Intravenous Gene Therapy With Pim-1 Via a Cardiotropic Viral Vector Halts the Progression of Diabetic Cardiomyopathy Through Promotion of Prosurvival Signaling

Rajesh Katare, Andrea Caporali, Lorena Zentilin, Elisa Avolio, Graciela Sala-Newby, Atsuhiko Oikawa, Daniela Cesselli, Antonio Paolo Beltrami, Mauro Giacca, Costanza Emanuelli, Paolo Madeddu

**Rationale:** Studies in transgenic mice showed the key role of Pim-1 (proviral integration site for Moloney murine leukemia virus-1) in the control of cardiomyocyte function and viability.

**Objective:** We investigated whether Pim-1 represents a novel mechanistic target for the cure of diabetic cardiomyopathy, a steadily increasing cause of nonischemic heart failure.

**Methods and Results:** In streptozotocin-induced type 1 diabetic mice, Pim-1 protein levels declined during progression of cardiomyopathy, along with upregulation of Pim-1 inhibitors, protein phosphatase 2A, and microRNA-1. Moreover, diabetic hearts showed low levels of antiapoptotic B-cell lymphoma-2 (Bcl-2) protein and increased proapoptotic caspase-3 activity. Studies on adult rat cardiomyocytes and murine cardiac progenitor cells challenged with high glucose confirmed the in vivo expression changes. In rescue studies, anti-microRNA-1 boosted Pim-1 and Bcl-2 expression and promoted cardiomyocyte and cardiac progenitor cell survival under high glucose conditions. Similarly, transfection with Pim-1 plasmid prevented high glucose-induced cardiomyocyte and cardiac progenitor cell apoptosis. Finally, a single intravenous injection of human *Pim-1* via cardiotropic serotype-9 adeno-associated virus (1×10^10 or 5×10^10 plaque-forming units per animal) at 4 weeks after diabetes induction led to sustained cardiac overexpression of Pim-1 and improved diastolic function and prevented left ventricular dilation and failure. Histological examination showed reduced cardiomyocyte apoptosis and fibrosis in association with increased c-kit^+^ cells and cardiomyocyte proliferation, whereas molecular analysis confirmed activation of the prosurvival pathway and conservation of sarcendoplasmic reticulum Ca^{2+}-ATPase and α-myosin heavy chain in Pim-1-treated hearts.

**Conclusions:** Pim-1 downregulation contributes in the pathogenesis of diabetic cardiomyopathy. Systemic delivery of human *Pim-1* via cardiotropic adeno-associated virus serotype-9 represents a novel and effective approach to treat diabetic cardiomyopathy. (Circ Res. 2011;108:00-00.)

**Key Words:** diabetic cardiomyopathies ■ diastolic dysfunction ■ Pim-1 kinase ■ gene therapy ■ cardiac stem cells

A common form of cardiomyopathy directly related to diabetes mellitus (DM), ie, diabetic cardiomyopathy, typically progresses from diastolic dysfunction to heart failure in the absence of coronary artery disease or hypertension.1–3 Studies in animal models illustrate the complexity of the underpinning pathogenic mechanisms (reviewed in Bugger and Abel4). Hence, a deeper understanding of targets for early therapeutic interventions is critically needed.

Recent work from Muraski and colleagues5 uncovered the pivotal role of Pim-1 (proviral integration site for Moloney murine leukemia virus-1), a member of the serine/threonine protein kinase family, in the cardiac cell response to stressors. Promotion of cardiomyocyte survival by Pim-1 is mediated by activation of B-cell lymphoma-2 (Bcl-2), phosphorylation/inhibition of Bcl-2–associated death promoter (Bad), and maintenance of mitochondrial integrity.6,7 By inducing c-Myc, nucleostemin, and cyclin E expression and p21 phosphorylation, Pim-1 increases the proliferative activity of cardiac progenitor cells (CPCs).8,9 Furthermore, Pim-1 acts as a positive modulator of contractility through elevation of sarcendoplasmic reticulum Ca^{2+}-ATPase.5 Pim-1 is positively regulated by the signal transducer and activator of

---

Original received December 13, 2010; revision received March 23, 2011; accepted March 25, 2011. In February 2011, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 13.7 days.

From the Departments of Experimental Cardiovascular Medicine and Vascular Pathology and Regeneration (R.K., A.C., G.S.-N., A.O., C.E., P.M.), School of Clinical Sciences, University of Bristol, Bristol, United Kingdom; Molecular Medicine Laboratory (L.Z., M.G.), International Centre for Genetic Engineering and Biotechnology, Trieste, Italy; and Department of Pathology (E.A., D.C., A.P.B.), University of Udine, Udine, Italy.

Correspondence to Professor Paolo Madeddu, MD, FAHA, Chair of Experimental Cardiovascular Medicine, University of Bristol, Bristol Royal Infirmary–Level 7, BS2 8HW Bristol, United Kingdom. E-mail madeddu@yahoo.com

© 2011 American Heart Association, Inc.

*Circulation Research* is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.110.239111
transcription 3 (STAT3) and protein kinase B (PKB/Akt),\(^6\) which act as modulators of insulin and nutritional status in the heart\(^10\) and are both downregulated in models of diabetic cardiomyopathy.\(^11\) On the other hand, Pim-1 is inactivated by protein phosphatase 2A (PP2A)\(^12\) and constitutes a direct inhibitory target of microRNA-1 (miR-1).\(^13\) However, no information is available on whether PP2A and miR-1 may inhibit Pim-1–associated signaling in the diabetic heart.

The objective of the present study was to explore whether an unbalanced prevalence of negative regulators of Pim-1 could result in the downregulation of prosurvival signaling in diabetic hearts. We also investigated whether forced expression of Pim-1 could inhibit the progression of cardiomyopathy. To this second aim, we used the adeno-associated virus serotype-9 (AAV9), which reportedly allows efficient global cardiac gene transfer after systemic injection.\(^14\)\(^\text{–}^\text{16}\)

### Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

### Ethics

Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and with approval of the British Home Office and the University of Bristol. Type-1 DM was induced in male CD1 mice (Charles River, Margate, United Kingdom) by injection of streptozotocin (STZ; 40 mg/kg body weight IP per day for 5 days).\(^11\) DM was confirmed by regular measurements of blood glucose levels (Online Figure I).

### Biochemical Measurements

Serum insulin (Millipore, Billerica, MA), cholesterol (Biovision, Mountain View, CA), triglycerides (Biovision), and cortisol (R&D Systems, Minneapolis, MN) were measured with ELISA kits.

### In Vivo AAV9–Pim-1 Gene Transfer

AAV9–Pim-1 was prepared by a cross-packaging approach, in which the human (h) Pim-1 gene (hPim-1) was packaged into adeno-associated virus capsid serotype-9 (AAV9), which reportedly allows efficient global cardiac gene transfer after systemic injection.\(^14\)\(^\text{–}^\text{16}\) Four weeks after DM induction, mice manifesting diastolic dysfunction, as assessed by pulsed Doppler analysis of mitral valve velocity,\(^11\) were randomly assigned to receive AAV9–Pim-1 (1×10\(^{10}\) or 5×10\(^{10}\) plaque-forming units [pfu] per animal) or AAV9–β-galactosidase (β-gal) through the tail vein under aseptic precautions; age-matched animals that received STZ–vehicle served as nondiabetic controls (n=16 in each group).

### Hemodynamic Measurements

Dimensional and functional parameters were measured with a high-frequency, high-resolution echocardiography system (Vevo 770, VisualSonics, Toronto, Canada; n=14 per group) as described previously.\(^11\)\(^\text{–}^\text{18}\) Left ventricular (LV) pressure (n=12 per group) was measured with a 1.4F Millar catheter (Millar Instruments, Houston, TX) before animals were euthanized. Myocardial blood flow was assessed with fluorescent microspheres, which were injected into the LV cavity (n=6 per group).\(^11\)\(^\text{–}^\text{18}\)

### Immunohistochemistry

For immunohistochemistry, 5-μm-thick LV cryostat sections were stained with specific antibodies and analyzed with fluorescence microscopy. Superoxides were revealed with dihydroethidium staining of 20-μm-thick LV cryostat sections (n=6 per group).\(^11\)\(^\text{–}^\text{18}\)

### In Vitro Studies

#### Isolation and Culture of Adult Cardiomyocytes and CPCs

Cardiomyocytes were isolated from the ventricles of 8-week-old male Wistar rats. Murine cardiomyocytes (HL-1 line), a generous gift from Dr William Claycomb (Louisiana State University Medical Center, New Orleans), were cultured as described previously.\(^18\) CPCs from murine hearts were extracted with a commercially available isolation kit (Millipore; Online Figure II).

