Metabolic Dysfunction Consistent With Premature Aging Results From Deletion of Pim Kinases

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Rationale: The senescent cardiac phenotype is accompanied by changes in mitochondrial function and biogenesis causing impairment in energy provision. The relationship between myocardial senescence and Pim kinases deserves attention because Pim-1 kinase is cardioprotective, in part, by preservation of mitochondrial integrity. Study of the pathological effects resulting from genetic deletion of all Pim kinase family members could provide important insight about cardiac mitochondrial biology and the aging phenotype.

Objective: To demonstrate that myocardial senescence is promoted by loss of Pim leading to premature aging and aberrant mitochondrial function.

Methods and Results: Cardiac myocyte senescence was evident at 3 months in Pim triple knockout mice, where all 3 isoforms of Pim kinase family members are genetically deleted. Cellular hypertrophic remodeling and fetal gene program activation were followed by heart failure at 6 months in Pim triple knockout mice. Metabolic dysfunction is an underlying cause of cardiac senescence and instigates a decline in cardiac function. Altered mitochondrial morphology is evident consequential to Pim deletion together with decreased ATP levels and increased phosphorylated AMP-activated protein kinase, exposing an energy deficiency in Pim triple knockout mice. Expression of the genes encoding master regulators of mitochondrial biogenesis, PPARγ (peroxisome proliferator-activated receptor gamma) coactivator-1α and β, was diminished in Pim triple knockout hearts, as were downstream targets included in mitochondrial energy transduction, including fatty acid oxidation. Reversal of the dysregulated metabolic phenotype was observed by overexpressing c-Myc (Myc proto-oncogene protein), a downstream target of Pim kinases.

Conclusions: Pim kinases prevent premature cardiac aging and maintain a healthy pool of functional mitochondria leading to efficient cellular energetics. (Circ Res. 2014;115:376-387.)

Key Words: aging ■ hypertrophy ■ metabolism ■ proto-oncogene proteins pim

Cardiac senescence and the aging phenotype are characterized by a multitude of changes on the cellular and organ level that occur during the lifetime of an organism. In contrast to rapid expansion of nascent cardiomyocytes taking place in postnatal development, de novo generation of myocytes in response to aging is limited. The onset of ventricular hypertrophy at the cellular and organ level is a hallmark of cardiac aging, which compensates for losses in cellular density and concomitant diminution of functional hemodynamic output. The consequence of pathological cardiac hypertrophy is eventual alterations in mitochondrial metabolism and energy homeostasis promoting glucose use over fatty acid oxidation (FAO), exacerbating disease. Preservation of mitochondrial integrity and function antagonizes aging because myocardial senescence is associated, in part, with decreased mitochondrial content and altered metabolic function. In This Issue, see p 325

Transcriptional coregulators, PPARγ coactivator-1 (PGC-1) α and β, serve as critical regulators of mitochondrial biogenesis and cellular ATP-producing pathways. PGC-1α and PGC-1β coactivate downstream transcription factors involved in mitochondrial biogenesis, such as estrogen-related receptor alpha (ERRα), nuclear respiratory factor 1 (NRF1), and mitochondrial transcription factor A (TFAM). PGC-1α is enriched and highly inducible in the heart. However, ablation of PGC-1α...
leads to compensatory upregulation of PGC-1β. PGC-1 coactivators regulate the mitochondrial FAO pathway, which serves as the primary supply for bioenergetic fuel in the healthy adult heart. Heart failure and hypertrophy prompt reprogramming of fuel use to rely predominantly on glucose metabolism similar to the fetal heart. Downregulation of PGC-1 signaling and the cognate downstream target PPARα contributes to the fuel shift toward fetal metabolism in the hypertrophied heart presenting as metabolic dysfunction. Furthermore, transgenic mice with single knockdown of PGC-1α or PGC-1β demonstrate age-dependent contractile dysfunction and impaired mitochondrial function, whereas mice lacking PGC-1α and PGC-1β die shortly after birth from heart failure.

Pim-1, a conserved serine/threonine protein kinase, exerts multiple protective effects on mitochondria and has recently been implicated in affecting metabolism through PGC-1α. In addition, Pim-1 stabilizes and phosphorylates c-Myc, a known regulator of mitochondrial metabolism and biogenesis. Pim-1 also affects mitochondrial dynamics through phosphorylation and cytosolic sequestration of Drp1. The Pim gene family consists of Pim-1, Pim-2, and Pim-3; 3 different genes transcribed from alternative start sites. All 3 Pim family members are constitutively active, exhibit similar substrate preferences, and differ primarily in tissue expression. Pim-1, the predominant isoform in the heart, can be genetically deleted in mice prompting compensatory upregulation of Pim-2 and Pim-3.

Pim-1 is highly expressed in postnatal hearts but diminishes precipitously during postnatal development. Recently, our group documented the remarkable ability of Pim-1 overexpression to rejuvenate aged human cardiac stem cells by decreasing senescent markers, promoting proliferation, and survival. Taken together, these studies implicate Pim kinases in the maintenance and preservation of a youthful cellular phenotype. Because Pim-1 promotes mitochondrial integrity and antagonizes acquisition of an aging phenotype, the relationship among Pim kinase, mitochondrial biogenesis, cardiac senescence, and metabolism resulting from loss of Pim kinase activity is investigated in this report.

Methods

Mice and Echocardiography

Echocardiography was performed under mild isoﬂurane sedation (0.5%–1.5%) using a Vevo 770 high-resolution system with wild-type FVB and Pim triple knockout (PTKO) mice. Cardiac function was analyzed in the parasternal long-axis view tracking the endocardium with the supplied analysis software to obtain left ventricle inner diameter during diastole, left ventricle anterior wall thickness during diastole, ejection fraction, and heart rate. We used the PTKO mouse model described by Mikk et al. Briefly, Pim-1 knockout mice were generated initially by homologous recombination using the targeting vector pGTP810 in FVB mice. Using Pim-1 knockout mice, PTKO mice were generated by deleting exons 1, 2, and 3 for Pim-2 with a PGK-β-gal cassette. Pim-3 exons 3, 4, 5, and 6 were deleted using a promoterless IRES-β-gal cassette.

ATP Assay

Lysates for ATP assay and citrate synthase assay were obtained from nontransgenic wild-type and PTKO mice. Briefly, mouse hearts were extracted after cervical dislocation and immediately snap frozen in liquid nitrogen. Hearts were then homogenized in isolation buffer: 70 mmol/L sucrose, 190 mmol/L mannitol, 20 mmol/L HEPES solution, 0.2 mmol/L EDTA solution. Homeogenized lysates were then used in an ATP assay (Invitrogen) according to the manufacturer protocol.

