Nuclear Plakoglobin Is Essential for Differentiation of Cardiac Progenitor Cells to Adipocytes in Arrhythmogenic Right Ventricular Cardiomyopathy


Rationale: Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a disease of desmosome proteins characterized by fibroadipogenesis in the myocardium. We have implicated signaling properties of junction protein plakoglobin (PG) in the pathogenesis of ARVC.

Objective: To delineate the pathogenic role of PG in adipogenesis in ARVC.

Methods and Results: We generated mice overexpressing PG, either a wildtype (PG WT) or a truncated (PG TR), known to cause ARVC, in the heart; and PG null (PG-/-) embryos. PG WT and PG TR mice exhibited fibro-adiposis, cardiac dysfunction, and premature death. Subcellular protein fractionation and immunofluorescence showed nuclear localization of PG WT and PG TR and reduced membrane localization of PG TR. Coimmunoprecipitation showed reduced binding of PG TR but not PG WT to desmosome proteins DSP and DSG2. Transgene PG WT and PG TR were expressed in c-Kit+;Sca1+ cardiac progenitor cells (CPCs) isolated from the hearts of PG WT and PG TR by fluorescence activated cell sorting. CPCs isolated from the transgenic hearts showed enhanced adipogenesis, increased levels of adipogenic factors KLF15, C/EBP-α and noncanonical Wnt5b, and reduced level of CTGF, an inhibitor of adipogenesis. Treatment with BIO activated the canonical Wnt signaling, reversed the proadipogenic transcriptional switch and prevented adipogenesis in a dose-dependent manner. Moreover, c-Kit+ CPCs, isolated from PG-/- embryos, were resistant to adipogenesis, expressed high mRNA levels of CTGF and other canonical Wnt signaling targets.

Conclusions: Nuclear PG provokes adipogenesis in c-Kit+ CPCs by repressing the canonical Wnt signaling and inducing a proadipogenic gene expression. The findings suggest that adipocytes in ARVC, at least in part, originate from c-Kit+ CPCs. (Circ Res. 2011;109:1342-1353.)

Key Words: cardiomyopathy ■ genetics ■ adipogenesis ■ Wnt signaling ■ progenitor cells

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an enigmatic disease characterized by fibroadipocytic replacement of cardiac myocytes, predominantly in the right ventricle. The phenotype is unique, progressive, and without an effective therapy. Progressive fibroadipogenesis, myocytes drop-out, and molecular remodeling lead to cardiac arrhythmias, ventricular dysfunction, and premature death. ARVC is an important cause of sudden cardiac death in the young.

Molecular pathogenesis of ARVC has remained elusive since its modern description more than 3 decades ago. Molecular genetic studies have partially elucidated genetic causes of ARVC and provided insights into its pathogenesis. Mutations in genes encoding junction protein plakoglobin (PG), plakophilin 2 (PKP2), desmoplakin (DSP), desmoglein 2 (DSG2), and desmocollin 2 (DSC2) are collectively responsible for about 50% of ARVC cases. Mutations in non-desmosome proteins TGFB3 and TMEM43 also have been reported in families with ARVC, ARVC, at least in a subset, is a disease of desmosome proteins.

To delineate the molecular pathogenesis of ARVC, we have focused on the signaling functions of PG, aka γ-catenin. PG, a member of the Armadillo arm proteins, has structural and functional similarities to β-catenin, the signal transducer of the canonical Wnt signaling through Tcf7l2/Lef1 transcription factors. PG is implicated in competitive interactions with β-catenin in regulating various pathways, such as binding to Tcf7l2, ubiquitination, incorporation into adherens junctions, and even desmosome assembly. The precise nature of these interactions is not

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From Center for Cardiovascular Genetics, Institute of Molecular Medicine and Department of Medicine, University of Texas Health Sciences Center at Houston, and Texas Heart Institute at St. Luke’s Episcopal Hospital, Houston, Texas. Achim Bell is currently affiliated with University of Mississippi Medical Center, Jackson.

Correspondence to A.J. Marian, MD, 6770 Bertner Street, Suite C900A, Houston, TX 77030. E-mail Ali.J.Marian@uh.tmc.edu

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adequately defined and appears to be context dependent. PG and β-catenin differ in their propensities binding to Tcf7l2.17,18 Accordingly, β-catenin exhibits a strong transcriptional activity through Tcf7l2 and provokes gene expression. In contrast, PG exhibits weak Tcf7l2-mediated transcriptional activity. Consequently, the net effect of binding of PG to Tcf7l2 is suppression of the canonical Wnt signaling. Canonical Wnt signaling not only regulates cell proliferation but also is a major transcriptional switch regulator of myogenesis versus adipogenesis.19 Thus, PG is a structural protein constituent of desmosomes as well as a signaling molecule that regulates the canonical Wnt signaling.