#### Prosurvival Effect of Pim-1 on Cardiomyocytes and CPCs Exposed to High Glucose

To simulate the diabetic condition, cardiomyocytes or CPCs were cultured in the presence of high d-glucose (HG; 30 mmol/L) or normal d-glucose (NG; 5 mmol/L) with d-mannitol (25 mmol/L) added as an osmotic control. Twenty-four hours before the HG challenge, cells were transfected either with hPim-1 plasmid (8 μg/1×10\(^6\) cells) or with anti-miR-1 (50 mmol/L; Applied Biosystems, Foster City, CA) with a commercially available transfection agent (lipofectamine 2000, Invitrogen, Carlsbad, CA). Expression of the hPim-1 transgene was identified with an antibody that specifically recognizes the human protein (Online Figure III). The transfection efficacy with this technique was 65±8%. Effective miR-1 inhibition was verified in pilot titration experiments (Online Figure IV). Null vector or scrambled sequence was used as negative control for Pim-1 plasmid and anti-miR-1, respectively. At the end of 48 hours of HG exposure, cells were collected for measurement of caspase-3/7, caspase-8, and caspase-9 activity (all from Promega, Madison, WI) and for immunoblotting and immunocytochemistry analysis. All experiments were performed in triplicate and repeated 5 times.

### RNA Isolation and Semiquantitative RT-PCR

Total RNA was isolated from flash-frozen LV myocardium at different time points after DM induction with TRIzol (Invitrogen) according to the manufacturer’s instructions. RT-PCR was performed according to the standard protocol described previously.\(^11\)\(^\text{–}^\text{18}\)

### Protein Extraction and Western Blotting

Proteins were extracted from LV samples, cardiomyocytes, and CPCs and used for Western blotting as described previously.\(^11\)\(^\text{–}^\text{18}\)

### Statistical Analysis

Comparison of multiple groups was performed by analysis of variance. Two-group analysis was performed by Student t test. Values were expressed as means±SEM. P<0.05 was considered significant.
Results

Upregulation of Pim-1 Inhibitors in Diabetic Hearts

We previously documented that diabetic cardiomyopathy is characterized from the early stage by reduction of Pim-1, along with downregulation of its activators, p-STAT3 (STAT3-p-Tyr705) and p-Akt (Akt-p-Ser473), and O-GlcNAc modification of Akt, which results in reduction of Akt activity.11 Here, we newly show that the decline of cardiac function in diabetic mice (Figure 1A) is associated with upregulation of PP2A (Figure 1B), a phosphatase that reportedly dephosphorylates and inactivates Pim-1.12 Furthermore, Pim-1 represents a validated inhibitory target for the muscle-specific miR-1,13 a microRNA implicated in cardiac remodeling, metabolic signaling, and arrhythmogenesis.19 Notably, we found that miR-1 is upregulated from the early phase (3-fold at 4 weeks after DM induction, \( P < 0.05 \) versus healthy mice) and increases further during progression of diabetic cardiomyopathy (6-fold at 20 weeks, \( P < 0.001 \); Figure 1C). Thus, an unbalance between negative and positive regulators leads to Pim-1 downregulation with advancing cardiomyopathy.

Forced Pim-1 Expression Prevents HG-Induced Cardiomyocyte Apoptosis

We next verified whether Pim-1 could counteract the negative influence of HG on cardiomyocyte viability. To this aim, adult rat cardiomyocytes were cultured under NG or HG conditions after transfection with Pim-1 plasmid or anti-miR-1. As expected, HG significantly reduced the expression of Pim-1 as assessed by immunocytochemistry (Figures 2A and 2D) and Western blot analysis with an antibody that recognizes both endogenous and transgenic Pim-1 (Figures 2B and 2E; Online Figure V, A), thus resulting in downregulation of the Pim-1 targets pBad and Bcl-2 (Figures 2B and 2E) and increased caspase-3/7 activity (Figures 2C and 2F; \( P < 0.01 \) versus NG for all comparisons). Transfection with hPim-1 or inhibition of miR-1 by anti-miR-1 rescued Pim-1 expression in HG-challenged cardiomyocytes (Figures 2A and 2B and Figures 2D and 2E, \( P < 0.01 \) versus HG with null vector or HG with scrambled sequence, respectively), which was associated with restoration of survival signaling, ie, pBad and Bcl-2 (Figures 2B and E), and inhibition of caspase-3/7 and caspase-9 activity (Figures 2C and F; Online Figure V, B; \( P < 0.01 \) versus HG for all comparisons). Interestingly, anti-miR-1, but not hPim-1, upregulates p-Akt under NG or HG conditions (Figures 2B and 2E), which suggests that miR-1 could inhibit Pim-1 directly, as well as via its upstream modulator, Akt. Similar results were observed in murine HL-1 cardiomyocytes transfected with Pim-1 plasmid or anti-miR-1 before exposure to HG (Online Figure VI).

Successful Transduction of Diabetic Hearts With Human Pim-1 After Systemic AAV9–Pim-1 Administration

To verify whether cardiac-specific expression of Pim-1 protects diabetic mice from cardiomyopathy, we constructed a new AAV9 vector carrying the hPim-1 gene. Effective transduction and duration of transgene expression were analyzed by Western blot (Figure 3A) and immunohistochemistry (Figure 3B). Hearts from mice that received the AAV9–\( \beta \)-gal (viral vector control) were negative for immunoreactive hPim-1, whereas intravenous injection of AAV9–Pim-1 resulted in robust and persistent expression of hPim-1 beginning 2 weeks after injection and becoming stable after 6 weeks (middle lane in Figure 3Ai). Using an antibody that does not distinguish between mouse (mPim-1) and human (hPim-1) Pim-1, we confirmed the decline of Pim-1 levels in hearts of AAV9–\( \beta \)-gal–injected diabetic mice (\( P < 0.01 \) versus time 0; upper lane in Figure 3Ai and Figure 3Aii). Conversely, we observed a marked increase in cardiac Pim-1...
after AAV9–Pim-1 injection that led to levels 10-fold higher than those of AAV9–β-gal–injected controls (P<0.001; upper lane in Figure 3Aii). Immunohistochemistry analysis confirmed the expression of hPim-1 in cardiomyocytes, both at the cytoplasmic and nuclear level (Figure 3B), and in vascular smooth muscle cells but not in endothelial cells (Online Figure VII and Online Videos I and II). Expression of hPim-1 was also detected in the limb adductor muscle, but not in the lung, liver, or spleen, which confirmed the musculotropic effect of AAV9 (Figure 3C; Online Figures VII and VIII).

**Effect of AAV9–Pim-1 Gene Therapy on General Health and Cardiac Function**

Diabetic mice showed a decrease in body weight, which was prevented by AAV9–Pim-1 gene therapy (Figure 4A). No treatment effect was observed in diabetic mice with regard to serum insulin, cholesterol, and cortisol; however, AAV9–
Pim-1 reduced triglyceride levels (Online Figure IX). Consistent with our previous study, echocardiography and pulsed Doppler analyses revealed a decrease in the E/A ratio at 4 weeks after DM induction, indicative of diastolic dysfunction, whereas contractility was preserved (Figures 4B and 4C). At this early phase of cardiomyopathy, diabetic mice were randomly allocated to treatment with AAV9–β-gal or AAV9–Pim-1 (1×10^10 or 5×10^10 pfu). Subsequent follow-up demonstrated the steady decline of the E/A ratio in AAV9–β-gal–treated diabetic mice, whereas forced expression of hPim-1 inhibited this trend, which resulted in improved diastolic function at late time points, with no difference between the 2 doses (Figure 4B, which shows the results of a low dosage; P<0.01 versus AAV9–β-gal).