Cell Culture and Viral Transduction

Immortalized murine embryonic fibroblast from wild-type (iWT MEFs) and PTKO (iPTKO MEFs) mice were provided by Dr Andrew S Kraft (Medical University of South Carolina, Charleston, SC) and were cultured as previously described. Briefly, immortalized MEFs were maintained in DMEM (Invitrogen 11965-092) containing 10% fetal bovine serum and 1% penicillin-streptomycin-glutamine. Adenovirus enhanced green fluorescent protein (EGFP) was created and expanded as previously described. The PGC-1α adenovirus is identical to that used in Lehman et al. 2000 JCI. Adenovirus c-Myc was purchased from Vector Biolabs (Philadelphia, PA; Catalog# 1285) and expanded. For adenoviral transduction, iWT MEFs and iPTKO MEFs were infected in 2% fetal bovine serum DMEM for 2 hours followed by 2 wash steps of phosphate buffered saline and incubated in 10% DMEM and collected 24 hours later for analysis. A 50 multiplicity of infection was used for adenovirus EGFP, c-Myc, and 200 for PGC-1α.

Neonatal rat cardiomyocytes (NRCMs) were isolated and maintained as previously published. Briefly, isolated NRCMs were maintained in 0.5% M199. siRNA transfections were performed as previously published using HiPerfect transfection reagent (Qiagen). siRNA for Pim-1, Pim-2, and Pim-3 were obtained from Sigma and used at a concentration of 50 mmol/L.

Mitochondrial Respiration Assay and Telomere Measurements

Mitochondrial isolation procedures were followed as previously published. Telomeres were measured in nontransgenic wild-type and PTKO hearts using quantitative fluorescence in situ hybridization or multiplex quantitative polymerase chain reaction method as previously described. Further details are in Online Data Supplement.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.0 (Graphpad Software). Statistical analysis was performed using Student t test. Echocardiography time course analysis was assessed by 2-way ANOVA with Bonferroni post hoc test. A P value of <0.05 was considered statistically significant. Error bars represent SEM. Significance indicators are *P<0.05, **P<0.01, and ***P<0.001.

Results

Cardiac Hypertrophy Followed by Premature Cardiac Failure Is Evident in PTKO Mice

The expression of Pim kinases is differentially regulated in the myocardium over lifespan. Pim-1 expression is downregulated by 53% starting at 100 days after birth and remains 50% decreased at 324 days when compared with postnatal day 3 during physiological cardiac aging (P<0.01; Online Figure IA). Pim-2 expression does not change with cardiac aging (Online Figure IA). Pim-3 expression is upregulated at postnatal day 14 by 2.2-fold and increases at 100 and 324 days after birth by 2.6-

Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>ERR-α</td>
<td>estrogen-related receptor alpha</td>
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<td>FAO</td>
<td>fatty acid oxidation</td>
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<tr>
<td>iPTKO MEFs</td>
<td>immortalized Pim triple knockout murine embryonic fibroblast</td>
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<td>iWT MEFs</td>
<td>immortalized wild-type murine embryonic fibroblast</td>
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<tr>
<td>NRCMs</td>
<td>neonatal rat cardiomyocytes</td>
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<tr>
<td>PGC-1α</td>
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3.9-fold, respectively, when compared with postnatal day 3 ($P<0.01$; Online Figure IA). During myocardial infarction, Pim-1 expression increases in the infarct area at day 1 by 2.59-fold and gradually diminishes over time by 40% within the infarct region (Online Figure IB). Pim-2 expression increases in the remote region at day 1 by 2.62-fold and also diminishes over time (Online Figure IC). Pim-3 expression starts to decrease at 1 day after infarction in both the remote and the infarct regions and by 2 weeks it is decreased by 78% in infarct and remote regions (Online Figure ID). Increased Pim expression early during myocardial infarction is consistent with a prosurvival role for Pim genes.

Pathological hypertrophic remodeling during cardiac aging is well documented, as is the ability of Pim-1 to antagonize myocardial hypertrophy in response to pressure overload. Expression of Pim-1, Pim-2, and Pim-3 kinases decrease by 62%, 81%, and 76% at 2 weeks, respectively, in response to transaortic constriction (Online Figure IE). Consistent with hypertrophic remodeling, PGC-1α expression is diminished with transaortic constriction by 59%, 72%, 88%, and 77% at 1 day, 4 day, 7 day, and 2 weeks, respectively (Online Figure IF). Therefore, blunting of cardiac hypertrophy previously reported after Pim-1 overexpression was compared with the effect of Pim kinase deletion in PTKO mice. Cell size area was increased by 1.4-fold in PTKO mice at 1 month ($P<0.001$; Figure 1A and 1B). Increases were significant for Nppa (atrial natriuretic peptide; 19-fold; $P<0.05$), Nppb (brain natriuretic peptide; 2-fold; $P<0.05$), Acta1 ($\alpha$-skeletal actin; 4-fold; $P<0.01$), and Myh7 ($\beta$-myosin heavy chain; 43-fold; $P<0.05$) after Pim

![Figure 1. Characteristics of cardiac hypertrophy in Pim triple knockout (PTKO) mice. A, Wheat germ agglutinin staining in cardiac sections demonstrating cell surface area of Nontransgenic (NTG) and PTKO mice at 1 month. B, Quantification of cell size area (CSA) in heart tissue sections at 1 month (n=4, NTG and PTKO mice). C–F, mRNA levels of fetal cardiac gene markers: atrial natriuretic peptide (Nppa), brain natriuretic peptide (Nppb), alpha skeletal actin (Acta1), and beta myosin heavy chain (Myh7), respectively. * P<0.05, ** P<0.01, and *** P<0.001.](http://circres.ahajournals.org/doi/10.1161/CIRCRESAHA.114.300194)
deletion consistent with reactivation of the fetal gene program (Figure 1C–1F). Phosphorylated ribosomal S6 and phosphorylated 4EBP1 were diminished by 53% and 52%, respectively, indicating decreased mTOR (mammalian target of Rapamycin) activity (P<0.001 and P<0.05; Online Figure IIA and IIB). However, consistent with hypertrophic remodeling phosphorylated Akt was increased by 1.4-fold, and RCAN1.4 (regulator of calcineurin) expression was upregulated by 4.4-fold in PTKO mice, a downstream readout for nuclear factor of activated T-cells (NFAT) transcriptional activation in pathological hypertrophy (P<0.001 and P<0.05; Online Figure IIC and IID).

Body weight and heart weight were decreased by 22% (P<0.001) and 27% in PTKO mice, respectively (P<0.001; Online Figure IIIA and IIIIB). However, heart weight/body weight ratio was unchanged between PTKO and wild-type mice (Online Figure IIIC). Smaller left ventricle interior diameter during diastole was revealed by longitudinal echocardiography from 1 to 6 months of age in PTKO mice (P<0.001; Figure 2A). Furthermore, left ventricle anterior wall thickness during diastole demonstrated a strong trend toward increased wall thickness at 1 month and became significant at 3 months, showing an increase of 20% which persisted at 6 months (P<0.05; Figure 2B). A 30% reduction in ejection fraction was evident at 6 months in PTKO mice, indicating premature cardiac failure (P<0.001; Figure 2C). Heart rate was maintained at consistent levels to ensure comparability between individual measurements (Figure 2D). Consistent with cardiac hypertrophy and heart failure, markers of fibrosis collagen 1α1 and collagen 3α1 were upregulated 1.6- and 2.5-fold, respectively (P<0.01; Figure 2E and 2F). Furthermore, enlargement of the left ventricle in PTKO mice was evident by Massons trichrome staining (Figure 2G and 2H). Collectively, these findings demonstrate hypertrophic remodeling in PTKO mice and progression toward failure at 6 months.