We have implicated signaling functions of PG in the pathogenesis of ARVC.14 Accordingly, impaired desmosome assembly in ARVC leads to nuclear localization of PG, repression of the canonical Wnt signaling and enhanced adipogenesis.14 Absence of PG at the cell junction has been advocated as a diagnostic marker for ARVC in humans.20 Through a series of genetic/fate mapping experiments, we have identified the second heart field cardiac progenitor cells (CPCs) as a cell source for excess adipocytes in ARVC. However, the direct role of PG in ARVC remains untested. We performed a series of experiments analogous to gain-and loss-of-function studies by overexpressing either a wildtype PG (PGWT) or a truncated PG (PGTR), known to cause ARVC in humans,11 in cardiac myocyte-lineage and genetically deleting PG in embryos (PG−/−). We isolated c-Kit+;Sca1+ CPCs from the hearts of PG transgenic mice and PG−/− embryos, determined differentiation of these CPCs to adipocytes, and delineated the responsible molecular mechanisms.

Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by the Institutional Animal Care and Use Committee.

Genetically Modified Mice

PGWT and PGTR (FVB background) were generated per conventional methods. To distinguish transgene PGWT from the endogenous PG (PGEndo), 3 sequential Flag epitopes were positioned at the N-terminal domain of PG. To generate the deletion construct (PGTR), known to cause ARVC in humans,11 in cardiac myocyte-lineage and genetically deleting PG in embryos (PG−/−). We isolated c-Kit+;Sca1+ CPCs from the hearts of PG transgenic mice and PG−/− embryos, determined differentiation of these CPCs to adipocytes, and delineated the responsible molecular mechanisms.

Survival, Gross Cardiac Morphology, and Histology

Survival rates were compared by Kaplan–Meier survival analysis. Ventricular/body weight ratios were measured in age- and sex-matched mice. Echocardiography was performed as described.15,22 Electrocardiograms were also recorded. Myocardial histology was examined by H&E and Masson Trichrome staining of thin sections.

Adipocytes were detected by Oil Red O (ORO) staining and C/EBP-α immunostaining, as published.15,22

Immunoblotting

Aliquots of ventricular tissue (50 mg) were homogenized using a Covaris Sonicator in RIPA buffer containing complete protease inhibitors. Protein concentration was determined by Lowry assay. Aliquots of 20 µg of protein extracts were electrophoresed for immunoblotting. Antibodies were anti-Flag, mutant PG-specific, and pan PG to detect PGWT, PGTR, and transgene plus PGEndo on the same blot, respectively.

Immunofluorescence

Thin myocardial sections and isolated cells were immunostained using anti-pan PG, Flag, PGTR, C/EBP-α, and c-Kit antibodies, as described.15,15 The samples were mounted in DAPI-containing Hard SetTM mounting medium.

Cell Protein Subfractionation

Nuclear, cytosolic, and membrane proteins were extracted, as published.15,22 Aliquots of 30 µg of proteins were used for immunoblotting and probed with pan-PG, PGTR, and Flag (PGWT) antibodies. Membranes were stripped in Restore Plus stripping buffer and were reprobed with antibodies against α-tubulin, Lamin A, and Cx43 to test purity of the separation.

Coimmunoprecipitation (Co-IP)

Co-IP was performed as described.15,22 Anti-DSP1/2, DSC2, and DSG2 antibodies were added to 500 µg aliquot of total protein extracts followed by precipitating the antibody-protein complexes by Protein A/G PLUS-Agarose beads and centrifugation. The final pellets were resuspended in a loading buffer and used for immunoblotting.

Isolation and Culture of CPCs from Adult Mouse Hearts

Hearts from 2- to 3-month-old mice were depleted from mature myocytes through digestion with 0.1% type 2 collagenase in α-MEM.
medium. Cell suspensions were sequentially passed through 70-μm, 40-μm, and 35-μm mesh strainers to eliminate residual mature myocytes and cell aggregates. The cell pellets were washed in MACS Buffer, and incubated with monoclonal antibodies to Sca1 and c-Kit. C-Kit+/H11001, Sca1+/H11001, and c-Kit+/H11001:Sca1+/H11001 cells were sorted using a FACS-Aria flow cytometer. The c-Kit+/H11001 cells were negative for markers of the myeloid, lymphoid, and erythroid lineages. After sorting, cells were seeded into 0.1% gelatin-coated plates and supplemented with 10% embryonic stem cell certified FBS, 10 ng/mL mouse basic fibroblast growth factor (bFGF), 1000 U/mL of mouse leukemia inhibitory factor (mLIF), and 1% antibiotic–antimycotic. All the experiments were conducted on cells yielded from the third to fifth passage.