Mice developed systolic dysfunction from 12 weeks of DM, as indicated by the reduction in LV ejection fraction, cardiac output, and fractional shortening (Figures 4Ci through 4Ciii). Furthermore, at late stages, they manifested LV chamber enlargement (Figures 4Civ and 4Cv) and a reduced ratio of LV anterior wall thickness to LV internal diameter at end diastole (Figure 4Cvi). Analysis with the Millar transducer showed an increase in LV end-diastolic pressure and a decrease in LV end-systolic pressure, dP/dt_max, and dP/dt_min in diabetic mice (Figure 4D), which confirmed the typical...
hemodynamic characteristics of heart failure. Analysis of pressure-volume loops further verified the marked dysfunction of diabetic hearts (Figure 4E). In contrast, forced expression of hPim-1 resulted in a global improvement of LV performance, pressure indices, volumes, and wall thickness/internal diameter ratio (Figures 4C through 4E; \( P<0.01 \) versus AAV9– \( \beta \)-gal–treated mice for all comparisons).

Anatomic Correlates of Pim-1–Induced Cardioprotection
Cardiomyocyte loss by apoptosis and interstitial fibrosis are typical features of diabetic cardiomyopathy and account for the inotropic deficit and diminished LV compliance, respectively. We confirmed that DM increases cardiomyocyte apoptosis, as assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling staining (Figure 5A; \( P<0.001 \) versus healthy mice), and interstitial fibrosis, as evidenced by Sirius red staining (Figure 5B; \( P<0.01 \) versus healthy mice), with both effects being remarkably blunted by AAV9–Pim-1 (Figure 5; \( P<0.01 \) versus AAV9– \( \beta \)-gal for both comparisons). Furthermore, diabetic cardiomyopathy consistently has been associated with microangiopathy in the absence of coronary artery disease.\(^{20}\) As shown in Figure 5C, myocardial
Figure 4. AAV9–Pim-1 improves DM-induced cardiac dysfunction. A, Line graphs showing changes in body weight among study groups. Time in weeks from STZ or STZ-vehicle. B, Representative pulsed Doppler images and line graphs showing effect of DM on E/A ratio, which is improved by AAV9–Pim-1 gene therapy. E and A waves represent mitral valve velocity during early diastolic filling and atrial contraction, respectively (n=14 mice per group). C, Indexes of LV function assessed by echocardiography at different time points.
perfusion was reduced by DM, and this defect was prevented by AAV9–Pim-1 (P<0.01 versus AAV9–β-gal). Concurrently, the high dose of AAV9–Pim-1 attenuated the DM-induced decrease in capillary density (P<0.01 versus AAV9–β-gal), and both doses prevented arteriole rarefaction (P<0.01 versus AAV9–β-gal; Figure 5D).

Molecular Mechanisms of Pim-1–Induced Cardioprotection

Next, we verified the signaling pathways implicated in the beneficial effect of Pim-1 on cardiomyocyte survival and inotropism. Forced expression of hPim-1 increased the levels of pBad, which inhibits proapoptotic caspase-3 by preserving the levels of antiapoptotic Bcl-2 (Figure 6A; P<0.01 versus AAV9–β-gal for all comparisons). A recent study showed that Pim-1 confers cardiomyocytes with mitochondrial resistance through oxidative stress challenge preservation of the inner mitochondrial membrane potential.7 We show for the first time that Pim-1 overexpression results in a remarkable reduction in reactive oxygen species levels in diabetic myocardium (Figure 6B; P<0.01 versus AAV9–β-gal). In contrast, AAV9–Pim-1 did not affect the DM-induced reduction in pAkt and Akt activity, which confirms that Pim-1 acts downstream of Akt (Figure 6C). Accordingly, there was no change in the other upstream regulators of Pim-1, such as STAT3, PP2A (Figure 6C), and miR-1 (data not shown) after AAV9–Pim-1 compared with AAV9–β-gal.

The sarcoplasmic reticulum regulates cardiac contractility through modulation of Ca2+ uptake, which is dysfunctional in DM.21 Importantly, forced expression of Pim-1 prevented the decrease of sarcoendoplasmic reticulum Ca2+-ATPase in diabetic hearts (Figure 6C). Furthermore, the inotropic decline of the diabetic heart was attributed to a switch in the relative proportion of the 2 isoforms of myosin heavy chain (MHC), with the α-MHC isoform becoming the dominant heart isozone.22 Importantly, Pim-1 gene therapy blunted the reduction of the α-MHC to β-MHC ratio in diabetic hearts (Figure 6C).
Pim-1 Overexpression Preserves CPC Viability and Proliferative Activity

Diabetic cardiomyopathy is reportedly associated with the progressive exhaustion of resident CPCs, which fail to compensate for the continuous loss of cardiomyocytes. Exposure of murine Sca-1+ CPCs to HG resulted in the reduction of Pim-1, pBad, and Bcl-2 and activation caspase-3 (Figure 7A; *P < 0.01 versus NG for all comparisons), which replicated the expressional changes induced by DM or HG in mature cardiomyocytes. In addition, HG caused a 60% reduction in Sca-1+ CPC proliferation (Figure 7B; **P < 0.001 versus NG). Importantly, overexpression of Pim-1 restored prosurvival signaling and the proliferative activity of CPCs in vitro (Figures 7A and 7B). The same effect was observed after inhibition of miR-1 by anti-miR1 (Figures 7C and 7D).

Immunohistochemistry analysis of diabetic hearts confirmed the reduction of c-kit+ cells and showed an increase in the fraction of senescent CPCs, assessed as c-kit+ Ki67− cells that showed high γ-H2A.X foci density. Pim-1 gene therapy specifically increased the prevalence of lineage-committed α-SA+ c-kit+ cells and reduced the proportion of c-kit+ cells that expressed the senescence marker (Figure 7E). Furthermore, DM induced a marked reduction in the proliferative ability of cardiomyocytes, as assessed by Ki-67 and PCNA, compared with healthy controls. In contrast, forced expression of Pim-1 caused a remarkable increase in the number of Ki-67+ (Figure 7F-i) and PCNA+ (Online Figure X) cardiomyocytes in diabetic hearts. Interestingly, Pim-1 also increased the number of Ki-67+ c-kit+ cells in the diabetic heart (Figure 7F-ii).

Discussion

This study illustrates a previously unrecognized mechanism that accounts for the propensity of cardiac cells to undergo apoptosis in a mouse model of type 1 DM, through inhibition of Pim-1 by miR-1 and PP2A and the lack of positive modulation by STAT3 and Akt. We also show that cardiac-specific Pim-1 gene therapy halts the progression of diabetic cardiomyopathy.

Previous work from Sussman’s group indicates that Pim-1 is a crucial component of the signaling machinery that...
Figure 6. Mechanisms of Pim-1–induced cardioprotection. A, Representative blots and bar graphs showing levels of pBad, Bcl-2, and cleaved caspase-3 at 16 weeks after gene therapy (n=6 per group). Actin was used as loading control. B, Representative fluorescent microscopy images and bar graphs showing effect of forced expression of Pim-1 on superoxide levels in myocardium evaluated by dihydroethidium staining (group size and treatment duration as above). Scale bars, 50 μm. C, Representative blots and bar graphs showing levels of pAkt, Akt kinase activity, pSTAT3, PP2A, SERCA2 (sarcoplasomic reticulum Ca²⁺-ATPase), and α-MHC and β-MHC (group size and treatment duration as above). Values are expressed as n-fold changes toward healthy and are means±SEM. **P<0.01 and ***P<0.001 versus healthy mice; ###P<0.01 and ####P<0.001 versus AAV9–gal–treated diabetic mice (Diabetic–gal).
Figure 7. A and C, Representative blots and bar graphs showing levels of Pim-1, pBad, Bcl-2, and activated caspase-3/7 in Sca-1+ murine CPCs exposed to HG after transfection with Pim-1 plasmid (Pim-1, 8 µg/1×10^6 cells; A) or anti-miR-1 (50 nmol/L; A), null vector (Null [A] or scrambled sequence [C]); or transfection vehicle (V). Scr indicates scrambled sequence. Actin was used as a loading control. Each experiment was repeated in triplicate. Values are expressed as n-fold changes toward NG for all parameters and are means±SEM. *P<0.05, **P<0.01, and ***P<0.001 versus NG; ##P<0.01 and ###P<0.001 versus HG-Null. B and D, Proliferation of CPCs from similar experiment was assessed by bromodeoxyuridine (BrdU) incorporation assay. Values are expressed as percentage increase. Group size and statistical analysis are the same as above. E and F, Representative confocal images and bar graphs showing c-kit+ α-sarcomeric actin+ Pim-1+ cells and c-kit+ Ki67+ H2A.X+ cells (E), Ki-67+ Pim-1+ cells (F-i), and Ki-67+ c-kit+ cells (F-ii) at 20 weeks after DM induction (16 weeks after AAV9). Scale bars are 10 µm for E and F-i and 50 µm for F-ii. *P<0.05, **P<0.01, and ***P<0.001 versus healthy mice; ##P<0.01 and ###P<0.001 versus AAV9-gal–treated diabetic mice (n=6 per group). Data from low dose of Pim-1 (1×10^10 pfu) treatment are shown, with no further benefit realized from high dosage (5×10^10 pfu).
counteracts cardiomyocyte apoptosis during the early phase of postischemic healing. In addition, Pim-1 is upregulated in failing hearts as an extreme, inefficient attempt to preserve cardiac function. At variance with those results, we report here that Pim-1 is downregulated beginning in the early phase of diabetic cardiomyopathy and steadily decreases through the progression of contractile dysfunction and heart failure.