Markers of Senescence Are Elevated With Loss of Pim Kinases

Senescent markers characterization was performed to determine the aging phenotype in PTKO mice. p16 expression was evident throughout the myocardium of PTKO mice at 3 months, indicating irreversible cellular senescence (Figure 3A). Increased p16 expression was confirmed by immunoblot in PTKO mice relative to nontransgenic controls (Figure 3B). p16 upregulation was validated by a concurrent reduction in p16 transcriptional repressors Id1 and Id2 by 60% and 64% in PTKO cardiac lysates, respectively (P<0.05, P<0.01; Online Figure IVA and IVB). Moreover, increased Ets-1 level (p16 transcriptional activator, 2.4-fold) also supports p16 induction (P<0.01; Online Figure IVC). p53 showed a 2.5-fold increase in PTKO mice at 3 months (P<0.01; Figure 3C) consistent with reduction of mouse double minute 2 homolog (MDM2) (p53 ubiquitin ligase MDM2; 57% lower; P<0.01; Figure 3D). Telomere mean fluorescence intensity was diminished by 41.5% in PTKO mice as measured by quantitative fluorescence in situ hybridization (P<0.001; Figure 3E and 3F). In addition, telomere length was decreased by 40% in PTKO mice by quantitative polymerase chain reaction analysis (P<0.001; Figure 3G). The collective phenotypic profile observed is consistent with expectations for premature cardiac senescence and predisposition toward heart failure in PTKO mice.

Knockdown of Pim-1, Pim-2, and Pim-3 in NRCMs stimulated an increase in p53 by 1.6-fold (P<0.05), whereas p16 expression was unchanged (Online Figure VA–VD). Furthermore, NRCMs treated with physiological (insulin) or pathological (phenylephrine) growth stimulus promoted an increase in p53 with loss of Pim-1, Pim-2, and Pim-3 by 2-fold (P<0.001) and 1.9-fold (P<0.01), respectively (Online Figure VA and VB). p16 gene expression was unchanged when compared with respective scrambled controls during stress (Online Figure VC and VD). Upregulation of Nppa by 1.4-fold (P<0.05), Nppb by 1.4-fold (P<0.05), and Myh7 by 2.5-fold was also noticed in NRCMs knocked down for Pim-1, Pim-2, and Pim-3, whereas α-skeletal actin displayed a strong increasing trend (Online Figure VE). Validation of siRNA efficiency resulted in 80%, 80%, and 67% decrease in gene expression of Pim-1, Pim-2, and Pim-3, respectively (P<0.001; Online Figure VF–VH). These data further endorse the premature cardiac aging phenotype of PTKO mice.

Mitochondrial Morphological and Functional Aberrations Occur With Loss of Pim

Pim-1 protects mitochondrial integrity and preserves mitochondrial morphology.10,11,19 Mitochondrial vacuoles and disrupted cristae structures were evident by electron microscopy in hearts of PTKO mice at 1 month after birth (Figure 4A). In addition, mitochondrial area and mitochondrial area per section were decreased by 11% and 10% in PTKO mouse hearts, respectively (P<0.05; Figure 4A). Validating these results, voltage-dependent anion channel expression was reduced by 16% in cardiac lysates of PTKO mice, indicating a reduction in mitochondrial mass (P<0.05; Figure 4B).

Loss of mitochondrial function is consistent with a reduction in energy reserve as revealed by analysis of ATP levels (Figure 4C). Corroborating these results, an increase in the ratio of phosphorylated AMP-activated protein kinase (AMPK) to total AMPK by 4-fold was noted in PTKO hearts by immunoblot (P<0.01; Figure 4D). Collectively, these results reveal alterations in mitochondrial morphology and functional capacity leading to energy deficiency resulting from loss of Pim kinases.

Metabolic Regulators Are Decreased With Deletion of Pim Kinases

Pim kinase deletion impairs expression of PGC-1α,15 with loss of PGC-1 coactivators promoting alteration of mitochondrial morphology.4 Therefore, the effect of Pim kinase deletion on expression of PGC-1 coactivators and downstream metabolic regulators was assessed. PGC-1α and PGC-1β expression were decreased by 42% and 82%, respectively, in PTKO cardiac lysates at 1 month (P<0.05; Figure 5A). Furthermore, downstream transcriptional targets of PGC-1 coactivators, including Erra, Nrf1, and Tfam (mitochondrial transcription factor A), were also decreased, respectively, by 44% (P<0.05), 83% (P<0.01), and 81% (P<0.01) in PTKO mice (Figure 5A). Peroxisome proliferative activated receptor gamma coactivator-related protein (PPRC1) was also diminished by 73% with Pim loss, evidently revealing lack of compensatory upregulation for related proteins (P<0.05; Online Figure VIA). Furthermore, decrease in PGC-1α (30%) and PGC-1β (20%) was confirmed at the protein level by immunoblot, corroborating gene expression analysis of PGC-1 coactivators (P<0.05; Figure 5B and 5C).
Mitochondrial biogenesis is also dependent on c-Myc expression that is a downstream target of Pim kinase.\textsuperscript{17,18} c-Myc protein levels were diminished by 36\% in PTKO mice ($P<0.01$; Figure 5D). The effect of decreased regulators of mitochondrial biogenesis is apparent by measurement of mitochondrial DNA content as a correlate for mitochondrial number that was reduced by 50\% in PTKO mice, consistent with decreased expression of PPAR$\gamma$ coactivators with Pim deletion ($P<0.05$; Figure 5E and 5F).

Decreased expression of PGC-1 coactivators alters the expression profile of cardiac metabolic genes,\textsuperscript{5,6,9} so representative genes involved in FAO, glycolysis, and oxidative phosphorylation were assessed. FAO-related enzymes located in the inner mitochondrial membrane were decreased, including Acate3 (acyl coenzyme A thioesterase 3; 14.9-fold; $P<0.001$), Acadv1 (acyl coenzyme A long chain; 6.9-fold; $P<0.001$), and Acadm (FAO-acetyl CoenzymeA dehydrogenase; 1.7-fold; $P<0.01$; Figure 6A). In contrast, transporters of fatty acids, such as Cpt1b

