3K-Tg mice, in which cardiac PI3K(p110 α) activity is

constitutively increased from birth, are protected from subsequent diabetes-induced upregulation of cardiac NADPH oxidase, accompanied by parallel benefits on LV diastolic dysfunction and cardiac remodelling (14). Conversely, mice with reduced cardiac PI3K(p110 α) from birth, via cardiac-specific dominant-negative PI3K (dnPI3K) expression, exhibit exaggerated diabetes-induced cardiomyopathy (14,17), associated with upregulation of LV NADPH oxidase. Hence, PI3K is cardioprotective, preventing diabetes-induced cardiac remodelling and dysfunction. However, whether a delayed intervention to increase PI3K activity in the heart might represent an effective treatment of established diabetes-induced LV oxidative stress and diastolic dysfunction (a clinically-relevant timepoint for treatment), has however not been sought.

In the present study, we investigated the effect of cardiac-targeted PI3K gene therapy in a setting of established diabetic cardiomyopathy (after LV diastolic dysfunction is manifest), at two separate time-points of follow-up. We demonstrate that recombinant adeno-associated virus 6-caPI3K (rAAV6-caPI3K) gene therapy attenuates diabetic cardiomyopathy, with notably greater effects at the later time point, revealing for the first time that a delayed PI3K-mediated therapeutic intervention is effective in the context of diabetes at a clinically-relevant time-point.

RESEARCH DESIGN AND METHODS

AAV generation and administration

The caPI3K construct, previously used to generate caPI3K-Tg mice (iSH2p110 provided by A/Prof Thomas F. Franke (18)), was cloned into an AAV plasmid with a CMV-promoter, packaged into pseudotype-6 capsids (rAAV6) (19) and the SV40-polyadenylation signal replaced with a synthetic poly-(A), as previously described (20). Administration of rAAV6-caPI3K (2×10^{11} vector genomes in 150 μ l of saline), transgene-null vector (rAAV6-null) or saline vehicle control (online supplements), were administered via a single tail-vein injection. This dose of the AAV6 vector with the CMV promoter was previously demonstrated to achieve robust expression of caPI3K selectively in cardiac muscle (20).

Animals

All animal procedures were approved by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee. 6-week-old FVB/N male mice received five consecutive daily i.p. injections of streptozotocin (STZ, 55mg/kg body weight, in 0.1mol/l citric acid buffer, pH4.5; Sigma-Aldrich, St Louis, MO, USA) to induce diabetes, or citric acid buffer vehicle (non-diabetic mice). Diabetes was confirmed by measuring blood glucose every 2-weeks from the saphenous vein using a glucometer (Roche, Basel, Switzerland). Only mice with STZ-induced blood-glucose levels >26 mmol/l were included in the diabetic groups in this study. After 8-weeks of untreated diabetes (or sham), a single injection of rAAV6-caPI3K or null vector was administered. To evaluate time-dependent effects, mice were followed for either 6-weeks (cohort 1) or 8-weeks (cohort 2) after AAV.

Echocardiography

To obtain measures of LV function, echocardiography was performed in anaesthetised mice (ketamine/xylazine/atropine: 60/6/0.7mg/kg i.p.) at baseline, after 8-weeks of diabetes, and

prior to catheterisation (Supplementary Figure 1) utilising a Philips iE33 ultrasound (North Ryde, NSW, Australia) with 15MHz linear (M-mode) and 12MHz sector (Doppler) transducer, as previously described (14). LV posterior wall thickness, LV chamber dimensions and fractional shortening were assessed from M-mode. LV filling was assessed using transmitral Doppler; the ratio of initial (E) and second (A) blood flow velocities (E/A ratio), E wave deceleration time and isovolumic relaxation time (IVRT) were measured.

Cardiac catheterisation

Haemodynamic variables were examined at end-point (20-22weeks-of-age) by catheterisation. LV systolic pressure, LV end-diastolic pressure (LVEDP), maximum and minimum (LV $\pm$ dP/dt) rates of LV pressure change, aortic blood pressure and heart rate were measured in anaesthetised mice (ketamine/xylazine/atropine: 100/10/1.2 mg/kg i.p.) using a 1.4Fr Millar MIKRO-TIP catheter with data acquisition via PowerLab System (AD Instruments, Bella Vista, NSW, Australia) (14).

Tissue collection and histological analyses

Following cardiac catheterisation, hearts were removed (21) and LV sections were cut (4 μ m) and stained with either haematoxylin and eosin (cardiomyocyte width and area), 0.1% Picrosirius Red (collagen deposition) or TUNEL for apoptosis (CardioTACs, Trevigen, MD, USA) (14,17).

LV superoxide levels

Superoxide levels were determined in fresh LV tissue using lucigenin-enhanced chemiluminescence (14). Briefly, small pieces (~1-2mm diameter) of LV were dark-incubated at 37°C for 1h with Krebs-HEPES Buffer, with or without β -nicotinamide adenine dinucleotide phosphate (NADPH 100 μ mol/L). Lucigenin (15 μ mol/L) was added and photons emitted were measured using a multimode plate-reader (Perkin Elmer, Waltham,

USA), and normalised to dry tissue weight (14). The detailed protocol is included in the online supplement.

Gene and protein expression

LV RNA and protein was extracted (21); gene expression was determined via real-time PCR using SYBR[®] Green chemistry (Applied Biosystems, Scoresby, Victoria, Australia). Primers were generated from murine sequences published on GenBank (Supplementary Table 1). Quantitative real-time analysis was performed using ABI Prism[®] 7700 Sequence Detection and the $\Delta\Delta C_t$ method was employed to obtain relative fold differences in expression. Western blotting utilised PVDF membranes, incubated with antibody of interest overnight (Supplementary Table 2), followed by HRP-conjugated anti-rabbit, goat or mouse antibody as appropriate (Cell Signaling, 1:3000) (14,21).

Statistical analysis

Results are expressed as mean $\pm$ SEM. Differences between groups were compared using two-way ANOVA with Fisher's *post-hoc* test. Statistical significance was considered at $P < 0.05$. All relative units are expressed as a fold change with the relevant control group normalised to 1.

RESULTS

Systemic characteristics

Hyperglycaemia was successfully induced by STZ and confirmed by elevated blood glucose and glycated haemoglobin (HbA1c) in diabetic mice (Table 1). Lower body weight and increased liver and kidney weight were also observed in diabetic mice. No differences were observed in null-vector or PI3K-treated mice after AAV in terms of glycaemia, body weight or tibia length (Table 1). These diabetes-induced differences in systemic characteristics were independent of gene therapy administered (rAAV6-caPI3K or null-vector); also observed in saline-treated diabetic mice (Supplementary Table 3). Systolic (SBP) and diastolic blood pressure (DBP) also tended to be lower in diabetic compared to non-diabetic mice (Table 2).

rAAV6-caPI3K transduction of the LV

The plasmid inserted into the AAV6 vector consisted of the CMV-promoter, the caPI3K construct, and a synthetic polyadenylation signal, as described (20) (Figure 1A). In a pilot study, we tested for the presence of a protein band ~40kDa larger than endogenous PI3K (110kDa subunit), due to the large size of the caPI3K-isoform. This provided evidence for successful LV expression of caPI3K protein in mice administered rAAV6-caPI3K. LV caPI3K was detected via western blot analysis, but was not evident in other organs (lung, kidney, liver and gastrocnemius muscle, Figure 1B). Furthermore, LV caPI3K protein was only detected in mice treated with PI3K gene therapy and not in null-vector-treated mice (Figure 1C). Interestingly, the endogenous level of LV PI3K(p110 α) protein (110kDa protein band) was lower in diabetic compared to non-diabetic mice in both cohorts, suggesting a possible endogenous suppression of LV PI3K(p110 α) levels with hyperglycaemia (Figure 1C). To investigate activation of the PI3K pathway, phosphorylation of protein kinase B (Akt, a key downstream target of PI3K(p110 α)) was

measured by western blot. LV Akt phosphorylation was increased (~50%) in non-diabetic mice treated with rAAV6-caPI3K versus null non-diabetic mice in both cohorts 1 and 2 (6 or 8-weeks after gene therapy, Figure 1D). LV Akt phosphorylation was also increased in PI3K-treated diabetic mice in cohort 2 compared with null-vector non-diabetic mice (Figure 1D).

rAAV6-caPI3K attenuates diabetes-induced cardiac dysfunction

Diabetes-induced LV diastolic dysfunction was evident prior to AAV administration, by a reduction in Doppler flow echocardiography-derived E/A ratio after 8-weeks of diabetes (Figure 2A). Mice administered rAAV6-caPI3K were protected from diabetes-induced E/A dysfunction in both cohorts. In contrast, LV diastolic dysfunction was still evident in null-vector-treated diabetic mice (Figure 2A). At study-endpoint, the prolongation of IVRT evident in null-vector-treated diabetic mice (vs non-diabetic pre-AAV), was prevented by rAAV6-caPI3K in cohort 1 ($P<0.05$) and attenuated in cohort 2 ($P<0.001$) (Figure 2B). Deceleration time, another hallmark of LV diastolic dysfunction, was prolonged in null-vector-treated diabetic mice at endpoint (Figure 2C), indicating an ongoing diabetes-induced decline in diastolic function. This was attenuated with PI3K gene therapy in both cohorts (Figure 2C). Echocardiography-derived peak A-wave velocity was increased by diabetes (Table 2), and limited by PI3K gene therapy. Saline-treated mice exhibited comparable LV diastolic dysfunction to null-vector-treated mice after 16-weeks of untreated diabetes (Supplementary Figure 2A-D). At study endpoint, cardiac catheterisation revealed reductions in LV-dP/dt and LV pressure in null vector diabetic mice (Figure 2D, Table 2), which were less marked in diabetic mice administered PI3K gene therapy.

Although no impairments in LV systolic function were evident after 8-weeks of STZ-induced diabetes (data not shown), fractional shortening was impaired by 16-weeks of diabetes, evident in null-vector-treated mice in cohort 2 (Figure 3A). Significantly, this

evidence of systolic dysfunction was prevented by PI3K gene therapy (Figure 3A). Diabetes also tended to decrease velocity of circumferential fiber shortening (V_{cfc}, Figure 3B) in cohort 2 (after 16-weeks of diabetes); these impairments were prevented by PI3K gene therapy. Consistent with this finding, LV+dP/dt was higher in diabetic mice with PI3K gene therapy in cohort 2 (Table 2). LV posterior wall thicknesses, LV end-systolic dimension and LV end-diastolic dimension, peak E wave velocity, peak aortic flow and LV end-diastolic pressure, were unaltered by diabetes or gene therapy (Table 2).

rAAV6-caPI3K protects against diabetes-induced adverse LV remodelling

LV collagen content was increased at study endpoint in null-vector-treated diabetic mice in cohorts 1 and 2 (Figure 4A). Delivery of rAAV6-caPI3K prevented this diabetes-induced increase in LV collagen content in both cohorts (Figure 4A), accompanied by parallel changes in connective tissue growth factor (CTGF) gene expression, which was increased in diabetic mice, attenuated by rAAV6-caPI3K gene therapy in cohort 1 ($P=0.05$) and abolished in cohort 2 (Figure 4B). Diabetes-induced cardiomyocyte apoptosis was evident by an increase in LV TUNEL-positive-stained cells in cohort 2 (Figure 4C), which was prevented by PI3K gene therapy. This was accompanied by an increase in LV Bax:Bcl2 protein (Figure 4D), a marker of apoptosis, in null-vector treated diabetic mice. Again, rAAV6-caPI3K administration prevented the increase of this marker, consistent with the PI3K-mediated increase in anti-apoptotic LV phosphorylated Akt. Heart weight was unaltered by diabetes or gene therapy (Table 1). Despite the absence of net cardiac hypertrophy, STZ-induced diabetes promoted cardiomyocyte hypertrophy in cohort 2, with an increase in cardiomyocyte area and width in null-vector diabetic mice, both of which were prevented by PI3K (Figure 5A). Markers of LV hypertrophy including β -myosin heavy chain and atrial natriuretic peptide (ANP), were increased by diabetes in cohort 1 and 2 (Figure 5B-C). PI3K gene therapy attenuated the increase in β -myosin heavy chain in cohort 1, with

a similar trend in cohort 2; the significant increase in ANP was prevented by PI3K in both cohorts (Figure 5).