The accrual of alterations of upstream Pim-1 activators and, as reported for the first time here, the upregulation of Pim-1 inhibitors PP2A and miR-1 explain the peculiar behavior observed in hearts of diabetic mice compared with ischemic or pressure-overload models. It is noteworthy that increased levels of ceramide in diabetic myocardium could be a possible mechanism behind the upregulation of PP2A, although this requires further investigation.

Muscle-specific microRNAs, such as the bicistronic miR-1 and miR-133 cluster, have been implicated in myocardial and cardiac stem cell development, cardiac hypertrophy, and remodeling. In particular, miR-1 negatively regulates the expression of hypertrophy-associated genes such as IGF-1 (insulin-like growth factor-1), calmodulin, and Mef-2a (myocyte enhancer factor 2a). Importantly, recent studies report that HG induces miR-1 activation in the rat cardiac H9c2 cell line via upregulation of miR-1 transcriptional regulator serum response factor. The present data demonstrate that miR-1 constantly increases from the early to the late phases of diabetic cardiomyopathy, thus mirroring an opposite trend of Pim-1. This inverse association is strengthened by the notion that Pim-1 is a direct target of miR-1, as demonstrated previously. Here, we newly show that miR-1 inhibition by anti-miR-1 rescues Pim-1 levels in cardiomyocytes and CPCs challenged with HG. Furthermore, both anti-miR-1 and Pim-1 plasmid restored the prosurvival signaling centered on phosphorylation of Bad and activation of Bcl-2 in cardiac cells. Intriguingly, removing the miR-1-dependent inhibition on Pim-1 resulted in elevation of phosphorylated Akt and abrogation of HG-induced cardiac apoptosis. This is consistent with the possibility that miR-1 impinges on cardiac cell viability through redundant molecular pathways. Accordingly, miR-1 reportedly inhibits IGF-1 and heat shock protein-60, thereby leading to negative modulation of phosphatidylinositol 3-kinase/Akt/Bcl-2. In line with the existence of an autoregulatory circuit upstream of Pim-1, active Akt phosphorylates and inhibits Foxo3a, thereby reducing the transcription of miR-1. Thus, in the diabetic heart, miR-1 could inhibit prosurvival signaling by directly interfering with Pim-1 transcription and overriding the Pim-1-activator, Akt.
The finding that Pim-1 declines from the early stages of diabetic cardiomyopathy prompted us to challenge a gene therapy approach that targets Pim-1 for prevention of DM-induced contractile dysfunction. Echocardiography and pulsed Doppler analyses have documented the functional benefit of Pim-1 gene therapy. Furthermore, histological studies have pinpointed the prevention of interstitial fibrosis and cardiomyocyte apoptosis as major determinants in the improvement of diastolic function and preservation of contractility. Adult cardiomyocytes express 2 MHC isoforms, α-MHC and β-MHC, with the former having higher ATPase activity. The ratio of α-MHC to β-MHC correlates directly with overall cardiac performance and is remarkably reduced early in the course of diabetic cardiomyopathy.22 Forced expression of Pim-1 resulted in restoration of proper α-MHC levels in diabetic hearts, which may contribute to improved contractile function. Furthermore, Pim-1 could enhance cardiac performance through preservation of sarcoendoplasmic reticulum Ca2++-ATPase activity, which is essential for maintenance of physiological levels of reactive oxygen species. The present results indicate a blunted increase of reactive oxygen species levels in Pim-1–overexpressing diabetic hearts, which suggests that Pim-1 can interfere with reactive oxygen species formation. This may additionally account for reduced cardiomyocyte apoptosis after Pim-1 gene therapy. Consistent with the present data, recent findings indicate that Pim-1 protects against oxidative stress–induced apoptosis by inhibiting apoptosis signaling kinase-1 and the caspase-3 cascade.26

CPCs genetically engineered to express hPim-1 showed improved viability and proliferative capacity, which are essential prerequisites for myocardial homeostasis and repair.27 We have shown here that Pim-1 gene therapy not only increased the prevalence of c-kit+ cells that were depleted by diabetes, but it also reduced the fraction of CPCs affected by senescence processes, which are a typical feature of diabetic cardiomyopathy. Pim-1 also increased the index of proliferation in diabetic heart. These data demonstrate the ability of Pim-1 to preserve the endogenous regenerative potential of CPCs in spite of persistent hyperglycemia. Furthermore, Pim-1 is necessary for vascular smooth muscle proliferation,28 and CPCs engineered with Pim-1 have been shown to promote neovascularization in a mouse infarct model.27 The present data showing the expression of Pim-1 in vascular smooth muscle cells provide a key for interpretation of the preserved arteriole density in Pim-1–transduced diabetic hearts. In contrast, endothelial cells were not transduced after Pim-1 gene therapy, which suggests that the improved capillary density may be due to transmission of prosurvival signals from cardiomyocytes to neighboring endothelial cells.

One important aspect of the present study is the use of a new cardiotropic vector. After systemic injection of AAV9–Pim-1, we found the hPim-1 transgene to be persistently and specifically expressed in cardiac cells and skeletal myocytes, thus reinsuring on possible off-target effects of the proto-oncogene. Biochemical measurements ruled out the possibility of an extracardiac influence on the beneficial effects seen after AAV9–Pim-1 treatment. The present study focused on the therapeutic action of Pim-1 in cardiomyopathy, yet based on preliminary results that showed preservation of skeletal myocyte morphology by Pim-1 (Online Figure VIII), it would be worthwhile to investigate the possible benefit of Pim-1 on skeletal muscles, in light of the frequent association of heart failure and skeletal myopathy.

The present study provides novel evidence for a role of Pim-1 in the pathogenesis of diabetic cardiomyopathy. Pim-1 can serve as a therapeutic agent for the treatment of DM-induced cardiac damage by blunting cell death, preserving intrinsic regenerative potential, and stabilizing the protein composition of the heart’s contractile machinery.

Acknowledgments

We thank Professor Saadeh Suleiman for his expert advice on the use of rat cardiomyocytes, Dr Federica Riu for her kind technical assistance in microRNA evaluation, Dr Susan Finerty for her kind technical assistance in preparation of AAV-Pim-1 plasmid, and Hua Lin for her kind technical assistance in the isolation of adult rat cardiomyocytes.

Sources of Funding

This study was supported by a project grant from the British Heart Foundation (PG/09/086) and RESOLVE (Resolve chronic inflammation and achieve healthy ageing, 202047).

Disclosures

None.

References

Novelty and Significance

What Is Known?

- Diabetic cardiomyopathy typically progresses from diastolic dysfunction to heart failure in the absence of coronary artery disease or hypertension.
- The provalin integration site for Moloney murine leukemia virus-1 (Pim-1) is decreased in the myocardium of diabetic mice beginning at the stage of diastolic dysfunction.
- Pim-1 plays an essential role in cardiomyocyte survival.

What New Information Does This Article Contribute?

- Pim-1 gene therapy counteracts the deleterious effect of diabetes on c-kit+ cardiac progenitor cells.