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**Figure 2. Premature cardiac failure at 6 months with Pim deletion.** A, Left ventricle inner diameter during diastole (LVIDd) in millimeters. B, Left ventricle anterior wall thickness during diastole (LVAWd) in millimeters. C, Percentage ejection fraction (EF \%). D, Average heart rate during echocardiography measurements. E and F, Gene expression analysis of collagen 1\alpha1 and collagen 3\alpha1 in cardiac tissue. G and H, Low-magnification image of nontransgenic (NTG) and PTKO hearts with trichrome staining. $n=8$ NTG and $n=9$ PTKO. *, **, and *** are significant compared with NTG mice at each individual time point. *$P<0.05$, **$P<0.01$, and ***$P<0.001$.
(carnitine palmitoyl transferase) and Cpt2 (carnitine palmitoyl transferase 2), were increased by 3.3-fold ($P<0.001$) and 2.3-fold ($P<0.01$), respectively, consistent with previous literature demonstrating upregulation of fatty acid transporters with prolonged AMPK activation (Figure 6A).$^{35}$ The rate-limiting step of glycolysis involves Hk2 (hexokinase 2) that was reduced by 15.3-fold ($P<0.001$; Figure 6B) in PTKO hearts. Conversely, downstream targets involved in the glycolytic pathway: Pfk
(phosphofructokinase), Pdha1 (pyruvate dehydrogenase E1 alpha 1), and Pfkfb2 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2) were highly upregulated by 3-fold (P<0.001), 3.6-fold (P<0.001), and 3.3-fold (P<0.001; Figure 6B). Furthermore, pyruvate respiration was increased, whereas succinate respiration was unaltered (P<0.05; Online Figure VIB and VIC), indicating a shift away from FAO and toward glucose metabolism similar to PGC-1α/β double knockouts. Genes involved in lipid sterol biosynthesis were also dramatically reduced in cardiac tissue of PTKO mice, indicating disruption in the PPAR signaling circuit (P<0.05; Online Figure VID). mRNA analysis for genes involved in oxidative phosphorylation, NDUFA (NADH dehydrogenase 1 alpha subcomplex subunit 9), NDUFV (NADH dehydrogenase ubiquinone flavoprotein 1), and Ldh2 (isocitrate dehydrogenase 2), revealed upregulation of 3.8-fold (P<0.001), 2.2-fold (P<0.001), and 2.9-fold (P<0.001), respectively (Figure 6C). However, Cytc (cytochrome C), a target of PGC-1α, was decreased by 8-fold (P<0.001; Figure 6C). To assess global changes, a microarray analysis of PPAR signaling genes involved in FAO, transport, and lipid sterol biosynthesis was performed, confirming altered expression of genes involved in the PPAR signaling pathway (Online Figure VII). Sixty-two PPAR-related signaling genes were detected in cardiac tissue, 32 showed a downregulation, 21 genes remained unchanged, and 8 genes were induced. Three of the 4

Figure 4. Mitochondrial morphological and functional aberrations with loss of Pim.
A, Electron microscopy scans of nontransgenic (NTG) (n=3) and Pim triple knockout (PTKO) (n=3) hearts at 1 month and quantification of mitochondrial area and mitochondrial area per section. B, Protein analysis of voltage-dependent anion channel (VDAC) in NTG (n=4) and PTKO (n=4) mouse hearts. C, Total ATP levels in NTG (n=13) and PTKO (n=10) heart lysates. D, Immunoblot for phosphorylated AMP-activated protein kinase (pAMPK) and total AMPK and analysis of pAMPK:AMPK ratio in cardiac samples of NTG (n=6) and PTKO (n=6) mice. * and ** are significant when compared with NTG mice. *P<0.05 and **P<0.01.
genes included in the targeted screen (Acadm, PGC-1α, Cpt1b, and Cpt2) demonstrated similar results to our targeted approach. Acadm and PGC-1α were downregulated, whereas Cpt1b was up-regulated. Cpt2 was upregulated in our screen and was unchanged in the microarray, this discrepancy might be because of differences in primer pairs because Cpt2 is predicted to have 5 alternatively spliced transcripts (NCBI: AceView). Taken together, these results demonstrate disruption of metabolic processes with Pim ablation mimicking the phenotype of PGC-1α/β double knockouts.

**c-Myc Rescues Expression of Metabolic Regulators**

The metabolic phenotype of PTKO mice correlates with loss of c-Myc expression secondary to Pim kinase ablation, so the participation of c-Myc was assessed by a rescue experiment involving overexpression of PGC-1α, c-Myc, and Pim-1 using iWT MEFs and PTKO (iPTKO MEFs) mice. Metabolic deficiencies in the iPTKO MEFs were confirmed to be comparable with those of PTKO cardiac lysates. Expression of c-Myc and PGC-1α was diminished by 82% and 55%, respectively, in iPTKO MEFs (P<0.05; Online Figure VIIIA) similar to PTKO mouse hearts. Phosphorylated AMPK:total AMPK ratio was increased in iPTKO MEFs by 2.3-fold consistent with decreased cellular energy stores (P<0.05; Online Figure VIIIB). Similarly, alterations of metabolic regulators and reduction in FAO enzymes were comparable between iPTKO MEFs and PTKO heart lysates (Online Figure VIIIC). Because the iPTKO (A)

**Figure 5.** PPAR coactivators and downstream targets are decreased with Pim deletion. A, mRNA levels of PGC-1α, PGC-1β, estrogen-related receptor alpha (Errα), nuclear respiratory factor 1 (NRF1), and mitochondrial transcription factor A (TFAM) in 1 month nontransgenic (NTG; n=6) and PTKO (n=6) hearts. B–D, Protein analysis of PGC-1α, PGC-1β, and c-Myc, respectively, in cardiac samples; NTG (n=6) and PTKO (n=7) hearts. E and F, Mitochondrial DNA analysis of Cox1 and Cytochrome B normalized to β-globin; NTG (n=4) and PTKO (n=4). * and ** are significant when compared with NTG mice. *P<0.05 and **P<0.01. CSQ indicates calsequestrin.
MEFs possess analogous metabolic derangements to PTKO heart samples, molecular restorations were attempted by overexpression of c-Myc, PGC-1α, or Pim-1. Overexpression of EGFP, Pim-1-EGFP, PGC-1α-EGFP, and c-Myc-EGFP adenovirus was confirmed in iPTKO MEFs (Online Figure VIIID). c-Myc overexpression in iPTKO MEFs increased the expression of metabolic regulators PGC-1α (1.7-fold; P < 0.05), PGC-1β (1.6-fold; P < 0.05), Errα (1.4-fold; P < 0.05), Nrf1 (1.2-fold; P < 0.05), and Avadhl (1.2-fold; P < 0.05; Figure 7A). Pim-1 overexpression increased the expression of metabolic regulators PGC-1α (1.6-fold; P < 0.05), PGC-1β (2.2-fold; P < 0.05), Errα (1.5-fold; P < 0.05), Nrf1 (1.3-fold; P < 0.05), and Avadhl (1.5-fold; P < 0.05; Figure 7B). PGC-1α overexpression in iPTKO MEFs restored gene expression of the PGC-1α downstream targets: Errα and Avadhl by 2.3- and 1.8-fold, respectively (P < 0.001; Figure 7C). Consequential to increased metabolic regulator expression, the ratio of phosphorylated AMPK:total AMPK decreased by 34% (P < 0.01) with c-Myc, 35% with Pim-1, and 41% (P < 0.01) with PGC-1α overexpression, indicating improvement in cellular energy stores after interventions to overexpress c-Myc, Pim-1, or PGC-1α (Figure 7D–7F). In summary, the phenotype resulting from loss of Pim kinases can be rescued with overexpression of c-Myc, Pim-1, or PGC-1α.