Isolation and Culture of PG+/+/ and PG−/− CPCs

PG−/− embryos were harvested at E11, separated from placenta and membranes under a dissecting microscope, and genotyped by direct PCR. PG−/− and PG+/− null embryos were used to isolate CPCs. To isolate CPCs, we incubated embryonal cells with mouse lineage antibodies cocktail and antibodies against Flk1, Sca1, and c-Kit. The FACS-Aria flow cytometer was used to identify and sort c-Kit−, Sca1−, and c-Kit+:Sca1+ cells from the population negative for markers of the myeloid, lymphoid, and erythroid lineages (CD34, CD45, CD20, CD45RO, CD8, and TER-119) and endothelial marker Flk1.

RT-PCR

Total RNA was isolated from CPCs using Qiagen RNasey Mini Kit. To eliminate genomic DNA, the extracts were treated with DNase 1 sequentially with 2 different DNase reagents. Aliquots of 2 μg of total RNA were reverse transcribed using SuperScript III First-Strand Synthesis System and oligo dT primers. The RT products were amplified by PCR using primers designed to specifically amplify PGEndo, PGWT, PGTR, α-MyHC, and Gapdh mRNAs (Online Figure II and Online Table I).

Induction of Adipogenesis

C-Kit+ progenitor cells from nontransgenic (NTG), PGWT, and PGTR mouse hearts and from PG+/+ and PG−/− embryos were plated at a density of 60,000 cells per well on 0.1% gelatin-coated cover glass and treated with Adipogenesis Induction Medium (α-MEM supplemented with 10% FBS, 1% antibiotic-antimycotic, 10 μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 1 μmol/L dexamethasone) for 5 days. The media was then changed to Maintenance Medium containing 10 μg/mL insulin. Two days later, cells were stained with ORO and C/EBP-α.
To quantify the number of adipocytes, the number of ORO-stained cells in 25 microscopic fields (40X magnification) and the number of C/EBPα-stained cells in 20 fields (63X magnification) per group were counted in 3 independent experiments.

Activation of Wnt Signaling by Pharmacological Inhibition of GSK-3β
CPCs from the heart of PGTR mice were plated on 0.1% gelatin-coated cover glass in proliferation media, supplemented with 2 μmol/L, 5 μmol/L, and 10 μmol/L of 6-bromoindirubin-3'-oxime (BIO), a known activator of the canonical Wnt signaling.23 Untreated cells and cells treated with BIO but not subjected to adipogenesis were included as control groups. At the end of the treatment, cells were stained with ORO and C/EBPα, and the percentages of positive cells were determined.

Real-Time PCR (qPCR)
Levels of mRNAs for selected molecular markers were quantified by qPCR using specific TaqMan Gene expression assays (Online Table I) and were normalized to Gapdh mRNA level.

Statistical Analysis
Statistical analysis was performed using STATA-Intercooled version 10.1 software (StataCorp LP, College Station, TX), as described.15 Data are expressed as mean ± 1 SD. Kaplan–Meier survival curves were compared by log-rank test.

An expanded Methods section can be found online at http://circres.ahajournals.org.

Results
Cardiac Lineage-Specific Expression of PG Leads to Increased Fibroadiposis, Cardiac Dysfunction, and Premature Death
We generated 2 lines of PGWT and 3 lines of PGTR transgenic mice using a 5.5-kbp α-MyHC promoter (a generous gift from Dr. Jeffrey Robbins, University of Cincinnati). The 11 novel amino acids in the PGTR were used as an epitope to generate mutant PG-specific (PGTR) antibody (Figure 1A). The custom-made antibody against this epitope reacted with PGTR but not PGWT (Online Figure I). Expression of the transgene protein in the heart was detected using Flag-specific (PGWT), PGTR-specific, and pan PG (transgene PGPGEndo) antibodies (Figure 1B). Relative expression levels of the PGWT and PGTR, quantified after probing the membranes with a Pan PG antibody, were 31% to 41% and 26% to 47% of total PG, respectively (Figure 1B and C). Lines with the higher expression of the transgene (41% in PGWT and 47% in PGTR) were used for further characterization.