Diabetes-induced increased LV superoxide is prevented by rAAV6-caPI3K

Diabetes-induced increase LV protein content of Nox2 and p22^{phox} was evident in cohort 2 (Figure 6A-B). The increased levels of these NADPH oxidase subunits were accompanied by an increase in PKC β 2 protein content in null vector-treated diabetic mice in both cohorts (Figure 6C). Administration of PI3K gene therapy attenuated each of these changes. Moreover, LV superoxide levels were increased 16-weeks after diabetes (cohort 2, Figure 6D), in conjunction with a trend for increased LV Nox4 gene expression (Figure 6E), both of which were limited by PI3K gene therapy.

rAAV6-caPI3K limits diabetes-induced ER stress

LV gene expression of markers of endoplasmic reticulum (ER) stress from cohort 2 was also determined, revealing a tendency for an increase in activating transcription factor-4 (ATF-4) and significant increases in C/EBP homologous protein (CHOP), glucose-regulated protein 94 (Grp94) and ER degradation-enhancing-mannosidase-like-1 (EDEM) in diabetic mice (Figure 7 A-D). PI3K gene therapy attenuated each of these increases. The impact of diabetes and of PI3K gene therapy on two targets of PI3K, namely C/EBP- β and p70S6K, were also determined. We demonstrated that the diabetes-induced increase in C/EBP- β was limited by PI3K gene therapy (Figure 7E). Diabetes also tended to reduce p70S6K phosphorylation; administration of PI3K gene therapy was associated with increased phosphorylation of p70S6K at both the Thr421/Ser424 and Thr389 sites, in mice in cohort 2 compared with diabetic null mice (Figure 7F).

DISCUSSION

Diabetes is a complex metabolic disease characterised by hyperglycaemia with many interacting factors leading to a broad range of complications, closely linked with oxidative stress (6). This includes, in particular, the development of diabetic cardiomyopathy, in which myocardial oxidative stress triggers abnormalities in both cardiac structure and function. The disorder often progresses to HF and ultimately death in affected patients (9,14,21,22). Importantly, even with intensive insulin therapy (or indeed the newer incretin-based therapies) diastolic function is not improved (23) nor heart failure risk reduced in patients, with T2D (24). Furthermore, not all patients currently achieve adequate glucose control (25). Intensive glucose-lowering in high-risk diabetic patients may even worsen the outcome of cardiovascular events (26). These factors, taken together with diabetes-induced insulin resistance and alterations in insulin receptor signalling (27,28), renders conventional therapies such as insulin inadequate for the treatment of diabetes-induced heart failure. The advantage of our approach is that by using caPI3K, PI3K is constitutively activated. Building on our prior evidence that PI3K is a negative regulator of NADPH oxidase in the heart (14), the present study now reveals the first evidence that delayed administration of rAAV6-caPI3K gene therapy attenuates further progression of, and mitigates, the damage associated with diabetic cardiomyopathy in mice with prior diabetes-induced LV diastolic dysfunction. LV diastolic dysfunction, often the first sign of diabetic cardiomyopathy (6), was observed after 8-weeks of STZ-induced diabetes, indicated by impairment in E/A ratio, consistent with previous findings (9,14,17,29). Once diastolic dysfunction was evident, a single injection of rAAV6-caPI3K was administered, and mice were followed for up to a further 8-weeks. In null-vector-treated mice, diabetes-induced impairments in E/A ratio, accompanied by prolongation of IVRT and deceleration time was clearly evident, after 16-weeks of diabetes. These functional abnormalities were prevented or substantially attenuated in mice treated

with rAAV6-caPI3K, with a more pronounced effect in cohort 2 (8-week post-AAV) compared to cohort 1 (6-week post-AAV). Collectively, this suggests that transduction with caPI3K(p110 α) has a favourable impact on diastolic function, at least in part via attenuating cardiomyocyte hypertrophy, oxidative stress, apoptosis and LV fibrosis. We previously demonstrated that caPI3K mice, where the transgene is turned on from birth by the α -myosin heavy chain promoter, were protected from subsequent development of diabetic cardiomyopathy (14). The present study provides the first evidence that a delayed treatment intervention to selectively enhance cardiac PI3K(p110 α) after diabetes-induced LV dysfunction has already commenced, provides substantial benefit in the diabetic heart.

Pathological cardiac hypertrophy, in which the heart and cardiomyocytes enlarge in response to insults such as pressure-overload (20,30), develops initially as a compensatory mechanism to maintain cardiac function. However, with time this transitions into decompensatory hypertrophy (31,32). The present study demonstrated increased LV hypertrophic gene expression (β -myosin heavy chain and ANP), accompanied by increased cardiomyocyte size after 16-weeks of diabetes, consistent with previous reports of STZ-induced diabetes (17,21). Gene therapy with rAAV6-caPI3K blunted these hypertrophic responses. In the present study however, the diabetes-induced cardiomyocyte hypertrophy in cohort 2 was not accompanied by increased heart weight, neither when normalised to body weight or tibia length. Net heart weight represents the combined effects of cardiomyocyte size, cardiomyocyte loss and cardiac deposition of extracellular matrix proteins (6,33). Hence it is likely that diabetes induced a loss of cardiomyocytes due to apoptosis, counteracting the potential enlargement for the concurrent remaining cardiomyocytes to yield a net increase in heart weight (6,22,34), consistent with pathological hypertrophy. Interestingly, physiological hypertrophy (increased cardiomyocyte and heart weight, in the absence of apoptosis or fibrosis) usually associated with increased PI3K(p110 α) expression, was not

observed in rAAV6-caPI3K gene therapy treated non-diabetic mice in this study. This may be due to a relatively modest level of caPI3K expression in the heart. In this study, administration of rAAV6-caPI3K induced a 50% increase in the phospho-Akt/Akt ratio in non-diabetic mice. In contrast, caPI3K-Tg mice, in which a 4-fold increase in phospho-Akt/Akt ratio is evident, display a ~20% increase in heart weight (35).

Hyperglycaemia-related cardiomyocyte apoptosis is also considered to play a key role in the development of diabetic cardiomyopathy (22,36,37). Levels of the cell survival kinase Akt are typically reduced in a setting of increased cell death (38,39). In this study, and our previous study using the same model of diabetic cardiomyopathy, LV Akt phosphorylation tends to increase in the heart of STZ-induced diabetic mice (21). This may represent a mechanism to protect the heart. Despite the increase in pAkt in diabetic mice, the Bax:Bcl2 protein ratio and TUNEL-positive apoptotic cardiac cells, were increased in diabetic mice and attenuated by PI3K gene therapy. Furthermore, PI3K gene therapy increased LV Akt phosphorylation further in diabetic mice in cohort 2 compared with diabetic null mice consistent with its anti-apoptotic properties. In the current study, diabetes-induced LV remodelling developed in association with increased LV ROS levels, consistent with previous reports (9,10,17). An increase in cardiac ROS in pathological settings is sufficient to drive myocyte hypertrophy and LV dysfunction, key features of diabetic cardiomyopathy (9,37,40). Increased ROS is regarded as a key player in the development of diabetic cardiomyopathy, leading to an anti-oxidant/oxidant imbalance, and hence oxidative stress (41,42). We demonstrate here that rAAV6-caPI3K gene therapy attenuated the diabetes-induced increase in LV Nox2, p22^{phox} and Nox4 expression, as well as LV superoxide levels. Diabetes-induced activation of the protein kinase C isoform PKC β 2 is one of the triggers of increased LV superoxide levels in the diabetic heart, and of subsequent pathological cardiac remodelling (15,43). The diabetes-induced increase in LV PKC β 2 in the present study was

abrogated by rAAV6-caPI3K, supporting previous observations in caPI3K-Tg mice (14). Our observations here and previous reports are consistent with PI3K inhibiting PKC β 2 induced pathology, as shown in a previous study in which caPI3K-PKC β 2 double transgenic mice displayed better cardiac function and less fibrosis than PKC β 2 mice alone (15). Thus, down-regulation of PKC β 2 may represent one possible mechanism for PI3K-mediated suppression of LV NADPH oxidase, and hence of superoxide levels, induced by hyperglycaemia. The ability of a single dose of rAAV6-caPI3K to blunt PKC β 2, Nox2, p22^{phox}, Nox4 and superoxide in the diabetic heart are likely to contribute to the observed protection against LV diastolic dysfunction and diabetes-induced impairments.

Increased ROS levels in the diabetic heart have also been linked to the development of ER stress, further contributing to cell death and cardiac dysfunction (44). In the current study, gene expression of the ER chaperone Grp94 was increased in the diabetic heart, in conjunction with a trend for increased ATF-4, which is thought to subsequently trigger transcription of CHOP, leading to cell death (45). In addition, EDEM, which can mediate cell recovery after injury (46), was also increased in the diabetic heart in the present study, perhaps as a compensatory cell defence mechanism attempting to restore normal ER function. In contrast, PI3K gene therapy alleviated each of these impairments in hearts from diabetic mice, possibly contributing to the associated protection against cardiac remodelling and apoptosis in the diabetic heart.

Diabetes-induced adverse cardiac remodelling was also associated with cardiac fibrosis, in addition to cardiomyocyte hypertrophy and apoptosis, which likely further exacerbates impairments in LV function. Diabetes-induced cardiac fibrosis is likely triggered by upregulation of LV ROS levels (9,36,37), via increases in LV transforming growth factor- β 1 and other pro-fibrotic signals (including CTGF, fibronectin and collagen isoforms) (6,47,48). In the present study, diabetes-induced increases in LV collagen content and CTGF

expression were observed, consistent with previous studies in both type-1 and type-2 diabetic mouse models (10,14,17,22). Together with increased LV ROS levels, this further confirms that diabetes induces pathological (as opposed to physiological) cardiac remodelling. Importantly, this diabetes-induced LV fibrotic response was abolished by rAAV6-caPI3K gene therapy. Previous studies have shown that deficiencies in cardiac PI3K activity exaggerate the fibrotic response to pathological insults such as pressure-overload, whereas caPI3K-Tg mice are protected from fibrosis (12,14,16). Whether rAAV6-caPI3K directly suppresses diabetes-induced cardiac fibrosis or the anti-fibrotic action is secondary to suppression of NADPH oxidase, is yet to be determined (14,49).