Discussion
Cardiac aging is characterized by pathological hypertrophic remodeling that is a primary indicator of and confirmed risk factor for heart failure. Pathological hypertrophy and progression toward heart failure were evident at 6 months in PTKO mice (Figures 1 and 2) consistent with previous published studies demonstrating antagonism of myocardial hypertrophy by Pim-1. Increased AKT phosphorylation and RCAN1.4 expression, a readout for activated NFAT, was evident with loss of Pim, which serve as molecular underpinnings for hypertrophic signaling (Online Figure II) and remodeling. In addition, fetal gene reprogramming typical for hypertrophic stimulation is blunted by Pim-1 overexpression. Promotion of hypertrophic effects at the molecular, cellular, and organ levels in PTKO hearts is relatively early in life (Figure 1), and enhanced senescence marker expression (Figure 3) is consistent with Pim kinase deletion manifesting with features of an accelerated aging phenotype.

Pathological hypertrophic remodeling causes an energetic fuel shift from FAO to glucose metabolism. Cellular metabolic changes are potent regulators of the aging phenotype and PTKO mice demonstrate an upregulation of glycolytic enzymes and diminished FAO enzymes, exhibiting metabolic alterations (Figure 6). Mitochondrial metabolism and energetics converge on the PPAR signaling circuit and the influence of Pim kinases on PGC-1α is established. Now for the first time to our knowledge, the connection between downregulation of PGC-1α and β and downstream targets affected by Pim kinase deletion has been revealed in the heart by our results (Figure 5). Furthermore, PGC-1α is important and necessary to maintain a pool of healthy mitochondria because PGC-1α regulates mitochondrial biogenesis. Disruption in mitochondrial biogenesis occurs during aging causing accumulation of unhealthy mitochondria and potential increases in toxic metabolic byproducts that potentially accelerate cellular aging. Diminished PPAR signaling occurs with aging in human and in murine animal models, supporting our contention that disrupted metabolism and diminished mitochondrial biogenesis through loss of PGC-1α in PTKO mice contributes to premature aging.

Downstream of Pim kinases is c-Myc, a potent regulator of mitochondrial biogenesis and PGC-1β. Diminished expression of c-Myc was evident in PTKO hearts and iPTKO MEFs (Figure 5D; Online Figure VIII). Acute overexpression of c-Myc in iPTKO MEFs promoted upregulation of PGC-1α and PGC-1β in addition to downstream regulators of mitochondrial biogenesis. c-Myc activation during pathological cardiac injury is shown to promote glucose metabolism by down regulating PPARγ coactivators. In comparison, the observed loss of c-Myc still prompted enhanced glycolytic enzyme expression in PTKO mice, suggesting alternative mechanism(s) for promotion of glucose metabolism in PTKO mice. The loss of Pim kinase consequently promotes diminished expression of c-Myc along with PPARγ coactivators presenting as cellular metabolic alterations consistent with acquisition of an aging phenotype.

Mitochondria play a central role in energy metabolism, with mitochondrial morphology intimately tied to mitochondrial function. Pim kinases protect mitochondrial integrity by
upregulating antiapoptotic B-cell lymphoma 2 (Bcl-2) family members (Bcl-2 and Bcl-XL [B-cell lymphoma-extra large]) and phosphorylating BAD on serine 112.16,34 Furthermore, Pim-1 regulates mitochondrial morphological dynamics by regulating Drp1 localization and phosphorylation.19 Imbalance in mitochondrial fission sensitizes mitochondria to cell death in addition to promoting formation of smaller mitochondria as seen in PTKO hearts (Figure 4A). Pim deletion also prompted altered mitochondrial morphology and disarray of myofibrils (Figure 4A). Mitochondrial morphology alterations can increase apoptotic sensitivity to cell death and propensity to permeabilize in response to stress. Disruption of mitochondrial dynamics occurring with Pim loss promotes instability in mitochondrial DNA,19,41 and reduction in mitochondrial DNA content precedes pathological hypertrophy.42 Consistent with these assertions, PTKO mice display diminished levels of mitochondrial DNA content before heart failure (Figure 5E and 5F) that can serve as an additional predisposing factor for premature aging at the mitochondrial level with loss of Pim.

Telomere attrition disrupts mitochondrial function through a molecular mechanism involving p53-mediated cellular senescence leading to a p53-dependent repression of PGC-1α.43 Telomere length decrease in PTKO mice (Figure 3E–3G) is a plausible inductive mechanism for upregulation of p53 activity and subsequent blunting of PGC-1α. Conversely, increased telomere length resulting from Pim-1 kinase overexpression is associated with decreased expression of senescent markers including p53 and increased cell cycling in human cardiac progenitor cells.24 Comparable beneficial effects of telomere length maintenance are also observed after Pim-1 kinase overexpression in murine cardiac progenitor cells.23 Furthermore, PTKO mice at 4 weeks exhibit an accelerated aging phenotype evident by telomere attrition of 40% by quantitative polymerase chain reaction and fluorescence in situ hybridization (Figure 3E–3G). This dramatic decrease would be comparable with the relative age of 30 months in a normal mouse.3 Increased cellular turnover during physiological aging leads to a reduction in telomere length with each cell division.44-46 Although Pim-1 deletion blunts cellular proliferation that may help slow telomeric shortening, any modest beneficial effect is apparently overwhelmed by the depression of TERT (telomerase reverse transcriptase) activity that compromises preservation of telomere length, leading to telomeric shortening in PTKO mice consistent with accelerated physiological aging. Therefore, the effect of Pim kinase on telomere biology highlights an important potential mechanism affecting mitochondrial function and causing cellular senescence that is the subject of ongoing investigation.

Figure 7. Rescue of metabolic genes with c-Myc, Pim-1, and PGC-1α overexpression. Overexpression of c-Myc, Pim-1, and PGC-1α adenovirus, respectively, in immortalized Pim triple knockout murine embryonic fibroblast (iPTKO MEFs) followed by (A–C) quantitative polymerase chain reaction of PPAR coactivators and downstream targets. D–F, Quantification of phosphorylated AMP-activated protein kinase (pAMPK)/total AMPK ratio. n=3 individual experiments and *, **, and *** are significant when compared with iPTKO MEFs infected with EGFP adenovirus mice. *P<0.05, **P<0.01, and ***P<0.001.
Pim kinases promote proliferation by regulating cell cycle proteins, such as p21, p27, and cdc25A, through phosphorylation.17,18 Furthermore, Pim phosphorylation and stabilization of c-Myc enhance cell cycle entry,19 leading to the plausible idea that Pim kinases antagonize the aging phenotype and favor ongoing mitotic activity by blunting the effect of cell cycle inhibitors. Indeed, downregulation of cell cycle inhibitors p16 and p53 leading to increased proliferation occurs in aged and senescent human cardiac progenitor cells overexpressing Pim-1 kinase.20 Conversely, in PTKO mice, the increased expression of p16 and p53 supports a role for the Pim kinase family in preserving phenotypic characteristics of cellular youthfulness (Figure 3; Online Figure V).