Six- to 12-month-old NTG, PGWT, and PGTR mice were euthanized for morphological and histological characterization. Hearts were enlarged in PGTR mice in comparison with NTG or PGWT mice (Figure 2A). The ventricular/body weight ratio was significantly increased in age- and sex-matched PGTR mice in comparison with NTG mice (5.1±1.1 versus 4.4±0.5 mg/g, respectively, P=0.006) or PGWT
Echocardiographic findings are presented (Figure 2D). Histological examination showed increased myocardial fibrosis and increased adipocytes in the myocardium of PGWT and PGTR mice (Figure 2B). Histological examination showed increased number of adipocytes in the heart in PGWT and PGTR mice (Figure 2B). Histological examination showed increased number of adipocytes in the heart in PGWT and PGTR mice (Figure 2B). Histological examination showed increased number of adipocytes in the heart in PGWT and PGTR mice (Figure 2B). Histological examination showed increased number of adipocytes in the heart in PGWT and PGTR mice (Figure 2B). Histological examination showed increased number of adipocytes in the heart in PGWT and PGTR mice (Figure 2B). Histological examination showed increased number of adipocytes in the heart in PGWT and PGTR mice (Figure 2B). Histological examination showed increased number of adipocytes in the heart in PGWT and PGTR mice (Figure 2B). Histological examination showed increased number of adipocytes in the heart in PGWT and PGTR mice (Figure 2B). Histological examination showed increased number of adipocytes in the heart in PGWT and PGTR mice (Figure 2B).

To recapitulate the human genotype, we attempted to generate mice that expressed only PGTR in the heart. However, expressing PGTR in the background of homozygous deficiency of PGEndo (PG+/−:PGTR) did not produce viable pups, presumably because of early lethality of PG−/− embryos. Bigenic mice expressing PGTR in the background of heterozygous deficiency of PGEndo (PG+/−:PGTR) were viable and exhibited a phenotype similar to that observed in the PGTR transgenic mouse, including increased fibroadipocytes (Online Figure IV). Echocardiographically, the bigenic PG+/−:PGTR mice showed increased LVEDD, LVESD, and LVM and reduced LVFS (Online Table III). Heterozygous deficiency of PG (PG+/−) was also associated with increased LVEDD, but these mice had preserved LVFS. In comparison with PG+/− mice, the bigenic PG+/−:PGTR mice exhibited significantly increased LVEDD, LVESD, and LVM and significantly lower LVFS (Online Table III).

### Impaired Binding of Mutant PGTR to Other Desmosome Proteins, Reduced Desmosome Incorporation, and Enhanced Nuclear Localization

Immunoreactive signal for PGTR was virtually absent at desmosomal sites and was primarily detected in the nucleus (Figure 3A). However, PGWT was detected in the desmosome as well as in the nucleus.

Immunoblotting of cell protein subfractions showed findings consistent with the results of the immunofluorescence staining. Accordingly, PGWT and PGTR were detected in the nuclear and cytosolic protein subfractions. PGWT was also present in the membrane fraction. In contrast, PGTR was minimally detectable in the membrane subfraction (Figure 3B).

Co-IP studies (Figure 3C) showed reduced binding of PGTR to its interacting desmosomal proteins DSP and DSG2, but preserved binding to DSC2. In contrast, binding of PGWT to DSP, DSG2, and DSC2 was preserved (Figure 3C). Likewise, both PGWT and PGTR were coprecipitated with TCF7l2 (Figure 3D).

### Enhanced Adipogenesis in CPCs Isolated from PGWT or PGTR

To test the direct role of PG in adipogenesis, we isolated c-Kit+/Sca1+, c-Kit−, and Sca1− cells from the hearts of age- and sex-matched NTG, PGWT, and PGTR adult mice by FACS. c-Kit−:Sca1− cells comprised about 0.1% ± 0.08% of cardiac myocytes depleted cell pools in the hearts in 4 independent isolations. The relatively high percentage of c-Kit−:Sca1− cells, while in accord with some of the previous data, likely reflects our 2-step gating enrichment strategy (Online Figure V). The percentage does not reflect the number of c-Kit−:Sca1− cells in vivo in the myocardium, which is expected to be much lower. There were no significant differences in the number of c-Kit−:Sca1− isolated from NTG, PGWT, and PGTR mouse hearts.

RT-PCR, performed using specific primers (Online Figure II and Online Table I), showed expression of PGEndo in CPCs isolated from NTG, PGWT, and PGTR mouse hearts (Figure 4A). Likewise, PGWT and PGTR were expressed in c-Kit−:Sca1− cells isolated from the hearts of PGWT and PGTR mice, respectively. In addition, α-MyHC was also expressed in the isolated CPCs (Figure 4A). The latter finding also corroborates expression of the PG transgenes regulated by the α-MyHC promoter.

Immunofluorescence staining of c-Kit−:Sca1− cells with specific antibodies against PGTR, PGWT, and pan PG also
confirmed expression of the PG in CPCs and suggested nuclear localization of transgene PG in these cells (Figure 4B and 4C). Moreover, immunofluorescence staining for PG and c-Kit, performed without cell permeabilization in order to preserve the transmembrane antigen c-Kit, confirmed coexpression of c-Kit and PG in CPCs (Figure 4C).