We have previously observed that LV diastolic dysfunction precedes systolic dysfunction in STZ-induced diabetic mice (14,17,21). Indeed LV systolic dysfunction is often not detected in the first 12-weeks of STZ-diabetes (9,10,14). We show evidence of impaired LV contractile function (progressive decline in fractional shortening and Vcfc) after 16-weeks of diabetes (cohort 2, in conjunction with cardiomyocyte hypertrophy and fibrosis). Significantly, administration of rAAV6-caPI3K gene therapy attenuated systolic dysfunction in cohort 2. These findings are consistent with observations in mice with pressure-overload, in which rAAV6-caPI3K therapy improved systolic function (20). Therefore, the ability of caPI3K(p110 α) to reverse pathological cardiac injuries, shifting the heart from a diseased phenotype to a more physiological phenotype, may thus be responsible at least in part for the improved LV contractile function observed in rAAV6-caPI3K treated diabetic mice in the current study (11,30,50).

Limitations and considerations

In this study, we utilised the STZ diabetic model to assess pathological changes in the diabetic heart. Although the model is largely considered a model of type 1 diabetes, it shares two key characteristics with type 2 diabetes, namely insulin resistance and impaired glucose handling [42, 43]. Therefore, using a delayed intervention of caPI3K(p110 α) gene therapy in this model, after the onset of diastolic dysfunction, may thus offer therapeutic insights for both type 1 and type 2 diabetes. Despite our promising findings, the use of gene therapy comes with its challenges and limitations. The limited packaging capability of the AAV and the large size of the caPI3K construct rendered achieving a higher titre and yield challenging for this particular gene therapy. Improvements in gene packaging would enable more efficient vector production. Furthermore, the use of gene therapy for the treatment of HF has recently encountered setbacks with recent outcomes of the CUPID trial, including poor incorporation of the vector into the myocardium, late therapeutic intervention and incorrect AAV dosing ratio (53). Nonetheless, these merely represent a modest (but not insurmountable) hurdle for gene therapy, identifying a gap for future refinements. Recently the results of an adenylyl cyclase-6 gene transfer trial were released, indicating promising outcomes for HF patients (54). However, the trial was limited to a small sample size, precluding definitive conclusions regarding its efficacy. Although issues to optimise the use of gene therapy remain, promising results and increasing interest has allowed gene therapy to gain significant momentum for therapeutic intervention. In the present study, AAV-mediated gene transfer of caPI3K exerted beneficial effects in preventing progression of diabetic cardiomyopathy.

Conclusions

This study has provided robust evidence that delayed treatment with rAAV6-caPI3K gene therapy regulates LV PI3K(p110 α) levels, and attenuates common characteristics of diabetic cardiomyopathy, despite persistent hyperglycaemia and pre-existent diastolic dysfunction,

with a more prominent effect in the longer follow-up group (summarised in Supplementary Figure 4). These findings, utilising a delayed gene delivery treatment intervention, offer distinct new therapeutic insights for managing the cardiac complication of diabetes to our previous prevention study in transgenic mice (in which PI3K activity was increased from birth, prior to the subsequent induction of diabetes). Here, diabetes-induced diastolic dysfunction (an early hallmark of diabetic cardiomyopathy) was evident before our PI3K treatment intervention was administered, simulating a desirable treatment time point for potential clinical management of diabetic cardiomyopathy. Our observations here thus contribute important pre-clinical groundwork for novel therapeutic approaches for reducing progression to HF and death in diabetes-affected patients, potentially both as a stand-alone therapy or as a supplement to standard care. Although gene therapy is currently considered a costly therapeutic intervention, increased demand for, and technological advancement in the development of gene delivery are likely treatment options in the future. Further studies to overcome these issues may prove beneficial for treating diabetic cardiomyopathy and other types of HF (particularly HF with preserved ejection fraction), and provide proof-of-concept for translation to the clinic.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest associated with this manuscript.

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CONTRIBUTION STATEMENT

RHR, JRM study conception and design; DP, MJD, CQ, SR, HK, HQ performed experiments; DP, HQ, KLW, PG designed and prepared AAV vectors; DP, MJD, HK, JRM, RHR analysed data; DP, MJD, XJD, JRM, RHR interpreted results; DP, JRM, RHR prepared figures; DP, MJD, JRM, RHR drafted manuscript; DP, MJD, CQ, SR, HK, HQ, XJD, KLW, PG, JRM, RHR edited and revised manuscript

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Table 1. Systemic characteristics of diabetic and non-diabetic mice at study endpoint.

	Cohort 1 (6 weeks post AAV)				Cohort 2 (8 weeks post AAV)			
	Non-Diabetic		Diabetic		Non-Diabetic		Diabetic	
	Null	PI3K	Null	PI3K	Null	PI3K	Null	PI3K
n	8	7	7	7	10	8	8	9
Body weight (g)	32.1 ± 0.7	32.0 ± 0.4	30.1 ± 0.5***	29.5 ± 0.5***	35.0 ± 1.0	34.5 ± 1.4	29.8 ± 1.0***	30.4 ± 0.7***
Blood glucose (mmol/L) [#]	11.5 ± 0.5	11.2 ± 0.4	31.4 ± 1.6***	32.1 ± 0.8***	11.7 ± 0.5	11.1 ± 0.4	31.3 ± 0.9***	30.3 ± 1.7***
HbA1c (%)	3.5 ± 0.2	3.9 ± 0.2	8.8 ± 0.6***	8.8 ± 0.5***	3.2 ± 0.1	3.4 ± 0.2	8.1 ± 0.8***	7.9 ± 0.4***
HbA1c (mmol/mol)	16.0 ± 1.9	19.8 ± 1.4	72.8 ± 6.5***	72.5 ± 5.6***	13.7 ± 1.1	15.0 ± 1.5	66.0 ± 8.9***	63.0 ± 4.8***
Heart weight (mg)	140 ± 6	137 ± 2	128 ± 3*	127 ± 2*	141 ± 3	143 ± 2	124 ± 4**	128 ± 3**
LV weight (mg)	101 ± 7	95 ± 2	90 ± 2	88 ± 3	97 ± 3	99 ± 2	84 ± 3	93 ± 2
Lung weight (mg)	157 ± 5	156 ± 6	161 ± 4	160 ± 6	168 ± 3	163 ± 4	158 ± 6	161 ± 5
Liver weight (g)	1.4 ± 0.1	1.3 ± 0.1	1.9 ± 0.1**	1.8 ± 0.0**	1.5 ± 0.1	1.5 ± 0.1	1.9 ± 0.1**	1.9 ± 0.0**

Kidney weight (mg)	216 ± 7	216 ± 7	271 ± 11**	275 ± 9**	223 ± 6	224 ± 6	295 ± 20**	267 ± 14**
Muscle (gastrocnemius) weight (mg)	167 ± 5	167 ± 3	150 ± 6	148 ± 5	177 ± 4	166 ± 4	136 ± 10	142 ± 6
Tibia length (mm)	17.5 ± 0.3	17.7 ± 0.1	17.3 ± 0.2	17.4 ± 0.2	17.3 ± 0.3	17.5 ± 0.2	17.4 ± 0.2	17.5 ± 0.1
Heart weight : tibia length (mg/mm)	8.0 ± 0.4	7.7 ± 0.1	7.4 ± 0.1*	7.3 ± 0.1*	8.2 ± 0.1	8.2 ± 0.1	7.1 ± 0.2**	7.3 ± 0.2**
Heart weight : body weight (mg/g)	4.3 ± 0.2	4.3 ± 0.1	4.2 ± 0.1	4.3 ± 0.1	4.0 ± 0.1	4.2 ± 0.1	4.1 ± 0.1	4.3 ± 0.1

Data are presented as mean ± SEM and analysed by two-way ANOVA followed by Fisher's *post-hoc* test. *P<0.05, **P<0.01, ***P<0.001 vs within-cohort non-diabetic, null-vector-treated mice. #Upper limit of detection for the glucometer was 33.3mmol/l; readings at or above this level were recorded, as 33.3 mmol/l.

Table 2. Echocardiography and cardiac catheterisation analysis of heart function in anaesthetised diabetic and non-diabetic mice at study endpoint.

	Cohort 1 (6 weeks post AAV)				Cohort 2 (8 weeks post AAV)			
	Non-Diabetic		Diabetic		Non-Diabetic		Diabetic	
	Null	PI3K	Null	PI3K	Null	PI3K	Null	PI3K
M-mode echocardiography								
n	7	7	7	6	9	9	6	9
Heart rate (bpm)	402 ± 22	385 ± 18	442 ± 16	443 ± 9†	417 ± 12	395 ± 20	432 ± 9	432 ± 19
LVEDD (mm)	3.9 ± 0.1	3.8 ± 0.1	3.6 ± 0.1	3.8 ± 0.1	3.8 ± 0.1	3.7 ± 0.1	3.6 ± 0.1	3.5 ± 0.1
LVESD (mm)	2.5 ± 0.1	2.4 ± 0.1	2.3 ± 0.1	2.2 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.4 ± 0.2	2.1 ± 0.1
LVPW (mm)	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.2	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
Doppler echocardiography								
n	8	7	5	6	9	8	6	8
Heart rate (bpm)	365 ± 14	361 ± 9	406 ± 11	352 ± 48	371 ± 11	353 ± 13	410 ± 15†	394 ± 12
Peak A wave velocity (cm/s)	41 ± 4	42 ± 3	63 ± 3**	54 ± 4*	45 ± 3	41 ± 3	59 ± 4**	54 ± 4†
Peak E wave velocity (cm/s)	84 ± 8	85 ± 4	85 ± 4	89 ± 6	82 ± 3	83 ± 4	75 ± 4	85 ± 2
Peak Aortic Flow velocity (cm/s)	85 ± 5	95 ± 6	110 ± 12*	97 ± 5	89 ± 4	94 ± 5	100 ± 6	103 ± 5

Cardiac catheterisation								
n	7	7	7	7	10	8	8	9
Heart rate (bpm)	376 ± 11	375 ± 8	402 ± 12	365 ± 13	374 ± 7	386 ± 12	393 ± 11	388 ± 13
LV Pressure (mmHg)	106 ± 4	103 ± 4	91 ± 1**	94 ± 2*†	114 ± 5	114 ± 5	90 ± 3**	101 ± 6
LVEDP (mmHg)	10 ± 1	10 ± 1	8 ± 1	9 ± 1	11 ± 1	11 ± 1	11 ± 1	10 ± 1
LV+dP/dt (mmHg/s)	8642 ± 345	7612 ± 420	8499 ± 465	8475 ± 412	8996 ± 399	10460 ± 533	7668 ± 623	10078 ± 910†‡
Aortic SBP (mmHg)	105 ± 8	108 ± 6	85 ± 3*	89 ± 3	122 ± 5	118 ± 5	91 ± 7**	99 ± 9**
Aortic DBP (mmHg)	68 ± 5	73 ± 4	56 ± 3	56 ± 5	80 ± 3	77 ± 2	57 ± 7**	63 ± 5**

LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension; LVPW, LV posterior wall; LVEDP, LV end-diastolic pressure. Data are presented as mean ± SEM and analysed by two-way ANOVA followed by Fisher's *post-hoc* test. *P<0.05, **P<0.01 vs within-cohort, non-diabetic null vector treated mice, †P< 0.05 vs within-cohort, non-diabetic PI3K treated mice, ‡P<0.05 vs within-cohort, diabetic null vector treated mice.