New strategies are needed for the treatment of diabetes-specific cardiomyopathy, an increasingly prevalent cause of heart failure. Here, we show that overexpression of Pim-1 or inhibition of miR-1 in adult cardiomyocytes prevents high glucose–induced damage through phosphorylation/inhibition of a proapoptotic protein, Bad, and activation of an antiapoptotic protein, Bcl-2. A single systemic injection of Pim-1 with cardiotoxic AA9V at the stage of diabetic dysfunction specifically transduced cardiac cells, which resulted in improvement of animal survival and preservation of cardiac and microvascular function via activation of prosurvival signaling mechanisms. Moreover, diabetes reduced the abundance of c-kit+ cardiac progenitor cells through activation of cell senescence. Importantly, Pim-1 gene therapy preserved the pool of cardiac-committed c-kit+ cells. This study provides a basis for novel approaches to halt the progression of diabetic cardiomyopathy.
Intravenous Gene Therapy With Pim-1 Via a Cardiotropic Viral Vector Halts the Progression of Diabetic Cardiomyopathy Through Promotion of Prosurvival Signaling
Rajesh Katare, Andrea Caporali, Lorena Zentilin, Elisa Avolio, Graciela Sala-Newby, Atsuhiko Oikawa, Daniela Cesselli, Antonio Paolo Beltrami, Mauro Giacca, Costanza Emanueli and Paolo Madeddu

Circ Res. published online April 7, 2011;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2011/04/07/CIRCRESAHA.110.239111

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2011/04/07/CIRCRESAHA.110.239111.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Supplemental Material

Expanded Methods

Production, purification, and characterization of rAAV vectors

In order to prepare AAV9-PI-M-1 viral vector, the complete coding sequence of human PIM-1 was amplified (KOD proofreading DNA polymerase, Novagen, USA) using primers GAATTCCGGATGCTTTGTCCAAAATC (BamHI-site) and GTGCAGCTGCGAGGCTGCTTGTCC (SalI-site) from a pCDNA3/hPIM33 plasmid (generous gift from Prof. Michael Lilly, University of California, USA) and cloned in the shuttle vector pAAV-MCS (Agilent Technology, USA) downstream of the cytomegalovirus promoter. Infectious vector stocks were then prepared by the AAV Vector Unit at ICGEB Trieste (http://www.icgeb.org/avu-core-facility.html), by a cross-packaging approach, whereby the vector genome was packaged into AAV capsid serotype 9. Methods for production and purification were previously described. AAV titers were in the range of $1 \times 10^{11}$ to $1 \times 10^{12}$ genome copies (g.c.) per milliliter.

Echocardiography

Measurements of dimensional and functional parameters were performed at baseline, at 4 weeks after diabetes (DM) induction (before treatment), and every 4 weeks thereafter, using a high-frequency, high resolution echocardiography system (Vevo 770, Visual Sonics, Toronto, Canada) (n=14 mice per group). Briefly, mice were anesthetized using tribromo-ethanol and transferred to an imaging stage equipped with a warming pad for controlled maintenance of mouse body temperature at 37°C and a built-in electrocardiography system for continuous heart rate (HR) and respiratory rate monitoring. Standard B mode (2D) images of the heart and pulsed Doppler images of the mitral valve inflow were acquired. The thickness of the left ventricle (LV) was measured at the level of the papillary muscles in parasternal short axis at end-systole and end-diastole. LV ejection fraction (LVEF) and fractional shortening (LVFS) were determined as described by De Simone et al. Measurement of intra-ventricular pressure

Terminal measurement of left ventricular pressure (LVP) was made at 20 weeks after DM induction (16 weeks after AAV9 treatment) (n=12 mice per group). The body temperature of the mice was maintained between 36º and 37ºC throughout the experiment using a homeothermic blanket warming system. A tracheotomy was made and the mouse was intubated using the 23 gauge catheter, secured in place with 6-0 silk suture. A high-fidelity 1.4F transducer tipped catheter (Millar Instruments, Houston, TX, USA) was zeroed in 37ºC saline. Calibration of the transducer was verified using a mercury manometer, as suggested by the manufacturer. The right carotid artery was isolated, and tow ties were gently pulled back, using hemostats, to block blood flow from vessel. When pulsatile flow was no longer visible, a small cut was made just below the distal tie, and the catheter was placed inside the carotid artery and secured in place. The transducer was advanced into the heart, where its position was confirmed by the rapid deflection of the diastolic pressure wave without any change in systolic pressure. Mice were allowed to stabilize for 10min. After stabilization, baseline data were collected, including the HR, LV end systolic pressure (LVP), LV end-diastolic pressure (LVEDP), and maximal rates of LV pressure rise (dP/dt$_{max}$) and fall (dP/dt$_{min}$). To calculate the pressure volume relationship, the recording from Millar catheter was synchronized with echocardiography measurements as per manufacturer instructions.

Measurement of blood flow using fluorescent microspheres

Myocardial perfusion was measured using fluorescent microspheres. A polyethylene (PE10) catheter was inserted through the right carotid artery for the reference blood withdrawal. Microspheres, 0.2µm in diameter (Molecular Probes, CA, USA) were injected
into the LV cavity over 1 min and flushed with 0.15 ml of 0.9% NaCl. Reference blood was collected via the carotid catheter starting 15 sec before to 1 min after the microsphere injection. The animals were sacrificed 2 min later and the heart was removed and separated into LV, right ventricle (RV) and septum. The kidneys were also collected and analyzed as internal control organs to demonstrate homogenous distribution of the microspheres throughout the bloodstream. Each sample was weighed, cut into small pieces and digested in 10 ml of 2 M ethanolic KOH containing 0.5% Tween 80 at 60°C for 48 h with constant shaking. After complete digestion of tissues, the microspheres were collected by centrifugation at 2000 x g for 20 min and sequential washing with 10 ml of deionized water with or without 0.25% Tween 80. Finally, microspheres were dissolved in 3 ml of 2-ethoxyethylacetate and the fluorescence intensity was determined using a fluorophotometer (Fluostar Optima, BMG labtech). Regional blood flow was calculated as the absolute blood flow in ml/min/g of tissue as described earlier.\textsuperscript{8,11}

**Immunohistochemical analysis of Pim-1 expression, assessment of myocardial capillary and arteriole densities, analysis of c-kit cells and evaluation of proliferation.**

LV cryosections with thickness of 5 µm were used for all immunohistochemical analysis unless specified. To detect the localization of Pim-1 after AAV9 injection, sections were incubated with rabbit monoclonal human Pim-1 antibody (Abgent, 1:100, overnight at 4°C) which is specific to human Pim-1, followed by Goat anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (1:100, Invitrogen, Molecular probes, 1:100, 1 h at room temperature). To recognize cardiomyocytes, sections were also stained with mouse monoclonal primary antibody for the cardiomyocyte marker α-sarcomeric actin (Abcam, 1:100, overnight at 4°C), which was revealed by counterstaining with the secondary antibody conjugated to Alexa 568 (Invitrogen, Molecular probes, 1:100, 1 h at room temperature).

For capillary density, sections were incubated with biotinylated Isolatect B4 (Invitrogen, Molecular Probes, 1:50, 2 h at 37°C in a humidified chamber), followed by streptavidin Alexa Fluor 488 (Invitrogen, Molecular probes, 1:100, 1 h at room temperature). Same sections were then probed with anti-mouse α-smooth muscle cell actin antibody conjugated with Cy3 (Sigma chemicals, 1:400, 1 h at room temperature) for detection of arteriole density. Capillaries and arterioles were calculated in at least 20 fields at X200 magnification and the final data expressed as the number of capillaries or arterioles per square millimeter. Arterioles were also categorized according to their luminal size.\textsuperscript{8,12}

For analysis of proliferation, sections were incubated overnight with either mouse monoclonal Ki-67 antibody (eBioscience, 1:100), or mouse monoclonal proliferating cell nuclear antigen (PCNA) antibody (Dako, 1:50), followed by goat-anti mouse secondary antibody conjugated with Alexa Fluor 568 (Invitrogen, Molecular Probes, 1:100). Pim-1 positive cells were then identified by overnight incubation of the same section with rabbit monoclonal human Pim-1 antibody (Abgent, 1:100), which was revealed by counterstaining with the goat anti-rabbit secondary antibody conjugated to Alexa 488 (Invitrogen, Molecular probes, 1:100). Finally, sections were stained with Dapi to recognize the nuclei. The data were expressed as number of Ki-67 or PCNA nuclei per 1 million cardiomyocytes.\textsuperscript{13}

For analysis of c-kit and γH2AX we used 5 µm thick paraffin embedded sections. After deparaffinization and antigen retrieval, sections were incubated with goat polyclonal c-kit antibody (R&D Systems, 1:50) for 2 h at 37°C followed by donkey α-goat secondary antibody conjugated with Alexa Fluor 488 (Invitrogen, Molecular Probes, 1:400). This is followed by incubation with mouse monoclonal antibody against γH2AX (Upstate technologies, 1:100) followed by donkey a-mouse secondary antibody conjugated with Alexa Fluor 555 (Invitrogen, Molecular Probes, 1:800). The additional co-staining for Pim-1 and Ki-67 were performed as explained above.