To determine whether expression of PG in CPCs enhanced adipogenesis, we stimulated c-Kit\textsuperscript{+/H11001}:Sca1\textsuperscript{+/H11001} cells isolated from NTG, PG\textsuperscript{WT}, and PG\textsuperscript{TR} hearts for adipogenesis and stained with ORO and C/EBP-α. CPCs isolated from PG\textsuperscript{WT} and PG\textsuperscript{TR} mice showed increased accumulation of fat droplets as well as a higher number of C/EBP-α expressing cells (Figure 5A), in comparison with NTG cells. Numbers of ORO and C/EBP-α positive cells are shown in Figure 5B and 5C. In addition, c-Kit\textsuperscript{+/H11001} but Sca1\textsuperscript{+} cells isolated from PG\textsuperscript{TR} mouse hearts also showed enhanced adipogenesis (Online Figure VIA and VIB). Moreover, CPCs isolated from PG\textsuperscript{TR} and PG\textsuperscript{WT} but not from NTG mice exhibited spontaneous adipogenesis in the absence of an adipogenic induction (Figure 5D through 5F).

To determine a transcriptional switch to adipogenesis regulated by the canonical Wnt signaling, we performed qPCR to quantify mRNA levels of KLF15, IGFBP5, and CTGF\textsuperscript{26,27}. KLF15 and IGFBP5 mRNA levels were increased markedly (3–5-fold and 10\textsuperscript{5}–144-fold, respectively) in PG\textsuperscript{WT} and PG\textsuperscript{TR} progenitor cells (Figure 5G). Likewise, mRNA level of Wnt5B, a noncanonical Wnt that promotes adipogenesis\textsuperscript{28} was increased significantly. In contrast, mRNA level of CTGF, a known inhibitor of adipogenesis and a target of the canonical Wnt signaling\textsuperscript{29} was significantly reduced.

To further substantiate suppressed canonical Wnt signaling by PG in mediating adipogenesis in CPCs, we activated the canonical Wnt signaling by treating c-Kit\textsuperscript{+/H11001}:Sca1\textsuperscript{+} cells with BIO, a known inhibitor of GSK3\textsuperscript{β}\textsuperscript{23}. Cyclin D1 and c-Myc mRNA levels, targets of the canonical Wnt signaling, were reduced in c-Kit\textsuperscript{+/H11001}:Sca1\textsuperscript{+} cells isolated from PG\textsuperscript{TR} hearts but not in cells treated with BIO, indicating suppressed canonical Wnt signaling (Figure 6A). Treatment with BIO normalized cyclin D1, c-Myc, and CTGF mRNA levels in c-Kit\textsuperscript{+/H11001}:Sca1\textsuperscript{+}
cells isolated from PGTR hearts (Figure 6A). In accord with molecular rescue of the canonical Wnt targets, treatment with increasing concentrations of BIO reversed adipogenesis in c-Kit\(^+\) Sca1\(^+\) cells in a dose-dependent manner, despite treatment with adipogenic inducers (Figure 6B through 6D). In contrast, CPCs not treated with BIO showed enhanced adipogenesis. Similar experiments in c-Kit\(^+\) only cells isolated from PGTR hearts replicated the findings in c-Kit\(^+\) Sca1\(^+\) cells on reversal of adipogenesis with BIO (Online Figure VIC and VID).

Resistance of PG\(^{-/-}\) CPCs to Adipogenesis
PG\(^{+/+}\) and PG\(^{-/-}\) c-Kit\(^+\) CPCs, isolated from E11 embryos (Online Figure VII and Online Table I) were morphologically identical (Figure 7A). Mouse embryonic fibroblasts (MEFs) isolated from PG\(^{+/+}\) and PG\(^{-/-}\) embryos showed differential adipogenesis when subjected to adipogenic induction. MEFs from PG\(^{-/-}\) showed about 20-fold reduction in the number of ORO stained cells in comparison with PG\(^{+/+}\) cells (Online Figure VIII).