The cardioprotective activity of Pim-1 through the preservation of mitochondrial structure and morphology is well established primarily based on the overexpression of Pim-1.16,19 However, the converse of this experimental approach implies that the loss of Pim kinase would promote deterioration of mitochondrial structure and function. Such diminution of Pim kinase occurs naturally with organ ischemic aging, but ours is the first assessment of germline deletion for all 3 Pim kinase family members on myocardial biology. Degenerative changes in mitochondria, such as altered structural integrity, shifts in metabolic fuel selectivity, and decreased ATP production, are hallmarks of cellular aging and senescence.50 Clearly, select essential features of the aging and senescent phenotype are recapitulated with accelerated timing in the PTKO line. Now a link can be placed to connect Pim kinases to aging and metabolic changes through the PPAR signaling circuit. Important to note as a limitation of this study is the PTKO mouse models are global knockouts and not cardiac specific. Therefore, in vivo findings although consistent with expectations remain correlative in nature, so these findings were buttressed by mechanistic molecular signaling in vitro studies with IPTKO MEFs. At present, the circumstantial evidence points toward alterations in mitochondrial biogenesis and metabolism coincident with premature cardiac aging of PTKO hearts. Looking forward to an interventional strategy, prevention of metabolic derangements is important to antagonize the cardiac aging phenotype. So too, delineating underlying causes of aging are important to develop therapeutic interventions to treat aging myocardopathy in the elderly population. The PTKO mouse model could serve in this capacity as an excellent model system to study cardiac aging and senescence. Collectively, this study gives credence to the possibility of using Pim-1 as a therapeutic agent for treatment of and intervention in the pathogenesis of cardiac aging.

Acknowledgments

We thank all members of the M.A.S laboratory for helpful discussion and comments.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- Mitochondrial dysfunction is associated with aging.
- Overexpression of Pim-1 kinase decreases the expression of senescent markers in human cardiac progenitor cells.
- Pim-1 kinase protects mitochondrial integrity and loss of Pim-1 kinase promotes mitochondrial fission.

What New Information Does This Article Contribute?

- Loss of Pim kinases causes premature cardiac aging.
- Mitochondrial and metabolic alterations are evident with loss of Pim and converges on PPARγ coactivator-1α signaling.
- Overexpression of Pim-1 rescued metabolic alterations in Pim triple knockout immortalized murine embryonic fibroblast.

Myocardial aging leads to deterioration of cardiac function promoting onset of heart failure, in part by, altered metabolism. Preventing deterioration of mitochondrial function is known to blunt cardiac aging and avert heart failure. Pim kinases promote cell proliferation, survival, and preserve mitochondrial integrity in the cardiac context. Therefore, Pim kinase-mediated protection of mitochondrial function was hypothesized to antagonize phenotypic consequences of aging associated with metabolic derangement. Cardiac metabolism depends on mitochondrial function that is regulated by PPARγ coactivator-1α signaling to stimulate fatty acid oxidation. Heart failure prompts decreased fatty acid oxidation and a concomitant increase in glycolytic metabolism that devolves into declining energy reserves. Loss of Pim kinases caused premature heart failure and was coincident with decreased PPARγ coactivator-1α signaling, altered cardiac metabolism, and decreased ATP levels. Conversely, overexpression of Pim-1, the major cardiac isoform, in Pim triple knockout immortalized murine embryonic fibroblast rescued metabolic gene expression and increased energy reserves. Therefore, taken together with ability of Pim-1 overexpression to blunt the expression of senescent markers in human cardiac progenitor cells and maintain mitochondrial integrity, our findings reveal novel beneficial properties imparted by Pim-1, lending further support for activation of Pim-1 as a therapeutic modality to inhibit cardiac aging and failure by preserving mitochondrial function and healthy metabolic activity.
Metabolic Dysfunction Consistent With Premature Aging Results From Deletion of Pim Kinases

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Supplemental Material

Metabolic Dysfunction And Premature Aging Are Caused By Pim Deletion

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Supplemental Methods

Mitochondrial respiration assay
Mitochondrial isolation procedures were followed as previously published. Hearts were removed while still contracting from mice anesthetized with ketamine/xylazine (50mg/kg and 10mg/kg, respectively) via intraperitoneal injection. Individual mouse hearts were rapidly minced in ice-cold isolation buffer (100 mmol/L KCl, 50 mmol/L MOPS pH 7.4, 1 mmol/L EGTA, 5 mmol/L MgSO4, 1 mmol/L ATP, 0.2% essentially fatty acid free BSA). Henceforth, all steps were performed at 0°C on wet ice. Tissue was homogenized in isolation buffer with a Polytron tissue grinder at 11,000 RPM for 2.5 s, followed by 3 strokes at 500 RPM with a Potter-Elvehjem PTFE tissue grinder. The homogenate was centrifuged at 600×g twice for 5 min and the supernatant was saved. Mitochondria were pelleted from the supernatant by centrifugation at 3,000×g twice, and the pellet was rinsed with isolation buffer. The final mitochondrial pellet was resuspended in 100 µl of resuspension buffer (220 mmol/L mannitol, 70 mmol/L sucrose, 2 mmol/L Tris base, and 20 mmol/L HEPES pH 7.4). Protein concentration was determined by Bradford assay using BSA standards. Oxygen consumption measurements were performed with an Oxygraph Clark type electrode (Hansatech Instruments) in respiration buffer (10 mmol/L MgCl2, 100 mmol/L KCl, 50 mmol/L MOPS pH 7.0, 1 mmol/L EGTA, 5 mmol/L KH2PO4, 0.2% essentially fatty acid free BSA). 200 µg of mitochondria were added to a final volume of 1 ml respiration buffer at 30°C. Complex I activity was measured using 2 mmol/L pyruvate and 2 mmol/L malate as substrates. Complex II activity was measured using 5 µmol/L rotenone with 5 mmol/L succinate as substrate.

Quantitative Fluorescence In Situ Hybridization (QFISH) Telomere Measurements
Telomere length was labeled and analyzed by quantitative in situ hybridization (QFISH) using DAKO’s PNA probe (K5325) and Leica confocal microscopy. Results were obtained by altering manufacturer’s protocol as follows. Slides were deparaffinized, rehydrated and treated with 3.7% Formaldehyde for 2 minutes, then subsequently washed with TBS. Next, followed antigen retrieval using 10mM Citrate pH 6.0 for 15 minutes. Slides were washed again with TBS and underwent proteolytic treatment using Proteinase K (Dako, S3004) diluted 1:10 in TBS, for 8 minutes. Preceding another wash, slides were dehydrated in cold ethanol. PNA probe was applied to dried sections, coverslipped using a 2mm round coverslip, and incubated on a 85°C hot plate for 5 minutes then allowed to hybridize at 37°C overnight. The following day, slides were immersed in Rinse Solution to remove coverslips and washed with 70% Formamide pH 7.2. Next, slides were washed in pre-heated Wash Solution at 65°C and quenched with 3% H2O2 for 20 minutes at room temperature. Slides were then washed, incubated with Sheep anti-FITC/HRP 1:200 in TNB for 1 hour at room temperature, washed again, amplified with Tyr/FITC 1:50 for 10 minutes, then dehydrated in cold ethanol. Lastly, slides were counter-stained and coverslipped using Topro 3 1:200 in Vectashield. Telomere signal in each nucleus was acquired using Leica software and divided by nuclear size followed by normalization to NTG controls. Additionally, signals were scanned using identical setting to control experimental variation.