**Pim-1 expression in vascular cells of the heart**

We used whole mount sections to identify the expression of Pim-1 in smooth muscle cells and endothelial cells of the heart at 16 weeks after gene therapy. Sixty microns thick
sections, were post-fixed with acetone (-20°C) for 10min and air dried at room temperature for 30min. Following permeabilization with 1% triton-X 100 and blocking of non-specific antigens, sections were incubated overnight at 4°C with biotinylated isoelectin-B4 to stain endothelial cells (1:50, Invitrogen), followed by incubation with streptavidin Alexa Fluor 568 (1:100, Invitrogen) for 3h at room temperature. For α-smooth muscle actin, sections were incubated with anti-mouse α-smooth muscle cell actin antibody conjugated with Cy3 (Sigma chemicals, 1:400, 3h at room temperature). Serial z-stack images of myocardium were generated using Leica SP5 AOBS confocal laser scanning microscope (Wolfson Bioimaging facility, University of Bristol).

Assessment of myocardial fibrosis
Myocardial fibrosis was analyzed by Sirius red staining followed by morphometric analysis using the Image Pro analysis software (MediaCybernetics, USA) and the data were expressed as the ratio between intensity of staining and area examined.8

TUNEL staining
Apoptosis was quantified on LV cryosections (5µm) by the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique (in situ cell death detection kit Fluorescein, Roche applied science, USA). Following treatment of slides with proteinase K (20µg/ml, 30min at 37°C), TUNEL assay was performed according to the manufacturer’s instruction. The same sections were then stained with DAPI to recognize nuclei. Cardiomyocytes were stained as described above. Twenty fields were randomly evaluated in each section at X400 magnification. The fraction of TUNEL positive nuclei over total cardiomyocyte nuclei was then calculated.8, 14

In situ detection of reactive oxygen species
Dihydroethidium staining for detection of superoxide
Superoxide production in the myocardium was determined using the fluorescent dye dihydroethidium (DHE, Invitrogen, Molecular probes). LV cryosections (20µm) were incubated with 5µmol/l DHE, at 37°C for 30min, in a humidified chamber. Images (X100 magnification) were captured on an Olympus fluorescence microscope fitted with camera (Media cybermatics) and the mean DHE fluorescence intensity of myocyte nuclei was calculated by dividing the combined fluorescence value of the pixels by the total number of pixels in 15 randomly selected field using Image-Pro advanced software.15

RNA isolation and semi-quantitative RT-PCR
Total RNA was extracted from flash-frozen LV samples (TRIzol, Invitrogen, UK) and CPCs (vide infra) and genomic DNA was eliminated using kit from Qiagen. One microgram of total RNA was reverse transcribed using Qiagen reverse transcriptase kit, followed by amplification of cDNA using quantitetc primers for troponin-T, α- and β- myosin heavy chain, tropomyosin and internal control 18S (all from Qiagen, UK). Each reaction was performed in triplicate. For detection of miRNA-1, 10ng of total RNA was reverse transcribed using TaqMan reverse transcriptase kit and specific reverse transcription primers for miR-1 and internal control U6 (all from Applied Biosystems, UK). Amplification of cDNA was performed using TaqMan universal PCR mix kit (Applied Biosystems, UK) in DNA Engine Opticon-2 System (BioRad, UK). For quantification, the amount of miRNA was normalized to the amount of U6 miRNA using the 2−DDCT method. Each reaction was performed in triplicate.16

Western blot analyses
Proteins were extracted from LV using ice-cold RIPA buffer. Protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad). Detection of proteins by western blot analysis was done following separation of whole tissue / cell extracts (50µg) on SDS-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (PVDF, Amersham-Pharmacia) and probed with the following antibodies: Ser473- phospho-
Akt (Cell Signaling, 1:1000), Akt (Cell Signaling, 1:1000), Tyr705 phospho-STAT3 (Cell signaling, 1:500), STAT3 (Cell Signaling, 1:1000), Pim-1 (Santacruz biotechnology, 1:250), human Pim-1 (Abgent, 1:500), Ser112- phospho-Bad (Cell Signaling, 1:1000), Bad (Cell Signaling, 1:1000), Bcl-2 (Cell Signaling, 1:1000), cleaved-caspase-3 (Cell Signaling, 1:1000), SERCA-2 (cell Signaling, 1:1000) and α- and β-cardiac myosin heavy chain (Abcam, 1:1000). Actin (Cell Signaling, 1:1000) was used as loading control. For detection, secondary antibody goat anti-rabbit or anti-mouse or donkey anti-goat conjugated to horseradish peroxidase (both from Santacruz biotechnology, 1:5000) were used, followed by chemiluminescence reaction (ECL, Amersham Pharmacia). Density of the bands was analyzed using Image-J (NIH, USA) software and data expressed as fold changes.

Assessment of Akt kinase activity in heart samples
Protein preparations (25 µg) from LV were assessed for Akt activity using the Akt/PKB Kinase Activity Assay Kit (Assay Design), according to the manufacturer’s instruction. Relative Akt kinase activity was expressed as fold change compared to healthy mice.

Isolation and culture of rat adult cardiomyocytes
The male Wistar rats were killed by cervical dislocation, the heart dissected and rinsed in cold solution A containing (in mM): 137 NaCl, 5 KCl, 1.2 MgSO4, 2.2 NaH2PO4, 20 N-hydroxyethylpiperazine-N’-2-ethanesulphonic acid (HEPES), 16 glucose, 5 Na pyruvate and 1.8 MgCl2 (pH 7.25 with NaOH) + 0.75 mM CaCl2. The heart was cannulated via the aorta and perfused for 4 min with solution A + 0.75 mM CaCl2 (all perfusing solutions were oxygenated and maintained at 37 °C). This was followed by a 4-min perfusion with solution A + 0.09 mM ethylene glycol-bis (β-aminoethylether) N,N,N’,N’’-tetraacetic acid (EGTA). Next the heart was digested with 50 ml of enzyme solution containing: solution A + 0.09 mM EGTA, 50 mg collagenase (Worthington Biochemical Corporation, Lakewood, New Jersey, USA. Type I), 5 mg protease (Sigma, Poole, Dorset, UK. Type IV), with (glutamate loaded) or without (control) 6.4 mM potassium L-glutamate until the tissue felt soft. There was a final 4-min perfusion with solution A + 0.15 mM CaCl2 before the ventricles were cut down and sliced. The sliced ventricles were suspended in approximately 20–25 ml solution A + 0.15 mM CaCl2 and shaken for 6 min at 37 °C. After filtration, cells were allowed to sediment, the supernatant was discarded, and the remaining cell layer suspended in solution A + 0.5 mM CaCl2. This sedimentation, removal of supernatant and resuspension step was repeated, but this time the cells were suspended in solution A + 1 mM CaCl2. This technique typically produced a yield of over 90% rod-shaped cells with the ability to exclude Trypan Blue.17 The resulting cells were then washed separately with medium 199 (Invitrogen) supplemented with 0.2% BSA, 10% FBS, 5 mM creatine, 5 mM taurine, 2 mM carnitine, 10 µM cytosine-D-arabinofuranoside (all from Sigma chemicals), ITS and antibiotics (both from Invitrogen). After the final wash cells were resuspended in the same medium and plated on laminin coated culture dish according to the experiments.18

Transfection with Pim-1 plasmid or anti-miR-1

Adult cardiomyocytes
After isolation 1X10^6 cardiomyocytes were seeded on a 24-well dish and allowed to settle for 4h. After 4h, cells were transfected with either hPIM-1 plasmid (8µg/1X10^6 cells) or anti-miR-1 (50nM, Applied Biosystems), using commercially available Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 24h, the medium was replaced with fresh medium and the cells were exposed to normal glucose (NG, 5mM) or high glucose (HG, 30mM) treatment for further 48h. For immunocytochemistry, 5X10^5 cells were seeded on 8-chamber multi-well dish and underwent similar transfection and
treatment procedure. At the end of treatment, cells were fixed with freshly prepared 4% PFA and probed overnight with either Pim-1 antibody that detects both rat and human Pim-1 (SantaCruz Biotechnology, 1:50) or Pim-1 antibody specific to human Pim-1 (Abgent, 1:50), followed by rabbit anti-goat secondary antibody conjugated to Alexa 568 (Invitrogen, Molecular probes, 1:100). For caspase activity assay, 5X10^3 cells were plated on 96-well dish and underwent similar transfection and treatment procedure. At the end of treatment, equal volume of caspase assay reagent was added to the wells and incubated in dark for 30min at room temperature. The luminescence was read using promega luciferase assay system and data expressed as fold changes to cells cultured in NG.