Figure 1. Successful incorporation of rAAV6-caPI3K into the LV, producing the constitutively active (ca) PI3K (p110 α) protein. (A) The AAV vector used to generate AAV6:caPI3K contained the inverted terminal repeats (ITRs), CMV promoter and synthetic polyadenylation sequence (SpA). (B) Expression of caPI3K transgene is restricted to LV (Lu, lung; Ki, kidney; Li, liver; Mu, muscle). (C) Quantification of endogenous PI3K(p110 α) in the LV (shown as p110 α). (D) Quantification of Akt phosphorylation. N, null-vector; P, rAAV6-caPI3K; ND, non-diabetic; D, diabetic. Data presented as mean $\pm$ SEM separated by cohort; *P<0.05, **P<0.01; n=4-7 per-group.

Figure 2. rAAV6-caPI3K gene therapy attenuates diabetes-induced LV diastolic dysfunction. Quantification of (A) E/A ratio, (B) IVRT and (C) deceleration time obtained from pulsed-wave Doppler echocardiography, before (8 weeks of diabetes) and after gene therapy in cohort 1 (6 weeks after AAV) and 2 (8 weeks after AAV). Representative mitral flow patterns from pulsed-wave Doppler echocardiography are shown. ND, non-diabetic; D, diabetic. (D) LV-dP/dt assessed by cardiac catheterisation. UN, undetermined. Data presented as mean $\pm$ SEM separated by cohort; *P<0.05, **P<0.01, ***P<0.001; n=5-10 per-group. Pre-AAV echocardiography data is combined from both null and PI3K treated mice for their respective cohort.

Figure 3. rAAV6-caPI3K gene therapy protects against diabetes-induced LV systolic dysfunction. Quantification of (A) fractional shortening and (B) velocity of circumferential fibre shortening (Vcfc) obtained from M-mode echocardiography. Representative M-mode echocardiogram is shown. ND, non-diabetic; D, diabetic. Data presented as mean $\pm$ SEM separated by cohort; *P<0.05, **P<0.01, ***P<0.001; n=6-9 per-group.

Figure 4. rAAV6-caPI3K gene therapy limits diabetes-induced LV fibrosis and apoptosis. (A) Percentage of picosirius red stained collagen. Representative LV picosirius red-stained images are shown (magnification x200, scale bar 60µm). (B) LV gene expression of CTGF. (C) Quantification of TUNEL-positive apoptotic cells in LV. Representative sections of LV TUNEL staining are shown (magnification x400, scale bar 60µm). (D) Quantification of Bax:Bcl2 LV protein content. N, null-vector; P, rAAV6-caPI3K; ND, non-diabetic, D, diabetic. Data presented as mean ± SEM separated by cohort; *P<0.05, **P<0.01, ***P<0.001; n=3-9 per-group.

Figure 5. rAAV6-caPI3K gene therapy limits diabetes-induced cardiomyocyte hypertrophy.

(A) Quantification of cardiomyocyte area and width. Representative LV H&E-stained images are shown (magnification x400, scale bar = 60µm); ND, non-diabetic; D, diabetic. LV gene expression of hypertrophic markers (B) β-myosin heavy chain and (C) ANP. Data presented as mean ± SEM separated by cohort; *P<0.05, **P<0.01; n=5-9 per-group.

Figure 6. rAAV6-caPI3K gene therapy inhibits diabetes-induced increases in LV superoxide.. LV densitometry of (A) Nox2, (B) p22^{phox} and (C) PKCβ2 protein levels. N, null-vector; P, rAAV6-caPI3K; ND, non-diabetic, D, diabetic. (D) Quantitative analysis of superoxide levels in fresh LV tissue using lucigenin chemiluminescence. (E) LV gene expression of Nox4 from cohort 2. Data presented as mean ± SEM separated by cohort; *P<0.05, **P<0.01; n=4-9 per-group.

Figure 7. rAAV6 caPI3K gene therapy limits diabetes-induced LV ER stress. LV gene expression of (A) ATF-4, (B) CHOP, (C) Grp94, (D) EDEM and (E) C/EBP-β from cohort

2. (F) LV densitometry of p70S6K phosphorylation from cohort 2. N, null-vector; P, rAAV6-caPI3K; ND, non-diabetic; D, diabetic. Data presented as mean $\pm$ SEM; *P<0.05, **P<0.01; ***P<0.001; n=6-9 per-group.