Telomere Length measurement (RT-PCR)
Telomere length was measured by real time PCR using a modified monochrome multiplex quantitative PCR method previously described. Albumin and the telomere template were amplified at the same time to account for the differences in DNA concentration per well and sample. To account for difference in reaction samples, 12
replicates of each sample were prepared using 10ng of DNA, 1X syber green, albumin, and telomere primers, bringing the reaction volume to 15uL. The thermal cycle protocol used is as follows, stage 1 (2 cycles of 15 mine at 95°C, 15 seconds at 94°C and 15 seconds at 49°C), stage 2 (15 seconds at 94°C, 10 seconds at 62°C and 15 seconds at 73°C with plate acquisition), stage 3 (32 cycles of 15 seconds at 94°C, 10 seconds at 62°C, 15 seconds at 73°C with signal acquisition, 10 seconds at 84°C, 15 seconds at 87°C with signal acquisition), and stage 4 (1 cycles of 0.05 seconds at 65°C with signal acquisition).

Sample preparation, Immunoblotting, RT-PCR, Mitochondrial DNA Content

Immunoblotting was performed using protein samples separated on 4-12% NuPAGE Novex Bis-Tris Gel (Invitrogen) by electrophoresis. Protein contents of gels were then transferred onto a polyvinylidene fluoride (PVDF) membrane, blocked in iBLOCK (Life Technologies), and exposed to primary antibodies over night. Alkaline phosphatase (AP), horseradish peroxidase (HRP), Fitc-, Cy5, or Cy3-conjugated IgGs (Jackson ImmunoResearch) were used as secondary antibodies. Fluorescence signal was detected and quantified by using a Typhoon 9400 fluorescence scanner together with ImageQuant 5.0 software (Amersham Biosciences).

mRNA was isolated using the Quick RNA Mini Prep kit (Zymo Research) according to the manufacturer protocol. cDNA was transcribed using the cDNA preparation kit (Biora). For RT-PCR sybr green (Biorad) was employed using the manufacturer protocol. Data were analyzed with the ΔΔC(t) method. A complete list of primers used for qPCR is provided (Table 2). Mitochondrial DNA (mtDNA) copy number was quantified by qPCR from isolated total DNA derived from heart tissue of PTKO mice (n=4) and wildtype mice (n=4) according to the manufacturer protocol of NucleoSpin tissue kit (Macherey-Nagel). 50 ng of total heart DNA was used and each sample was measured in duplicates. Two different primer pairs were used to quantify and confirm relative mtDNA copy number: COXI and Cytochrome b (mitochondrial) and β-Globin/H-19 for genomic DNA. All sequences are listed in Table 2. Data obtained by qPCR were analyzed by the ΔΔCT method.

Transmission Electron Microscopy and Mitochondrial Area

Mitochondria were examined by transmission electron microscopy. Animals were euthanized by cervical dislocation before dissection. The heart was excised and submerged immediately in 2% glutaraldehyde in 0.1M cacodylate buffer with 1% sucrose. Cardiac tissue was cut with a razor blade into 1 mm² pieces and incubated in fixative solution on ice for 10 minutes. All of the following steps, through the 100% ethanol dehydration step, were performed on ice. After incubation, each sample received a fresh replacement of fixative solution and was fixed for 1 hour. Following fixation, the samples were washed three times (10 minutes each wash) in 0.1M cacodylate buffer with 1% sucrose. The samples were then postfixed with 1% osmium tetroxide in 0.1M cacodylate buffer with 1% sucrose for 1 hour in the dark and washed three times (10 minutes each wash) in water. The samples were dehydrated through a graded series of ethanol solutions from 30% to 50%, 70%, 85%, 95% and 100% (10 minutes each). Two successive 10 minute incubations in 100% ethanol were performed before the samples were transferred into 100% acetone at room temperature followed by an additional 10 minute incubation step in 100% acetone. Tissue samples were infiltrated with 1:2 EPON/acetone resin followed by 2:1 EPON/acetone resin for 8 hours each. Infiltration with pure EPON was completed overnight. Tissue samples were transferred into a flat embedding mold in pure EPON and polymerized at 60°C for 48
hours. Thin sections (60nm thick) from the embedded samples were cut, collected on 100 mesh copper grids, and stained with uranyl acetate and lead citrate. Images were captured using a FEI Tecnai 12 transmission electron microscope operated at 120kV. Images were acquired at 11000X. Measurements of mitochondrial area per section were calculated as follows using ImageJ software: mitochondrial area was measured followed by normalization to area of section. Sample size was n=3 for each group analyzed.

Sample preparation, Immunoblotting, qPCR, Mitochondrial DNA Content, Microarray Analysis

Immunoblotting was performed using protein samples separated on 4-12% NuPAGE Novex Bis-Tris Gel (Invitrogen) by electrophoresis.

mRNA was isolated using the Quick RNA Mini Prep kit (Zymo Research) according to the manufacturer protocol. cDNA was transcribed using the cDNA preparation kit (Bio-Rad). For RT-PCR sybr green (Biorad) was employed using the manufacturer protocol. Data were analyzed with the ΔΔC(t) method. A complete list of primers used for qPCR is provided (Table II). Further details are provided in supplemental information.

Microarray analysis was performed according to the manufacturer protocol using PPAR signaling array from Bar Harbor technologies (catalog number: 00198247) on CFX Connect thermal cycler (Bio-Rad).

Immunohistochemistry and Cell Counts

Heart sections were deparaffinized followed by antigen retrieval in citrate (10 mM, pH 6.0). One-hour block in TNB buffer was followed by incubation in primary antibody overnight. Primary antibodies and concentration used are listed (Table I). Three washes to remove unbound primary antibody in Tris/NaCl were followed by secondary antibody incubation for 1.5 hours at room temperature. Specimens were mounted with Vectashield (Vector Laboratories). To pro to label nuclei was added in the last wash step after incubation with secondary antibodies at 1:5000 for 20 minutes. Images were obtained on a Leica DMRE confocal microscope. Area assessments for myocyte size were performed using the outlining tool from ImageJ after images of cardiac specimens stained with wheat germ agglutinin. Sections were stained with sarcomeric actin to visualize cardiomyocytes and myocardial area. Infarct size was calculated as proportion of infarcted area relative to total area of the respective section with at least 6 high power fields were analyzed per heart for non-transgenic (NTG) or Pim Triple KnockOut (PTKO) mouse heart samples. Image acquisition and size measurements were performed under sample-blinded conditions.