**HL-1 cardiomyocytes**
The day before transfection 1X10^5 cells were seeded on a 24-well dish, so that they become 95% confluent at the time of transfection. Twenty four hours later, the cells were transfected with either hPIM-1 plasmid (8µg/1X10^6 cells) or anti-miR-1 (50nM, Applied Biosystems) as described above. Five thousand cells were seeded on a 8-chamber slide for immunocytochemistry and 1X10^3 cells were seeded on 96-well dish for caspase activity assay. Transfection protocol as explained above.

**Experiments on CPCs**

**Extraction of CPC from murine hearts**
Cardiac progenitor cells were extracted from adult murine hearts using commercially available cardiac stem cells isolation kit (Millipore) according to the manufacturer instruction. In brief, five adult mice hearts were minced, pooled and digested with enzyme solution. The CPCs were then isolated by differential centrifugation using the gradient solutions provided with the kit. The purity and cardiomyocytes differentiation potential of CPCs was confirmed using flow cytometry analysis after staining the cells with Stem cell antigen-1 (Sca-1) (Supplemental Figure 2). Using this technique, we extracted up to 1X10^6 Sca-1^pos cells from 5 murine hearts. The extracted cells were then plated and cultured with commercially available stem cell maintenance medium (Millipore). For differentiation, cells were plated on poly-L-ornithine coated culture dish and maintained for 12 days using the cardiomyocytes differentiation medium provided in the kit. For transfection with hPIM-1 or anti-miR-1, the protocol used for HL-1 cells was followed.

**BrdU incorporation assay**
To study the effect of hPIM-1 on CPC proliferation, we used the BrdU incorporation assay. Following transfection with PIM-1 plasmid or anti-miR-1 as explained above, CPCs were exposed to normal glucose (NG, 5mM) or high glucose (HG, 30mM) for 48h. BrdU (10µM) was added to the medium during the whole exposure period. BrdU incorporation by CPCs was measured using a BrdU immunofluorescence assay kit from Roche, according to the manufacturer’s instructions. Briefly, CPCs were fixed and made permeable with FixDenat solution for 1h, then incubated with monoclonal anti-BrdU peroxidase-conjugated antibody (anti-BrdU-POD) for 90min. Bound anti-BrdU-POD was detected by a substrate reaction, then quantified by an ELISA plate reader. Each experiment was performed in triplicate and repeated 5 times.

**Statistical Analysis**
Results are expressed as mean ± standard error. The hemodynamic and echocardiographic measurements were compared by use of repeated measures Two-Way ANOVA (factorial design: two independent variables, (1) treatment and (2) presence or absence of DM), followed by pair-wise comparison using the Holm-Sidak method. For the histological, morphometric and biochemical analysis, difference between multiple groups were analyzed using one-way ANOVA and difference between two groups using t-test (paired or unpaired as appropriate). For myocardial BF and expression studies, when
normality test failed, differences between groups were analyzed using Siegel-Tukey test. A P value of <0.05 was considered statistically significant.
References


2. Lilly M, Kraft A. Enforced expression of the Mr 33,000 Pim-1 kinase enhances factor-independent survival and inhibits apoptosis in murine myeloid cells. *Cancer research*. 1997;57:5348-5355.


Supplemental Figure Legends

Online Figure I: Serum glucose levels
Bar graphs show the serum glucose levels in healthy or diabetic mice during the 20 weeks study. **P<0.01 vs. healthy at corresponding time point. Diabetic-β-gal – diabetic mice treated with AAV9-β-gal; Diabetic-Pim-1 (1X10^10) – Diabetic mice treated with low dose (1X10^10 pfu/animal) AAV9-hPIM-1; Diabetic-Pim-1 (5X10^10) - Diabetic mice treated with high dose (5X10^10 pfu/animal) AAV9-hPIM-1.

Online Figure II: Characterization of murine CPCs
A. Flow cytometry identification of Sca-1^pos murine CPCs, showing the gating procedure and purity of selected cells. B. Immunocytochemistry microphotographs of cardiomyocytes derived from Sca-1^pos cells following culture in differentiation medium and staining for cardiac specific markers such as α-sarcomeric actin and connexin-43. Scale bars are 50µm. C. Bar graphs showing the expression of troponin-T, α- and β–myosin heavy chain (MHC) and tropomyosin in cultured CPCs before and after differentiation, evaluated by semi-quantitative RT-PCR. Values are means±SEM. Each experiment repeated in triplicates.

Online Figure III: Transfection efficacy of human Pim-1 plasmid in adult rat cardiomyocytes
Representative images of the hPIM-1 plasmid transfected (A) and non-transfected (B) adult rat cardiomyocytes stained with human specific Pim-1 antibody. Scale bars are 50µm.

Online Figure IV: Titration of miR-1 optimal inhibitory dose
Bar graphs showing the inhibition of miR-1 expression (verified by RT-PCR) using different concentrations of anti-miR-1 in cardiomyocytes exposed to high glucose (HG). Scrambled sequence (Scr) used as control. *P<0.05 and ***P<0.001 vs. normal glucose (NG) and ###P<0.001 vs. corresponding dose of HG-Scr. Each experiment repeated in triplicates. Values are means±SEM.

Online Figure V:
A. Representative western blotting membranes showing Pim-1 expression (34kD) in adult rat cardiomyocytes exposed to normal glucose (NG) or high glucose (HG) after transfection with hPIM-1 plasmid (Pim-1) or null (Null), detected using antibody that recognizes both rat and human Pim-1. B. Caspase-8 and -9 activity - Bar graphs showing the caspase-8 and -9 activity in adult rat cardiomyocytes cultured in normal glucose (NG) or high glucose (HG) after transfection with hPIM-1 plasmid (Pim-1, 8µg/1X10^6 cells), Null vector (Null) or transfection vehicle (V). Values expressed as fold change to NG-V group and are means±SEM. *P<0.05 and **P<0.01 vs. NG-V and #P<0.05 vs. HG-null.

Online Figure VI: Pim-1 protects HL-1 cardiomyocytes from high glucose
A. Representative florescent microscopic images showing the expression of Pim-1 in murine HL-1 cardiomyocytes exposed to normal (NG) or high glucose (HG). HG-challenged cardiomyocytes were transfected with hPIM-1 plasmid (Pim-1, 8µg/1X10^6 cells), Null vector (Null) or transfection vehicle (V). Scale bars are 50µm. B. Representative western blots and bar graphs from a similar experiment showing the expression of pAkt, Pim-1, phospho and total Bad and Bcl-2. Actin was used as loading control. C. Bar graphs showing the levels of activated caspase-3/7. D. Representative florescent microscopic images showing the expression of Pim-1 in cardiomyocytes exposed to HG following transfection with anti-miR-1 (50nM), control scramble sequence (Scr) or vehicle (V). Scale bars are 50µm. E. Representative western blots and bar graphs from a similar experiment. F. Bar graphs showing the levels of activated caspase-3/7. Each experiment was repeated five times in
triplicate. Values are expressed as n-fold changes toward NG for all parameters and are means±SEM. *P<0.01 and **P<0.001 vs. NG and #P<0.05, ##P<0.01 and ###P<0.001 vs. HG-null or HG-scr.

Online Figure VII: AAV9-hPIM-1 transduce vascular smooth muscle cells
Representative confocal microscopic images showing the expression of hPim-1 in vascular smooth muscle cells (A, identified by co-staining with α-smooth muscle actin). No expression was detected in endothelial cells (B, identified by co-staining with isolectin-B). Scale bars are 50µm.