The number of c-Kit\(^+\) cells (Lin\(^-\) and Flk1\(^-\)) was significantly reduced in the PG\(^{-/-}\) embryos in comparison with PG\(^{+/+}\) embryos. Representative plots showing the gating setting used to identify and sort Lin\(^-\) Flk1\(^-\) c-Kit\(^+\) cells are shown in Figure 7B. On average, c-Kit\(^+\) cells represented about 2% to 8% of the Lin\(^-\) Flk1\(^-\) cells in the PG\(^{+/+}\) embryos, in contrast to 0.1% to 1.5% of the Lin\(^-\) Flk1\(^-\) cells in the PG\(^{-/-}\) embryos, a several-fold reduction. In addition, PG\(^{-/-}\) CPCs were resistant to adipogenesis, because only rare ORO positive cells were found despite adipogenic induction. In contrast, adipogenesis was effectively induced in the PG\(^{+/+}\) CPCs (Figure 7C and 7E). Consistent with resistance to adipogenesis, mRNA level of CTGF, an inhibitor of adipogenesis,\(^{27}\) was increased by 22-fold in PG\(^{-/-}\) c-Kit\(^+\) CPCs (Figure 7F). To determine whether resistance to adipogenesis in PG\(^{-/-}\) c-Kit\(^+\) CPCs was associated with activation of canonical Wnt signaling, we measured mRNA levels of Sox2, c-Myc, and cyclin D1, downstream targets of this pathway and cyclin E1 and PCNA, markers of cell proliferation. Sox2, c-Myc, and cyclin D1 as well as cyclin E1 and PCNA mRNA levels were increased significantly (Figure 7F). Collectively, these data link inhibition of adipogenesis in PG\(^{-/-}\) c-Kit\(^+\) CPCs to activation of the canonical Wnt signaling.
The findings provide direct evidence for the essential signaling functions of nuclear PG in repressing the Wnt/β-catenin signaling pathway in CPCs and inducing a transcriptional switch to adipogenesis in ARVC. In the presence of PG, c-kit⁺/Sca1⁺ CPCs exhibited enhanced differentiation to adipocytes. In contrast, in the absence of PG these cells were resistant to adipogenesis. These findings in conjunction with previous data in cardiac myocytes-restricted Dsp-deficient mice and genetic fate mapping experiments identify PG as a mediator of differentiation of a subset of CPCs from a myogenic to an adipogenic fate in ARVC. We expressed PG under the transcriptional regulation of Myh6, which is conventionally considered a postnatal gene. However, Myh6 is also expressed during early embryonic period and in c-kit⁺ CPCs. In support of the embryonic expression of Myh6, cardiac myocytes lineage-restricted deletion of Dsp leads to near total embryonic lethality at E12–14. Notwithstanding the existing data, we detected expressions of CTGF mRNA and PG, regulated by Myh6 promoter, in CPCs isolated from PGWT and PGTR groups. In contrast, mRNA levels of KLF15 and IGFBP5 were increased dramatically. Likewise, mRNA level of Wnt5b, a noncanonical Wnt known to induced adipogenesis, was also increased significantly.

**Figure 5. Adipogenesis in c-Kit⁺:Sca1⁺ cells.** A, Represents ORO- and C/EBPα-stained panels showing detection of fat droplets and expression of C/EBPα in c-Kit⁺:Sca1⁺ cells isolated from the heart of NTG, PGWT, and PGTR mice. We examined 2 x 10⁶ cells for ORO and 200 cells for C/EBPα staining. B, The number of c-Kit⁺:Sca1⁺ cells stained positive for fat droplets was 150 ± 18 and 162 ± 21 cells per field in PGWT and PGTR groups, respectively, in comparison with 15 ± 10 cells per field in the NTG group. C, Shows percentage of c-Kit⁺:Sca1⁺ cells that were positive for C/EBPα expression. D, ORO- and C/EBPα-stained sections showing spontaneous adipogenesis in c-Kit⁺:Sca1⁺ CPCs in the transgenic hearts. E and F, Represent quantitative assessment of ORO- and C/EBPα-stained cells in the absence of adipogenic induction. G, mRNA levels of selected markers of adipogenesis in c-Kit⁺:Sca1⁺ cells normalized to Gapdh mRNA levels and shown relative to NTG. CTGF mRNA level, an inhibitor of adipogenesis, was reduced significantly in c-Kit⁺:Sca1⁺ cells in the PGWT and PGTR groups. In contrast, mRNA levels of KLF15 and IGFBP5 were increased dramatically. Likewise, mRNA level of Wnt5b, a noncanonical Wnt known to induced adipogenesis, was also increased significantly.

**Discussion**

The findings provide direct evidence for the essential signaling functions of nuclear PG in repressing the Wnt/β-catenin signaling pathway in CPCs and inducing a transcriptional switch to adipogenesis in ARVC. In the presence of PG, c-kit⁺:Sca1⁺ CPCs exhibited enhanced differentiation to adipocytes. In contrast, in the absence of PG these cells were resistant to adipogenesis. These findings in conjunction with previous data in cardiac myocytes-restricted Dsp-deficient mice and genetic fate mapping experiments identify PG as a mediator of differentiation of a subset of CPCs from a myogenic to an adipogenic fate in ARVC. We expressed PG in CPCs under the transcriptional regulation of Myh6, which is conventionally considered a postnatal gene. However, Myh6 is also expressed during early embryonic period and in c-kit⁺ CPCs. In support of the embryonic expression of Myh6, cardiac myocytes lineage-restricted deletion of Dsp under the transcriptional regulation of the α-MyHC promoter leads to near total embryonic lethality at E12–14. Notwithstanding the existing data, we detected expressions of α-MyHC and PG, regulated by Myh6 promoter, in CPCs isolated from PGWT and PGTR groups. Coexpression of PG and α-MyHC in c-Kit⁺ cells indicates a subset of cells that are in transition from a progenitor state to an early myogenic lineage.