Masson Trichrome staining was performed as previously described and micrographs were acquired using Leica DMRE confocal microscope.
Supplementary Figure I: Expression of Pim kinases during aging and pathological challenge. A) Gene expression analysis of Pim 1,2, and 3 during physiological cardiac aging. B-D) Pim1, Pim2, and Pim3 gene expression respectively post myocardial infarction. E) Time course analysis of Pim kinase gene expression post transaortic constriction. F) PGC-1α gene expression post transaortic constriction. *p < 0.05; **p < 0.01; ***p < 0.001. #p < 0.05; ##p < 0.01; ###p < 0.001.
Supplementary Figure II: Hypertrophic pathways in PTKO mice. A-B) Immunoblot of mTORC1 targets phosphorylated and total ribosomal S6 and phosphorylated and total 4EBP1 with loss of Pim. C) Phosphorylated Akt levels in PTKO mice assessed by immunoblot. D) Gene expression of RCAN1.4 in PTKO mice. n=6 NTG and n=6 PTKO. *, **, *** is significant compared to NTG. *p < 0.05; **p < 0.01; ***p < 0.001.
Supplementary Figure III: Biometric parameters in PTKO mice. A-C) Body weight, heart weight, and heart weight to body weight ratio in NTG and PTKO mice at six months respectively. n=8 NTG and n=9 PTKO. D) Myocyte number in PTKO hearts assessed by confocal microscopy at 1 month. n=4 NTG and n=4 PTKO. *** is significant compared to NTG mice. ***p < 0.001.
Supplementary Figure IV: Pim deletion activates p16 pathway. A & B) Immunoblot of p16 transcriptional repressors Id1 and Id2 in NTG (n=3) and PTKO (n=3) mice. C) Protein analysis of p16 transcriptional activator Ets-1 in NTG (n=3) and PTKO (n=4) cardiac lysates. * and ** is significant compared to NTG mice. *p < 0.05 and **p < 0.01.
Supplementary Figure V: Increased senescence occurs in NRCMs with Pim kinase knockdown. A&B) Gene expression of p53 in NRCMs with siRNA to Pim123 followed by insulin or phenylephrine stimulation respectively (n=4). C&D) mRNA analysis of p16 following Pim123 knockdown and growth stimulation (n=4). E) Analysis of fetal gene program in NRCMs with Pim kinase knockdown (n=4). F-H) Validation of siRNA efficiency to Pim kinas in NRCMs by quantitative PCR. *p < 0.05, **p < 0.01, and ***p<.001 when compared to scrambled alone. # p<.05 when compared to scrambled Ins or PE.
Supplementary Figure VI: Functional mitochondrial alterations and reduced PPAR signaling in PTKO mice. A and B) Mitochondrial respiration assay using glutamate and succinate as substrates in NTG (n=4) and PTKO (n=4) one month isolated mitochondria. C) mRNA levels of PPRC1 in NTG (n=6) and PTKO mice (n=6) at one month D) mRNA analysis of genes downstream of PPAR signaling involved in lipid sterol biosynthesis in NTG (n=6) and PTKO (n=6) one month hearts. *, **, *** is significant compared to NTG. *p < 0.05; **p < 0.01; ***p < 0.001.
Supplementary Figure VII: Microarray analysis of genes involved in PPAR signaling. Microarray analysis of cardiac mRNA from NTG and PTKO mice at 1 month. 62 PPAR-related signaling genes were detected in cardiac tissue, 32 showed a downregulation, 21 genes remained unchanged, and 8 genes were induced. Three out of the four genes included in the targeted screen (Acadm, PGC-1α, Cpt1b, and Cpt2) demonstrated similar results to our targeted approach. Acadm and PGC-1α showed decreased gene expression, Cpt1b exhibited increased expression. Genes included in the microarray panel are involved in fatty acid oxidation, fatty acid transport, cholesterol synthesis and lipid/sterol biosynthesis.
Supplementary Figure VIII: iPTKO MEFs demonstrate similar metabolic dysfunction as PTKO mice. A) Immunoblot of c-Myc and PGC-1α in iWT MEFs and iPTKO MEFs (n=3) B) Phosphorylation levels of AMPK and quantification of pAMPK : AMPK in iPTKO MEFs compared to iWT MEFs (n=3). C) q-PCR of genes involved in the PPAR signaling circuit (n=3). D) Protein analysis of adenoviral overexpression of PGC-1α, c-Myc, Pim1, and EGFP. *, **, *** is significant compared to iWT MEFs. *p < 0.05; **p < 0.01; ***p < 0.001.
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<tr>
<td>PDHA1 Pyruvate dehydrogenase E1 alpha 1</td>
<td>GGGACGTCTGTGAGAGAGC TGTGTCATGGTAGCGGTAA</td>
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<td>PFKFB2 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2</td>
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<td>Cytc Cytochrome C</td>
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<td>Ndufa NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 9 (Ndufa9)</td>
<td>ATCCCTTACCCTTTGCCACT CCGTAGACCTCAATGGACT</td>
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<tr>
<td>Ndufv NADH dehydrogenase (ubiquinone) flavoprotein 1 (Ndufv1)</td>
<td>TGTGAGACCCTGTCAATGGGA CATCTCCCTCACAAATCGG</td>
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<td>PPRC1 Peroxisome proliferator-activated receptor gamma</td>
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</tr>
<tr>
<td>Gene Name</td>
<td>Forward Primer Sequence</td>
<td>Reverse Primer Sequence</td>
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<td>coactivator-related protein 1</td>
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<td>Mvk mevalonate kinase</td>
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<td>Acsi3 Long-chain-fatty-acid—CoA ligase 3</td>
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<td>Elov1 Fatty acid elongase 1</td>
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<td>Hsd17b12 hydroxysteroid (17-beta) dehydrogenase 12</td>
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<td>Sc5d sterol-C5-desaturase</td>
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<td>Acss2 acyl-CoA synthetase short-chain family member 2.</td>
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<td>Nppa (ANP) Atrial natriuretic peptide</td>
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<td>Nppb (BNP) Brain natriuretic peptide</td>
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<td>αSKA alpha skeletal actin</td>
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<tr>
<td>Gene</td>
<td>For primer</td>
<td>Rev primer</td>
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<td>Collagen 3α1</td>
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**Primers for quantification of Mitochondrial DNA**

<table>
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<tr>
<th>Gene</th>
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<th>Rev primer</th>
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<tbody>
<tr>
<td>CoxI (mitochondrial)</td>
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<td>TGGGGCTCCGATTATTAGTG</td>
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<td>Cytochrome B (mitochondrial)</td>
<td>ATTCCTTGCATGTGACGAG</td>
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<td>H19 (nuclear)</td>
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B1 globin (nuclear) | For primer | GCACCTGACTGATGCTGAGAA
| Rev primer | TTCATCGCGCTTCACCTTTCC