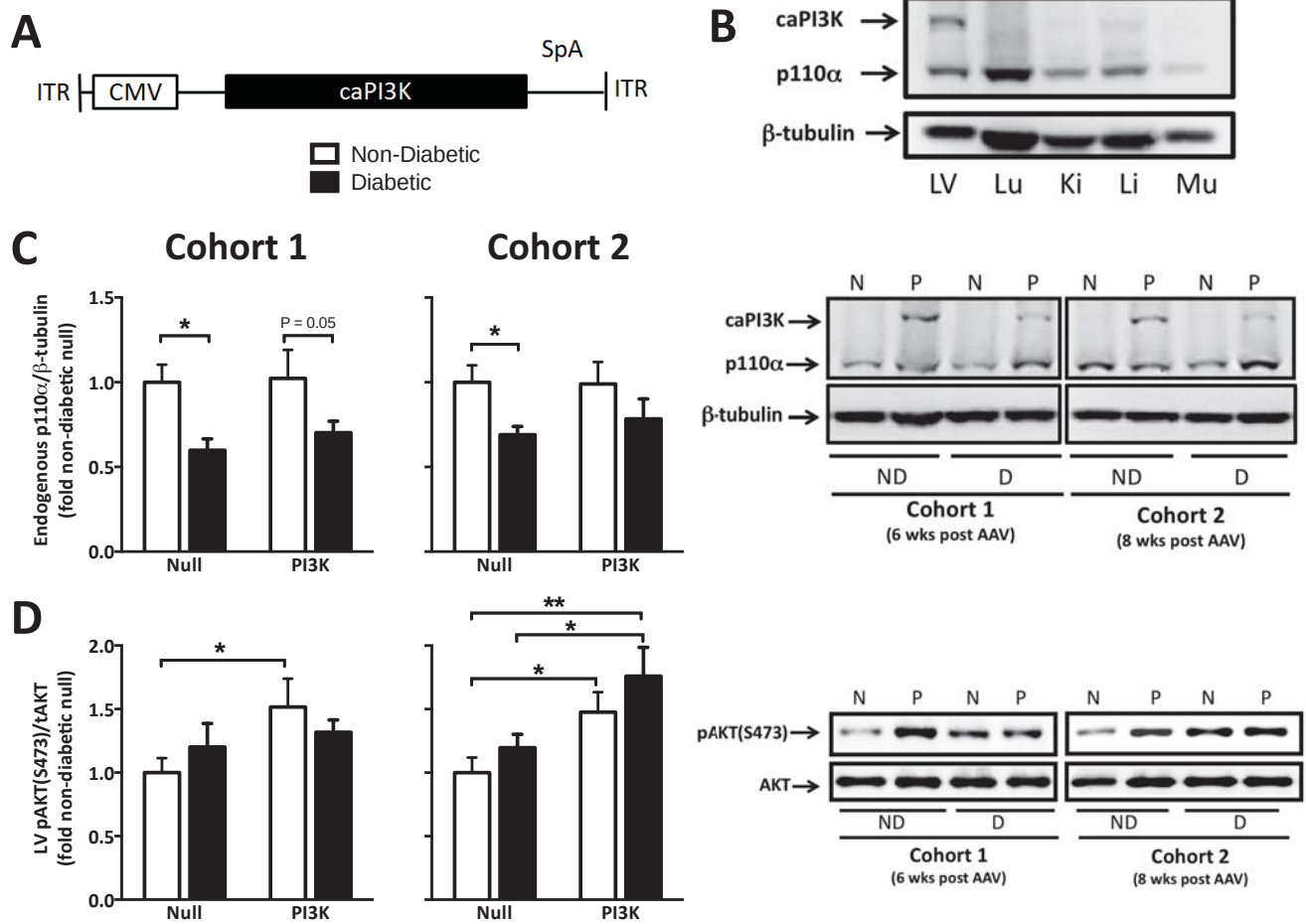


Figure 1

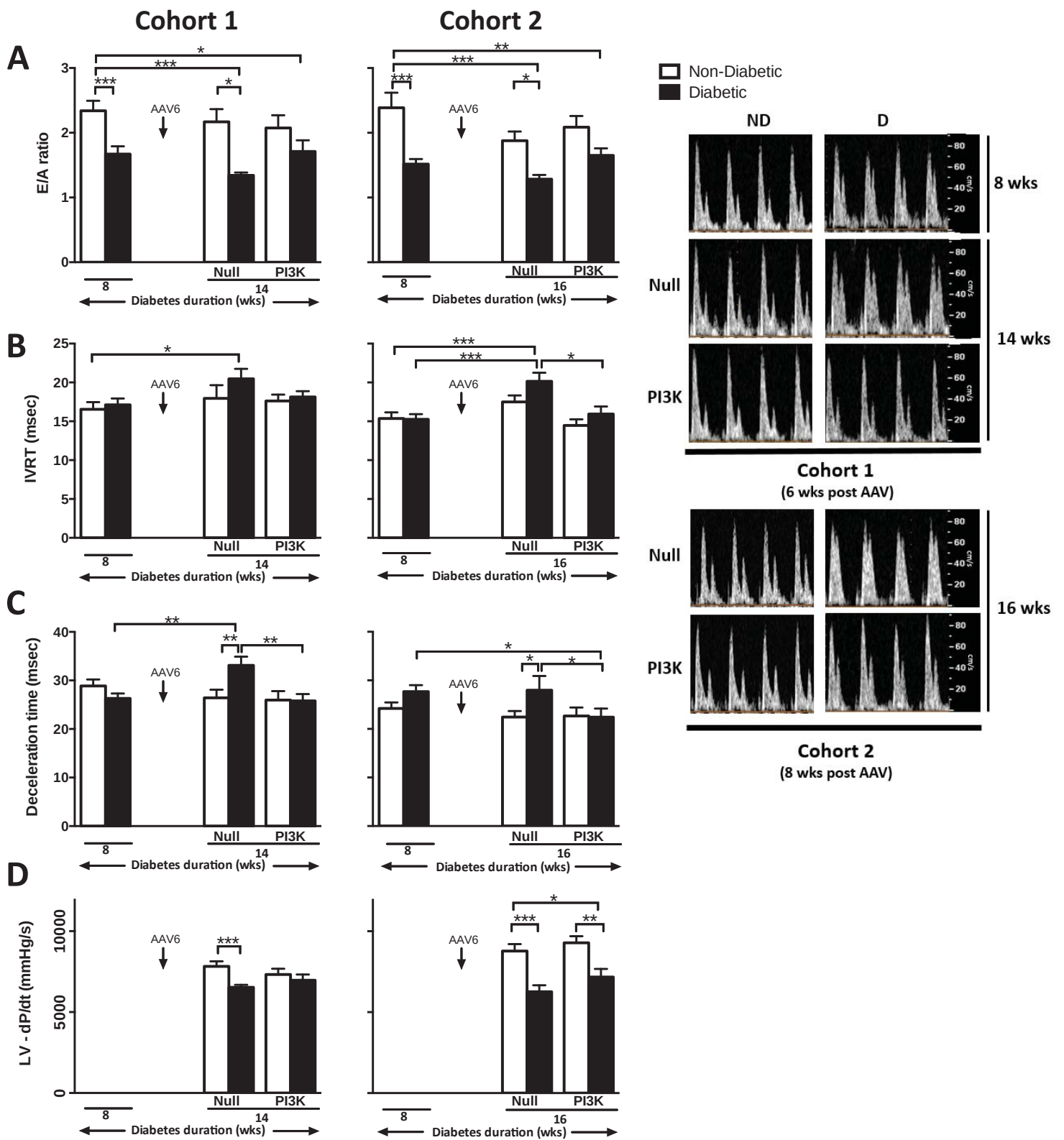


Figure 2

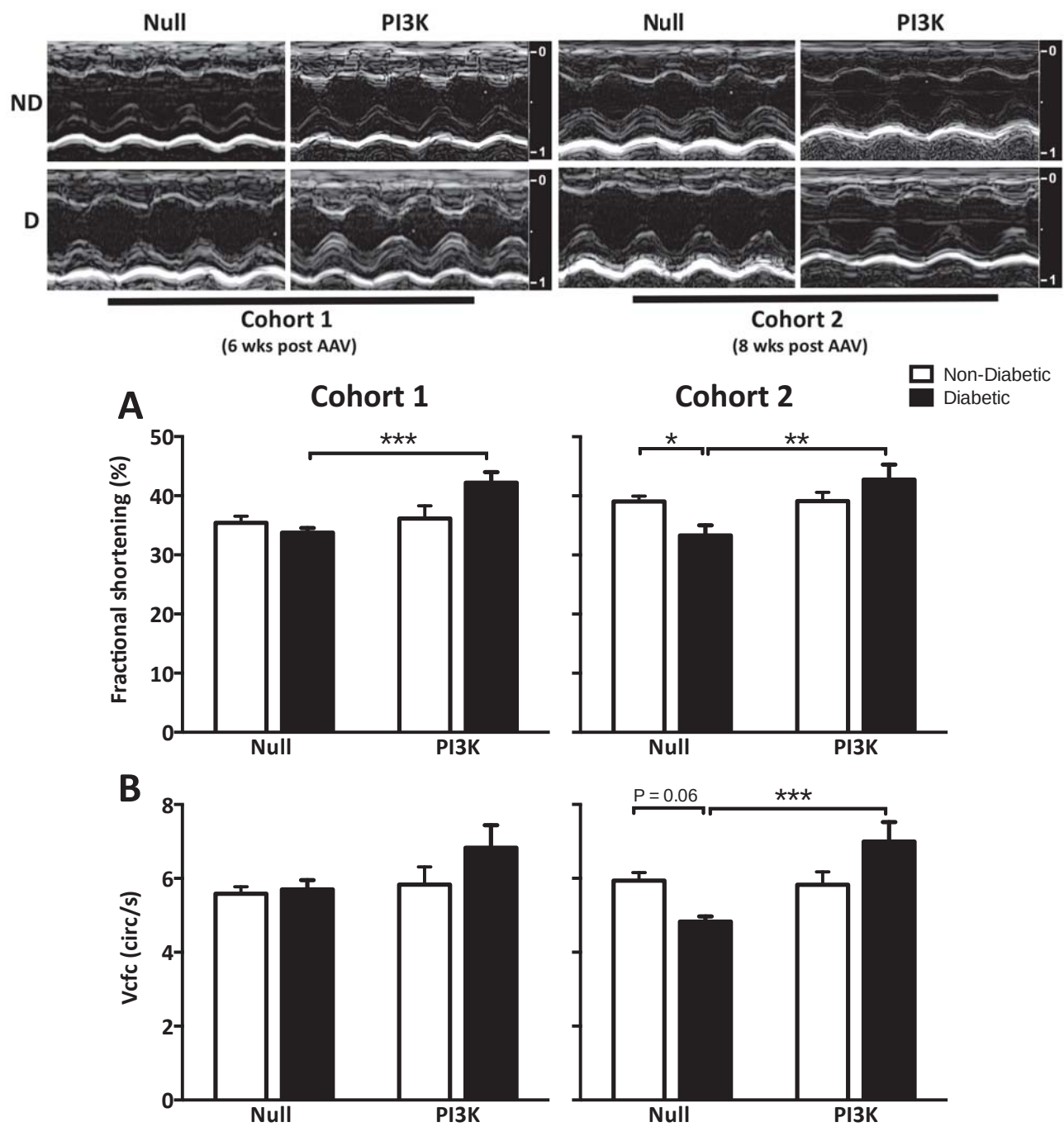
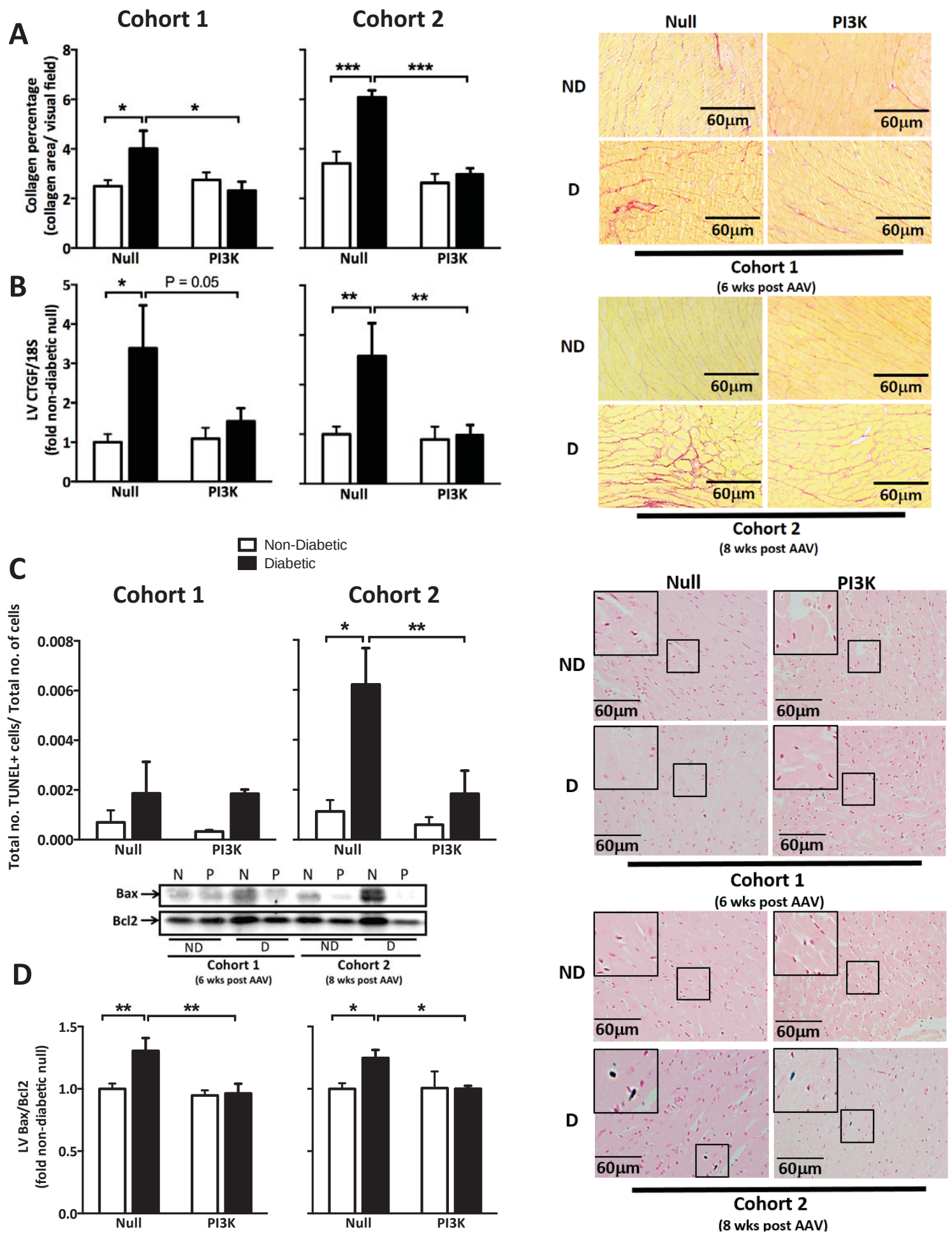