Unlike our transgenic PGWT model, PG is not overexpressed in human ARVC. We overexpressed PGWT in the heart in order to determine the signaling functions of PG that is not incorporated into desmosomes (nonjunctional PG), as in human ARVC, PG is displaced from the junction. We placed a Flag epitope to track and distinguish the transgene PGWT from the endogenous PG. Co-IP and immunofluorescence studies showed proper desmosome localization and binding of Flag-tagged PG to selected desmosome proteins. Unlike the PGWT, the mutant PGTR was not epitope tagged, because of generation of a PGTR-specific antibody. The
PG\textsuperscript{WT} and PG\textsuperscript{TR} both exhibited excess fibroadipocytes, suggesting that the observed phenotype is not because of the Flag epitope. Furthermore, we and others have previously shown that nontagged endogenous PG could translocate to nucleus, suppress the canonical Wnt signaling, and provoke adipogenesis.\textsuperscript{14} Nevertheless, we also generated bigenic PG\textsuperscript{WT}/H\textsubscript{11001}/H\textsubscript{11002}:PG\textsuperscript{TR} mice, which exhibited excess fibroadipocytes in the heart and cardiac dysfunction, as also observed in the single transgenic PG\textsuperscript{TR} mice. Collectively, these data indicate that findings reflect signaling functions of PG.

PG\textsuperscript{TR} transgenic mice express a truncated PG, known to cause Naxos disease in humans.\textsuperscript{11} The PG\textsuperscript{TR} transgenic mice exhibit a phenotype that resembles ARVC. However, the genotype of PG\textsuperscript{TR} mice is not identical to the genotype of humans as Naxos disease is caused by a homozygous deletion mutation in PG. Expression of PG\textsuperscript{TR} under the transcriptional control of \textit{Myh6} did not rescue early embryonic lethality of PG deficiency,\textsuperscript{21} indicating earlier expression of PG than \textit{Myh6}. The PG\textsuperscript{WT}/PG\textsuperscript{TR} mice, however, exhibited a phenotype similar to PG\textsuperscript{TR} mice. Nevertheless, because of the differences in the genotypes of mouse models and human patients, application of the findings to human ARVC remains to be determined. In addition, the findings may not pertain to the pathogenesis of ARVC caused by genes coding for nondesmosome proteins or various phenotypic features of ARVC, such as gap junction remodeling, cardiac arrhythmias, and myocardial fibrosis, which may result from interactions of PG with other cellular proteins, not analyzed in the present study. Finally, alternative approaches to activate the canonical Wnt signaling, other than inhibition of GSK3\textsuperscript{β} with BIO, might provide further insights.

Cardiac dysfunction in PG\textsuperscript{TR} and PG\textsuperscript{WT}/PG\textsuperscript{TR} was similar and likely results from poor assembly of the truncated PG into desmosome, reflected by reduced levels of PG\textsuperscript{TR} in cell membrane. In contrast, PG\textsuperscript{WT} mice exhibited only mild left ventricular dilatation. Despite disparities in cardiac dysfunction, PG\textsuperscript{TR} and PG\textsuperscript{WT} mice exhibited premature mortality and cardiac arrhythmias. The molecular mechanisms responsible for cardiac arrhythmias and increased mortality in PG\textsuperscript{WT} remain to be explored. The findings also suggest partial dissociation of cardiac dysfunction from fibroadipogenesis in ARVC, as the former primarily reflects impaired desmosome assembly at least in early stages of ARVC, and the latter differentiation of CPCs to adipocytes. In advances stages of

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\caption{Rescue of adipogenesis in c-Kit\textsuperscript{+}:Sca1\textsuperscript{+} cells on activation of the canonical Wnt signaling. A, mRNA levels of selected markers of the canonical Wnt signaling in c-Kit\textsuperscript{+}:Sca1\textsuperscript{+} cells not treated or treated with BIO. Cyclin D1, c-Myc and CTGF mRNA levels were reduced in c-Kit\textsuperscript{+}:Sca1\textsuperscript{+} isolated from PG\textsuperscript{TR} mice. Treatment with BIO normalized their mRNA levels. B, Treatment of c-Kit\textsuperscript{+}:Sca1\textsuperscript{+} cells isolated from the heart of PG\textsuperscript{TR} mice with 3 doses of BIO prevented adipogenesis in CPCs despite adipogenic induction. C and D, Quantification of ORO and C/EBP\textalpha positive cells in c-Kit\textsuperscript{+}:Sca1\textsuperscript{+} cells treated with BIO and untreated cells.}
\end{figure}
ARVC, adipocytes infiltration of the myocardium might also contribute to cardiac dysfunction.2