Online Figure VIII: Evaluation of skeletal muscle
A. Representative confocal microscopic images showing the transduction of hPim-1 in the skeletal muscle following systemic injection of AAV9-hPIM-1. Scale bars are 50µm. B. Bar graphs showing the muscle area among study groups. *P<0.01 and ***P<0.001 vs. healthy and ##P<0.01 vs. AAV9-β-gal-treated diabetic (β-gal). Scale bars are 2mm. Values are means±SEM. n=6 per group.

Online Figure IX: Biochemical measurements
Bar graphs showing the levels of serum insulin, cholesterol, triglycerides and cortisol at the end of the study in animals treated with AAV9-hPIM-1 or AAV9-β-gal. *P<0.05, **P<0.01 and ***P<0.001 vs. healthy and #P<0.05 vs. AAV9-β-gal-treated diabetic (Diabetic-β-gal). Diabetic-Pim-1 (1X10^{10}) – Diabetic mice treated with low dose (1X10^{10} pfu/animal) AAV9-hPIM-1; Diabetic-Pim-1 (5X10^{10}) - Diabetic mice treated with high dose (5X10^{10} pfu/animal) AAV9-hPIM-1. Values are means±SEM. n=6 per group.

Online Figure X:
Representative confocal images and bar graphs showing the PCNA^{pos}Pim-1^{pos} cells at 20 weeks after DM induction (16 weeks after AAV9). Scale bars are 50µm. *P<0.01 vs. healthy and ###P<0.001 vs. AAV9-β-gal treated diabetic (n=6 per group). Data from low dose of AAV9-hPIM-1 (1X10^{10} pfu) treatment is shown with no further benefit from high dosage (5X10^{10} pfu).

Legends for Online Videos

Online video I
Serial z-stack images of myocardium showing the expression of hPIM-1 (in green), α-smooth muscle actin (in red) and Dapi (in blue). AAV9-hPIM-1 treatment transduced Pim-1 in vascular smooth muscles (identified by the positive staining for α-smooth muscle actin) of the heart.

Online video II
Serial z-stack images of myocardium showing the expression of hPIM-1 (in green), isolectin-B (in red) and Dapi (in blue). No expression of Pim-1 was identified in the endothelial cells (identified by the positive staining for isolectin-B) of the heart.
Online Figure I: Serum glucose levels
Bar graphs show the serum glucose levels in healthy or diabetic mice during the 20 weeks study.

***P<0.001 vs. healthy at corresponding time point. Diabetic-β-gal - diabetic mice treated with AAV9-β-gal; Diabetic-Pim-1 (1X10^10) - Diabetic mice treated with low dose (1X10^10 pfu/animal) AAV9-hPIM-1; Diabetic-Pim-1 (5X10^10) - Diabetic mice treated with high dose (5X10^10 pfu/animal) AAV9-hPIM-1.
Online Figure II

A. Flow cytometric characterization of murine CPCs

B. Cardiomyocytes derived from murine CPCs differentiation

C. Gene expression before and after differentiation

Online Figure II: Characterization of murine CPCs
A. Flow cytometry identification of Sca-1^pos murine CPCs, showing the gating procedure and purity of selected cells. B. Immunocytochemistry microphotographs of cardiomyocytes derived from Sca-1^pos cells following culture in differentiation medium and staining for cardiac specific markers such as α-sarcomeric actin and connexin-43. Scale bars are 50μm. C. Bar graphs showing the expression of troponin-T, α- and β-myosin heavy chain (MHC) and tropomyosin in cultured CPCs before and after differentiation, evaluated by semi-quantitative RT-PCR. Values are mean±SEM. Each experiment repeated in triplicates.
Online Figure III: Transfection efficacy of human PIM-1 plasmid in adult rat cardiomyocytes
Representative images of the hPim-1 plasmid transfected (A) and non-transfected (B) adult rat cardiomyocytes stained with human specific Pim-1 antibody. Scale bars are 50µm.

Online Figure IV: Titration of miR-1 optimal inhibitory dose
Bar graphs showing the inhibition of miR-1 expression (verified by RT-PCR) using different concentrations of anti-miR-1 in cardiomyocytes exposed to high glucose (HG). Scrambled sequence (Scr) used as control. *P<0.05 and ***P<0.001 vs. normal glucose (NG) and ###P<0.001 vs. corresponding dose of HG-Scr. Each experiment repeated in triplicates. Values are means±SEM.
Online Figure V

A. Representative western blotting membranes showing Pim-1 expression (34kD) in adult rat cardiomyocytes exposed to normal glucose (NG) or high glucose (HG) after transfection with hPIM-1 plasmid (Pim-1) or null (Null), detected using antibody that recognizes both rat and human Pim-1.

B. Caspase-8 and -9 activity - Bar graphs showing the caspase-8 and -9 activity in adult rat cardiomyocytes cultured in normal glucose (NG) or high glucose (HG) after transfection with hPIM-1 plasmid (Pim-1, 8µg/1X10^6 cells), Null vector (Null) or transfection vehicle (V). Values expressed as fold change to NG-V group and are means±SEM. *P<0.05 and **P<0.01 vs. NG-V and #P<0.05 vs. HG-null.
Online Figure VI

A.

B.

C.

Katare et al, CIRCRESAHA/2010/239111/R2

15
Online Figure VI: Pim-1 protects HL-1 cardiomyocytes from high glucose

A. Representative fluorescent microscopic images showing the expression of Pim-1 in murine HL-1 cardiomyocytes exposed to normal (NG) or high glucose (HG). HG-challenged cardiomyocytes were transfected with hPim-1 plasmid (Pim-1, 8μg/1×10⁶ cells). Null vector (Null) or transfection vehicle (V). Scale bars are 50μm. B. Representative western blots and bar graphs from a similar experiment showing the expression of phospho and total Akt, Pim-1, phospho and total Bad and Bcl-2. Actin was used as loading control. C. Bar graphs showing the levels of activated caspase-3/7. D. Representative western blots and bar graphs from a similar experiment. F. Bar graphs showing the levels of activated caspase-3/7. Each experiment was repeated five times in triplicate. Values are expressed as n-fold changes toward NG for all parameters and are means±SEM. *P<0.01 and **P<0.001 vs. NG and *P<0.05, **P<0.01 and ***P<0.001 vs. HG-null or HG-scr.
Online Figure VII

A. α-smooth muscle actin

B. Isolectin-B

Online Figure VII: AAV9-hPIM-1 transduce vascular smooth muscle cells
Representative confocal microscopic images showing the expression of hPim-1 in vascular smooth muscle cells (A, identified by co-staining with α-smooth muscle actin). No expression was detected in endothelial cells (B, identified by co-staining with isolectin-B). Scale bars are 50μm.
Online Figure VIII

A. Representative confocal microscopic images showing the transduction of hPim-1 in the skeletal muscle following systemic injection of AAV9-hPIM-1. Scale bars are 50μm. B. Representative hematoxylin and eosin stained skeletal muscle images and bar graphs showing the muscle area among the study groups. **P<0.01 and ***P<0.001 vs. healthy and ###P<0.01 vs. AAV9-β-gal-treated diabetic (β-gal). Scale bars are 2mm. Values are means±SEM, n=6 per group.
Online Figure IX: Biochemical measurements
Bar graphs showing the levels of serum insulin, cholesterol, triglycerides and cortisol at the end of the study in animals treated with AAV9-hPIM-1 or AAV9-β-gal. *P<0.05, **P<0.01 and ***P<0.001 vs. healthy and #P<0.05 vs. AAV9-β-gal-treated diabetic (Diabetic-β-gal). Diabetic-Pim-1 (1X10^{10}) - Diabetic mice treated with low dose (1X10^{10} pfu/animal) AAV9-hPIM-1; Diabetic-Pim-1 (5X10^{10}) - Diabetic mice treated with high dose (5X10^{10} pfu/animal) AAV9-hPIM-1. Values are mean±SEM.
Online Figure X: Representative confocal images and bar graphs showing the PCNA\textsuperscript{pos}pim-1\textsuperscript{pos} cells at 20 weeks after DM induction (16 weeks after AAV9). Scale bars are 50\,\mu m. *P<0.01 vs. healthy and ***P<0.001 vs. AAV9-\beta-gal treated diabetic (n=6 per group). Data from low dose of AAV9-hPIM-1 (1\times10^{10} \text{ pfu}) treatment is shown with no further benefit from high dosage (5\times10^{10} \text{ pfu}).