Figure 3



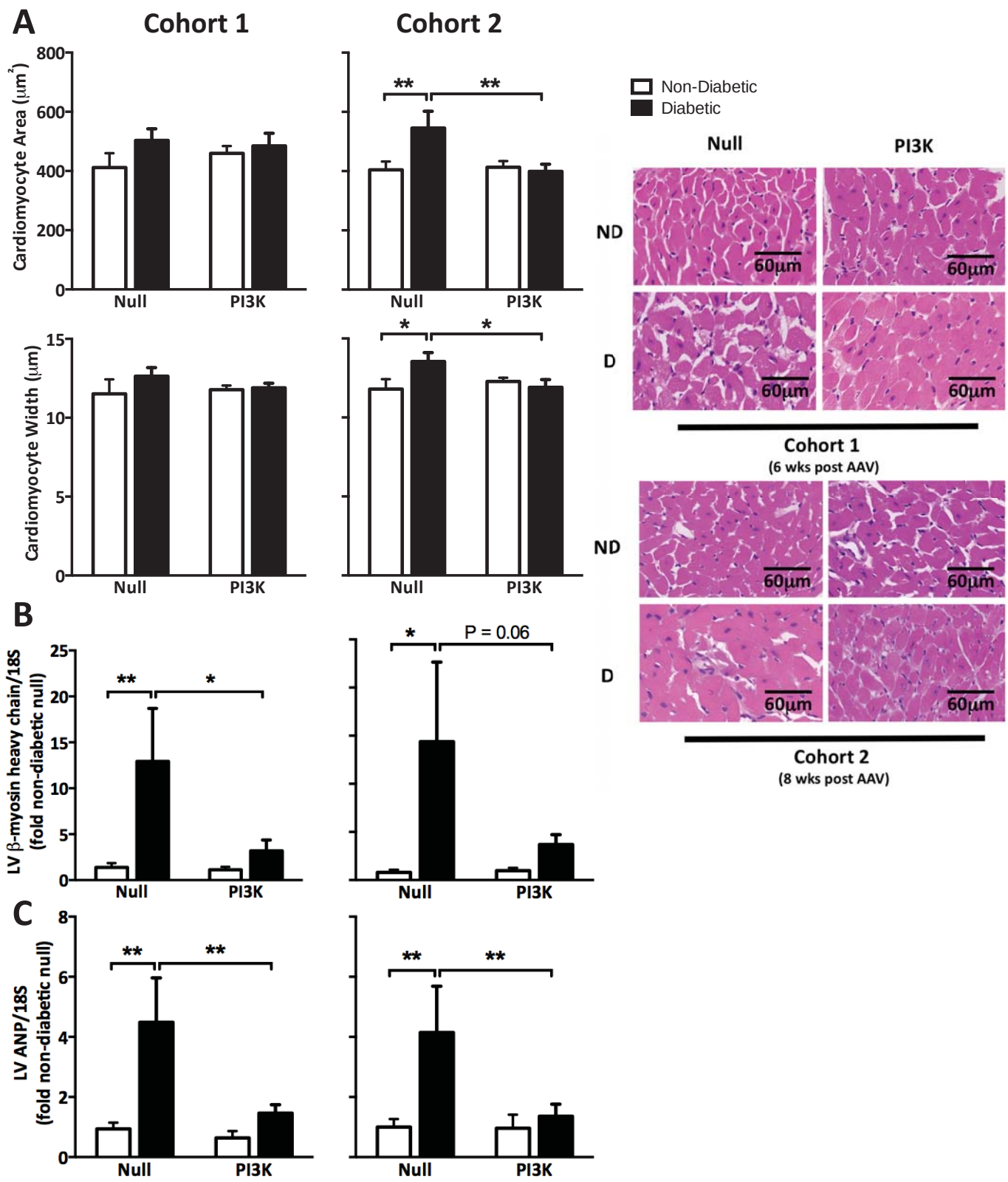


Figure 5

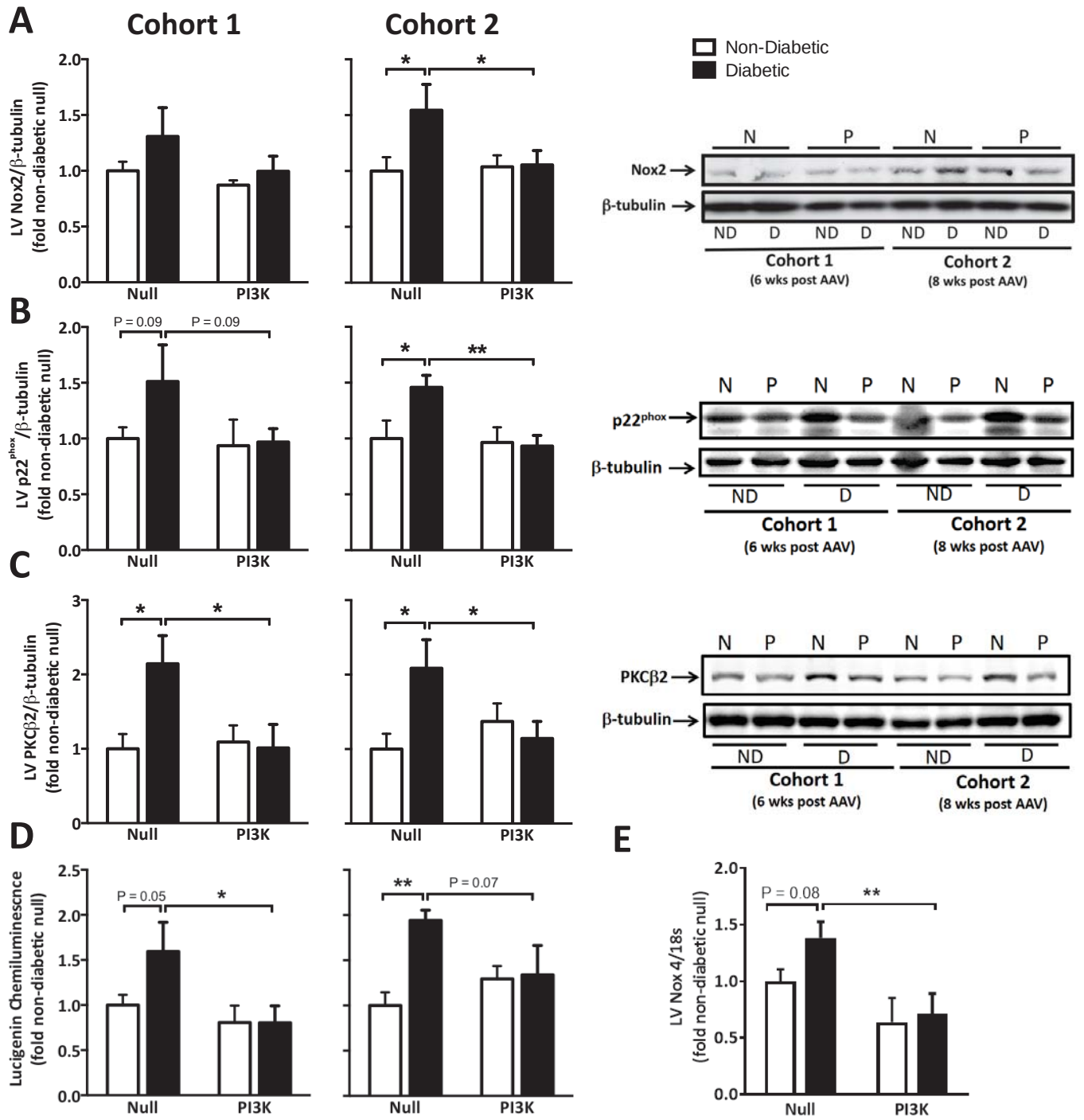


Figure 6

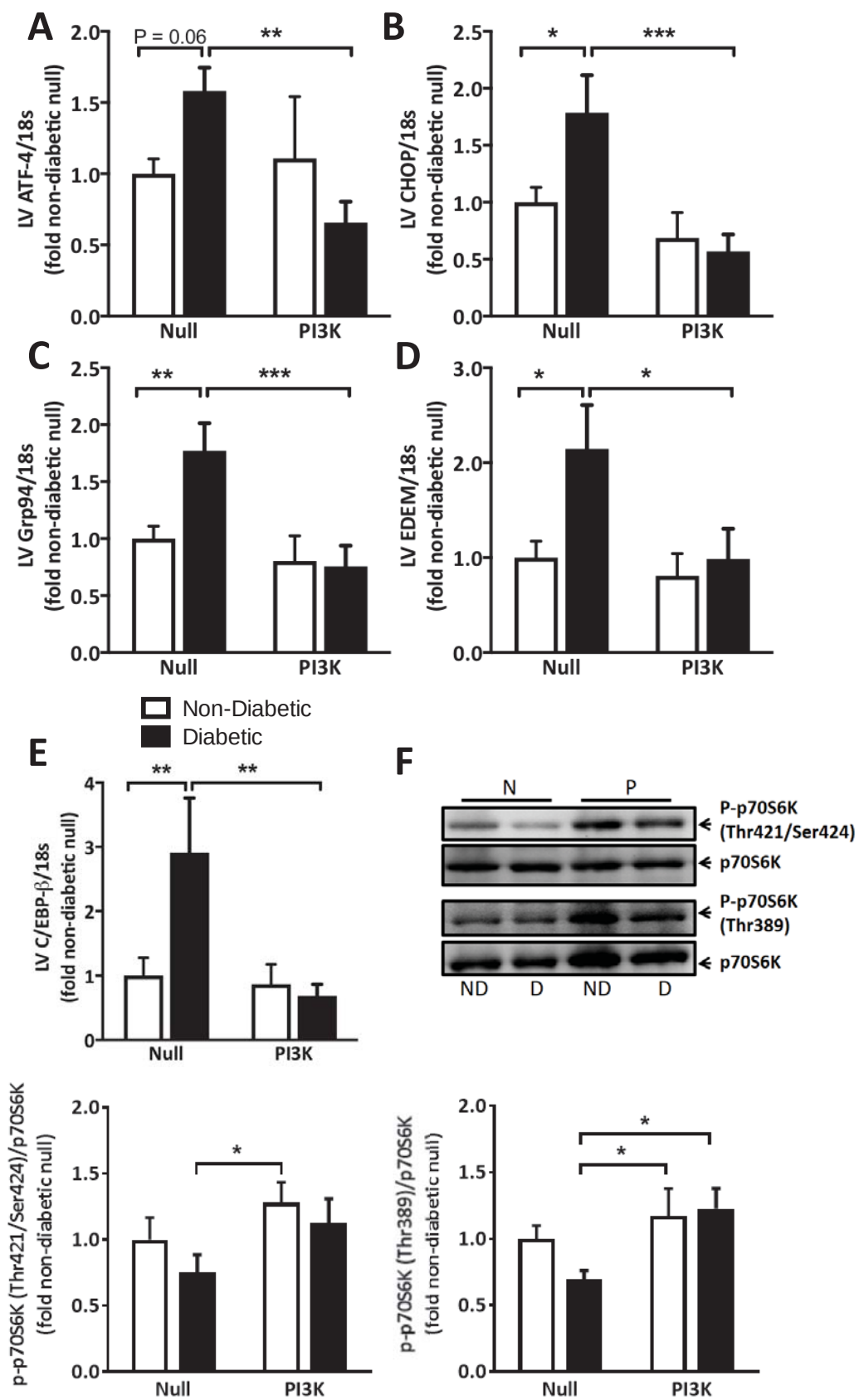


Figure 7

**Phosphoinositide 3-Kinase (p110 α) Gene Delivery Limits Diabetes-induced
Cardiac NADPH Oxidase and Cardiomyopathy in a Mouse Model with
Established Diastolic Dysfunction**

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*D Prakoso and MJ De Blasio are joint first authors (both contributed equally to this work)

[#]JR McMullen and RH Ritchie are joint senior authors (both contributed equally to this work)

Online Supplement

Supplemental methods

Measurement of LV superoxide levels using lucigenin chemiluminescence

Lucigenin-enhanced chemiluminescence was used to estimate NADPH-driven superoxide levels in fresh mouse LV tissue. Briefly, tissues from the LV apex were dissected into 4 small pieces (1–2mm in diameter) and dark-equilibrated at 37 °C for 1 h. Three LV pieces were incubated with 100 μ M β -NADPH containing Krebs-HEPES buffer (NaCl 99mM, KCl 4.69 mM, CaCl₂ 2.5 mM, MgSO₄ 1.20 mM, NaHCO₃ 25mM, KH₂PO₄ 1.03 mM, D (+) glucose 5.6 mM and Na-HEPES 20mM) pH 7.4, in separate wells of an opaque 96-well plate (Crown Scientific, Victoria, Australia). The remaining piece of LV tissue was used as a no-substrate control, incubated in Krebs HEPES buffer only (without β -NADPH). Lucigenin (Bis-N-methylacridinium nitrate; Sigma Aldrich) was added into each of the 4 wells (final concentration 5 μ M, in NADPH-containing Krebs buffer) and the photons emitted (as a result of the reaction between $\cdot\text{O}_2^-$ and lucigenin) were measured (30 x 1-minute reads per plate) using an EnSpire® Multimode Plate Reader (Perkin Elmer, Waltham, USA). Tissues were then dried at 37°C for 48h for determination of tissues weight, to allow calculation of $\cdot\text{O}_2^-$ generation as counts/second/gram of dry tissue weight. Background luminescence (an additional row in each plate, serving as a no-tissue control) was subtracted from the average of the 30 x 1 minute readings. Each measurement was expressed as relative light units per second (RLU/s) normalised to dry tissue weight.

Supplementary Tables

Supplementary Table 1. Primer sequences for quantitative real-time PCR.

Gene name	Primer Concentration (μ M)	Primer sequences (5'–3')	
		Forward	Reverse
Connective tissue growth factor (CTGF)	10	TGGCGAGATCATGAAAAAGA	AGATGTCATTGTCCCCAGGA
β -myosin heavy chain (β -MHC)	10	TCTCCTGCTGTTTCCTTACTTGCTA	GTACTCCTCTGCTGAGGCTTCCT
Atrial natriuretic peptide (ANP)	10	TGGGACCCCTCCGATAGATC	AGCGAGCAGAGCCCTCAGT
Activating transcription factor-4 (ATF-4)	10	GGGTTCTGTCTTCCACTCCA;	AAGCAGCAGAGTCAGGCTTTC
C/EBP homologous protein (CHOP)	10	CCACCACACCTGAAAGCAGAA	AGGTGAAAGGCAGGGACTCA
Glucose regulated protein 94 (Grp94)	10	AAGAATGAAGGAAAAACAGGACAAAA	CAAATGGAGAAGATTCCGCC
ER degradation-enhancing-mannosidase-like 1 (EDEM)	10	CTACCTGCGAAGAGGCCG	GTTTCATGAGCTGCCCCACTGA
NADPH oxidase-4 (Nox4)	10	CTGGAAGAACCCAAGTTCCA	AAAGGCACAAAGGTCCAGAA
CCAT-enhanced-binding proteins- β (C/EBP- β)	10	ACGACTTCCTCTCCGACCTCT	AGGCTCACGTAACCGTAGTCG
18S (housekeeper)	1	TGTTACCATGAGGCTGAGATC	TGGTTGCCTGGGAAAATCC

Supplementary Table 2. Primary antibodies utilised for western blot.

Primary Antibody	Concentration	Supplied and Catalogue
Anti-PI3K(p110 α)	1:1000	Cell Signaling #4249
Anti-phospho(p) Akt (Ser473)	1:1000	Cell Signaling #9271
Anti-Akt	1:1000	Cell Signaling #9272
Anti-GP91phox (Nox2)	1:1000	Santa Cruz #sc-5827
Anti-PKC β 2	1:1000	Santa Cruz #sc-210
Anti-p22phox	1:1000	Santa Cruz #sc-2078
Anti-Bax	1:1000	Cell Signaling #2772
Anti-Bcl2	1:1000	Cell Signaling #2876
Anti-Phospho-p70S6 Kinase (Thr389)	1:1000	Cell Signaling #9205
Anti-Phospho-p70S6 Kinase (Thr421/Ser424)	1:1000	Cell Signaling #9204
Anti-p70S6 Kinase	1:1000	Cell Signaling #9202
Anti- β -tubulin	1:1000	Cell Signaling #2146

Supplementary Table 3. Systemic characteristics at study endpoint for non-diabetic and diabetic mice treated with saline in both cohort 1 and 2 (14 wks and 16 weeks of diabetes). Saline was administered 8 weeks after induction of diabetes or sham.

	Cohort 1 (14wks of diabetes)		Cohort 2 (16wks of diabetes)	
Phenotype	Non-Diabetic	Diabetic	Non-Diabetic	Diabetic
n	5	4	6	5
Body weight (g)	35.4 ± 1.2	30.5 ± 0.8*	36.5 ± 1.3	30.6 ± 2.0*
Blood glucose (mmol/L)	13.7 ± 0.5	33.3 ± 0.0***	11.9 ± 0.8	32.7 ± 0.4***
HbA1c (%)	3.4 ± 0.3	8.3 ± 0.3***	3.4 ± 0.2	8.0 ± 0.3***
HbA1c (mmol/mol)	15.8 ± 2.3	67.5 ± 2.8***	15.4 ± 2.1	64.0 ± 3.1***
Heart weight (mg)	144 ± 6	126 ± 3*	143 ± 2	127 ± 8*
LV weight (mg)	103 ± 7	86 ± 4	105 ± 2	88 ± 8
Liver weight (g)	1.6 ± 0.1	1.7 ± 0.7	1.5 ± 0.0	2.0 ± 0.1**
Kidney weight (mg)	237 ± 13	302 ± 27**	225 ± 10	295 ± 9**
Lung weight (mg)	161 ± 7	162 ± 4	166 ± 4	165 ± 7
Gastrocnemius muscle weight (mg)	167 ± 9	141 ± 2*	175 ± 4	136 ± 11*
Tibia length (mm)	17.8 ± 0.1	17.4 ± 0.1	17.5 ± 0.2	17.6 ± 0.3
Heart weight: tibia length (mg/mm)	8.0 ± 0.3	7.2 ± 0.2	8.2 ± 0.1	7.2 ± 0.3
Heart weight: body weight (mg/g)	4.0 ± 0.1	4.1 ± 0.1	3.9 ± 0.1	4.2 ± 0.2

Data are presented as mean ± SEM and analyzed by two-way ANOVA followed by Fisher's *post-hoc* test. *P<0.05, **P<0.01, ***P<0.001 vs within-cohort non-diabetic mice.

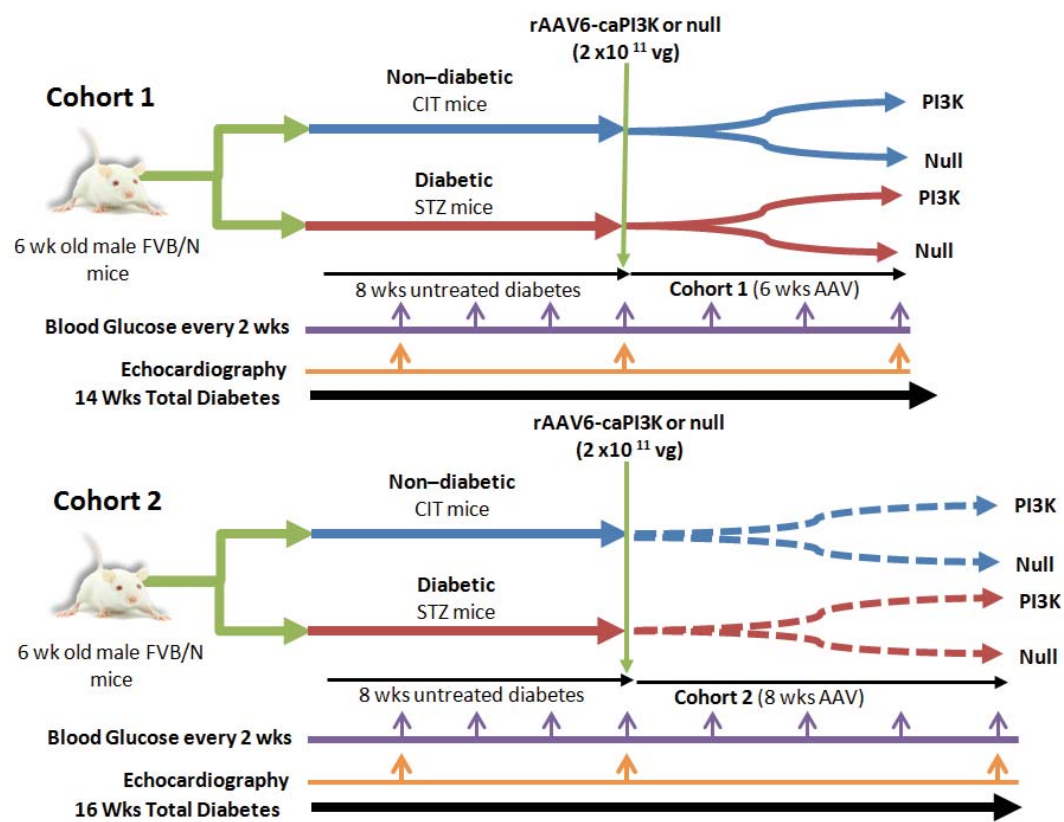
Supplementary Figures

Supplementary Figure 1. Schematic representation of the experimental design. Diabetes was induced in male FVB/N mice by streptozotocin (STZ, 55mg/kg/day i.p. for 5 days) and confirmed by hyperglycemia (>26 mM). After 8 weeks of untreated diabetes, LV diastolic function was determined on E/A ratio (using echocardiography). A single tail vein injection of rAAV6-caPI3K (2×10^{11} vector genomes in 150 μ l saline or null vector control) was administered to both diabetic and non-diabetic, and mice were followed for either a further 6 weeks (cohort 1) or 8 weeks (cohort 2) after AAV.

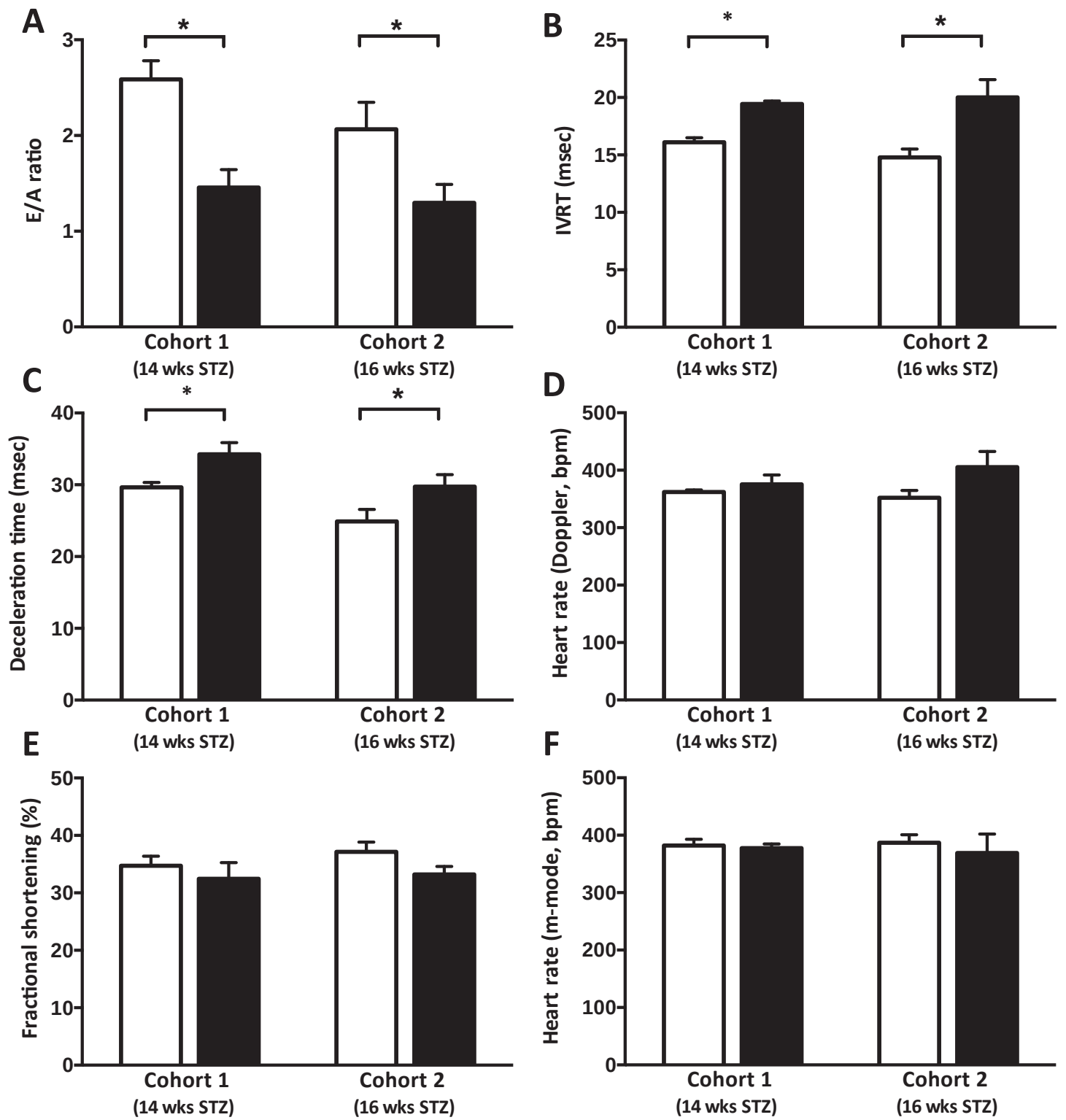
Supplementary Figure 2. Echocardiographic measurement of non-diabetic and diabetic mice administered saline after 8 weeks of diabetes. (A) E/A ratio, (B) IVRT, (C) deceleration time and (D) heart rate obtained from endpoint Doppler echocardiography from both cohort 1 and 2 (14 and 16 weeks of diabetes, respectively). (E) Fractional shortening and (F) heart rate obtained from endpoint M-mode echocardiography from both cohorts. Data are presented as mean $\pm$ SEM and analysed by two-way ANOVA followed by Fisher's *post-hoc* test; $n = 4-6$ per-group. * $P < 0.05$.

Supplementary Figure 3. Cardiac catheterisation measurement of non-diabetic and diabetic mice administered saline. (A) LV-dP/dt, (B) LV+dP/dt, (C) LV end diastolic pressure, (D) heart rate, (E) aortic systolic blood pressure and (F) aortic diastolic blood pressure (obtained from catheterisation) for both cohorts 1 and 2 (14 and 16 weeks of diabetes respectively). Data are presented as mean $\pm$ SEM and analysed by two-way ANOVA followed by Fisher's *post-hoc* test; $n = 4-6$ per-group. * $P < 0.05$, ** $P < 0.01$.

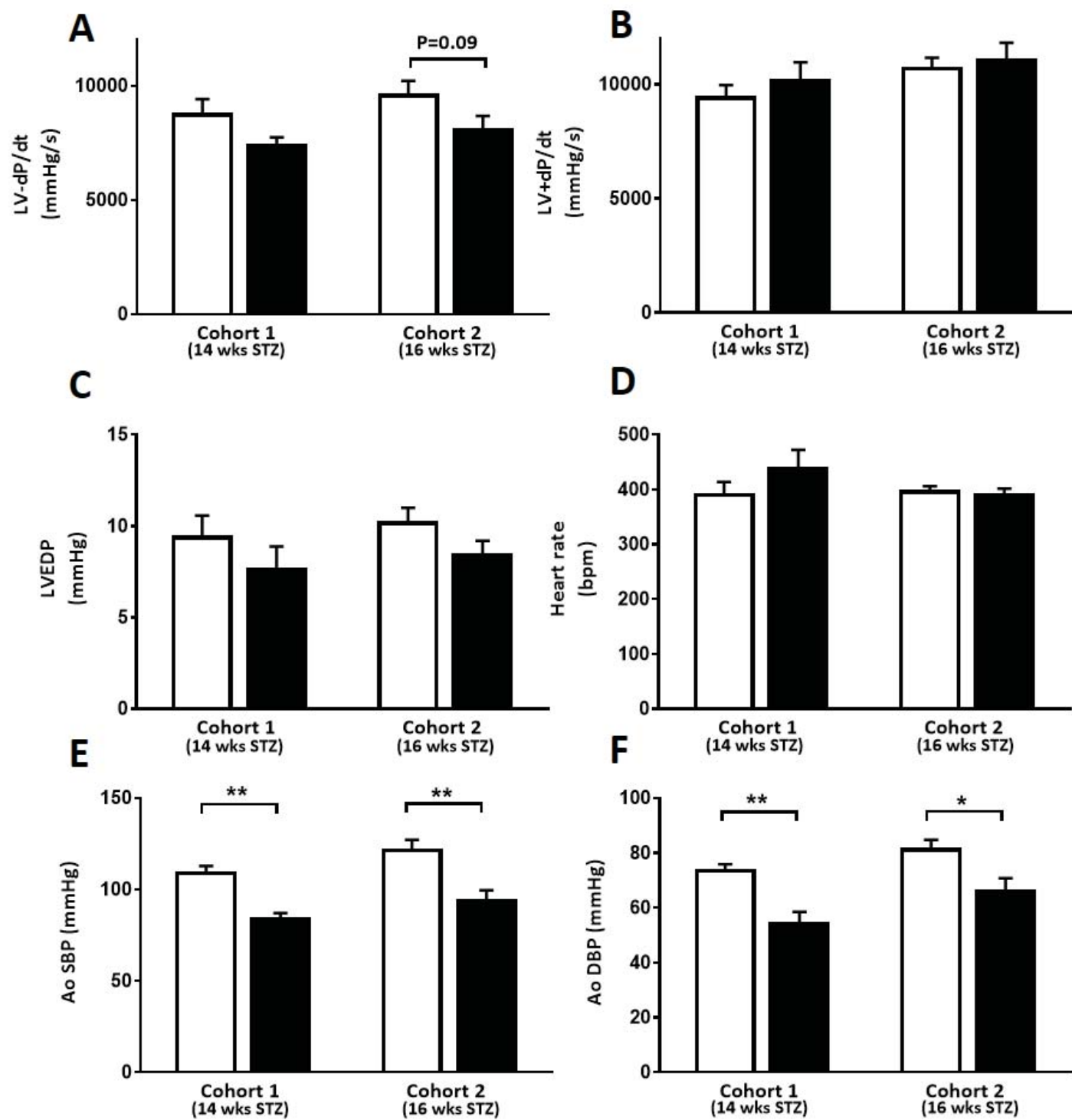
Supplementary Figure 4. Schema depicting proposed mechanism of rAAV6-caPI3K gene therapy. Diabetes-induced hyperglycemia increases production of superoxide in cardiomyocytes. As diabetes progresses, accumulation of superoxide leads to cardiomyocyte hypertrophy and increased apoptosis. The increased in cardiomyocyte apoptosis, permits accumulation of extracellular matrix and cardiac fibrosis. These factors combined cause a decline in LV diastolic function, evident after 8 weeks of diabetes. Administration of rAAV6-caPI3K leads to the LV production of caPI3K protein, activating pro-survival kinase Akt and inhibiting PKC β 2 (which could otherwise exacerbate cardiomyocyte superoxide production). Without intervention, this diabetes-induced cardiac pathology could progress to a decrease in LV systolic function which, in combination with other co-morbidities, may lead to heart failure and death.



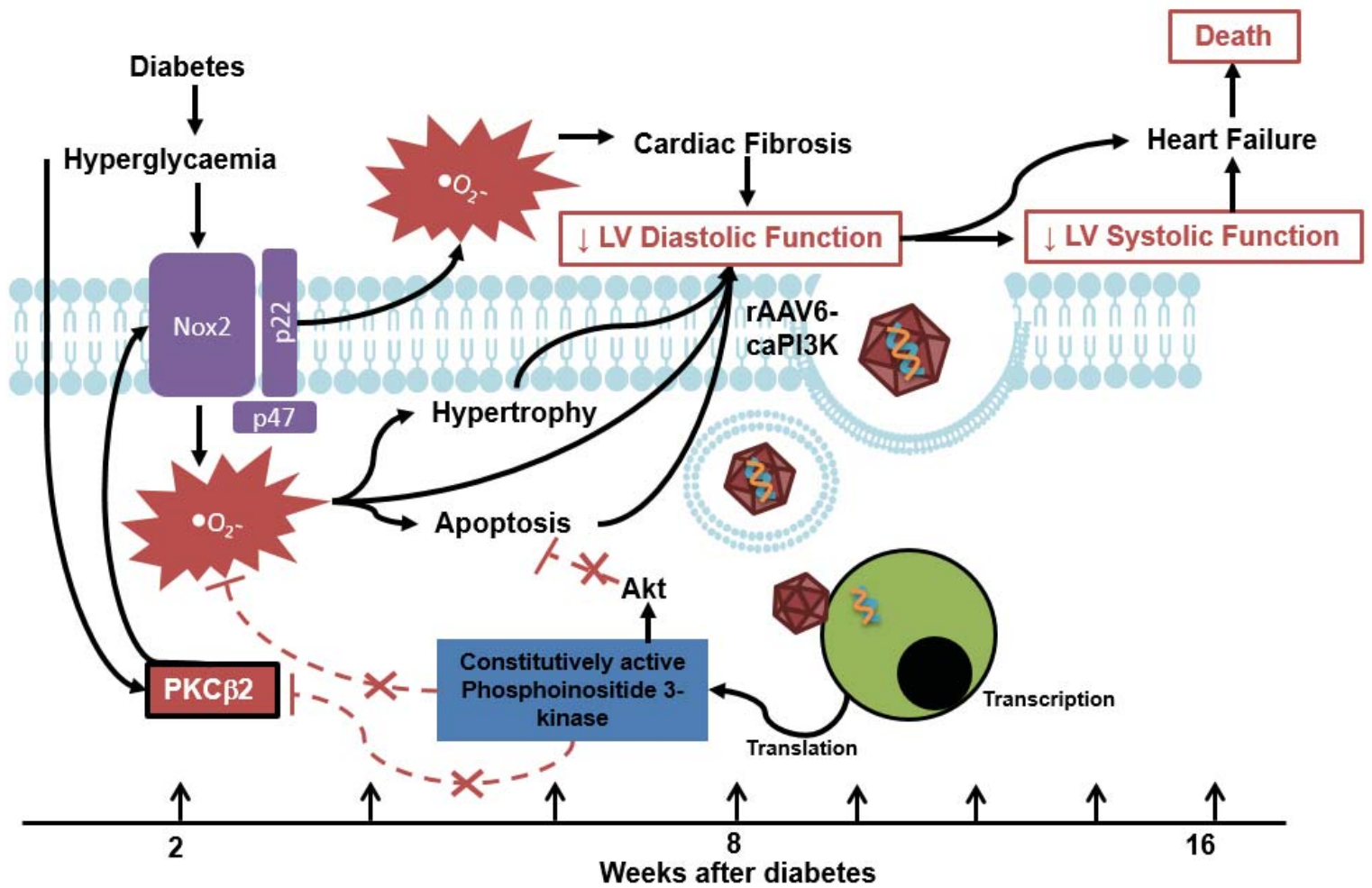
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4