PG seems to regulate—probably through the canonical Wnt pathway—expression of several adipogenic factors. Changes in CTGF mRNA level inversely paralleled expression of PG. CTGF, also known as CCN2, is a known target of the canonical Wnt signaling29 and yet regulates Wnt signaling by interacting with Wnt coreceptor, LRP6.32 These data identify CTGF as a plausible molecular mediator of adipogenesis in ARVC. Likewise, expression level of KLF15, known to inhibit cardiac myocytes hypertrophy and repress transcriptional activator myocardin,33,34 was significantly increased in PG−/− CPCs. KLF15 is highly expressed in brown adipose tissue and is markedly up-regulated during differentiation of preadipocytes to adipocytes.26 KLF15 is also known to inhibit CTGF in cardiac fibroblasts.35 Moreover, IGFBP5 mRNA level was increased by more than 100-fold in CPCs that expressed transgene PG. IGFBP5 is a target of Akt1/FOXO transcription factors and also is regulated by the Wnt/β-catenin signaling pathway (reviewed by Beattie et al36). IGFBP5 is implicated in a diverse array of biological functions, including muscle atrophy, cell senescence, survival, and differentiation as well as fibrosis and inflammatory response through IGF-1-dependent and -independent mechanisms (reviewed by Beattie et al36). While these biological processes have been implicated in the pathogenesis of ARVC, specific biological roles of IGFBP5 in ARVC, and the molecular basis of increased mRNA levels merit additional studies. Additional interventions targeted to CTGF, KLF15, and IGFBP5 in animal models of ARVC would be necessary to substantiate the essential roles of these molecules in the pathogenesis of ARVC.

In summary, through a series of gain- and loss-of-function studies, we have shown the central role of nuclear PG in mediating adipogenesis of ARVC and identified CTGF, KLF15, IGFBP5, and Wnt5b as potential mediators of the unique and enigmatic phenotype in ARVC. These data in conjunction with our previous studies in Dsp-deficient mice.
and genetic fate mapping studies\textsuperscript{14,15} highlight the signaling functions of nuclear PG in repressing the canonical Wnt signaling and mediating differentiation of a subset of c-Kit\textsuperscript{+} CPCs to adipocytes.

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Disclosures

None.

References


Novelty and Significance

**What Is Known?**

- Arrhythmogenic right ventricular cardiomyopathy (ARVC), an important cause of sudden cardiac death in the young, is an enigmatic disease characterized by fibroadipocytic replacement of cardiac myocytes, particularly in the right ventricle.
- Mutations in genes encoding desmosome proteins are responsible for at least 50% of the ARVC cases.
- Partial nuclear displacement of plakoglobin (PG) from desmosomes leading to suppression of the canonical Wnt signaling in the second heart field cardiac progenitor cells (CPCs) is a putative mechanism for ARVC.

**What Is New?**

- The c-Kit⁺ CPCs isolated from the heart of transgenic mouse models of ARVC expressed PG, exhibited suppressed canonical Wnt signaling, and showed enhanced adipogenesis. Activation of the canonical Wnt signaling rescued the adipogenic phenotype.
- In contrast, c-Kit⁻ CPCs isolated from PG null embryos showed activation of the canonical Wnt signaling and were resistant to adipogenesis.
- CTGF, KLF15, Wnt5b, and IGFBP5 were transcriptional switch regulators of adipogenesis targeted by the PG-canonical Wnt signaling pathway in the c-Kit⁺ CPCs.

These findings provide insights into the molecular pathogenesis of ARVC and implicate the signaling functions of nuclear PG as a responsible mechanism. Our studies show that in ARVC, nonjunctional PG translocates to the nucleus where it activates a transcriptional program that switches the differentiation of c-Kit⁺ CPCs from a myogenic to an adipogenic fate.
Nuclear Plakoglobin Is Essential for Differentiation of Cardiac Progenitor Cells to Adipocytes in Arrhythmogenic Right Ventricular Cardiomyopathy
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Nuclear Plakoglobin Is Essential for Differentiation of Cardiac Progenitor Cells to Adipocytes in Arrhythmogenic Right Ventricular Cardiomyopathy

Raffaella Lombardi, MD, PhD, Maria da Graca Cabreira-Hansen, PhD, Achim Bell, PhD*, Richard R. Fromm, James T. Willerson, MD, AJ Marian, MD

Center for Cardiovascular Genetics, Institute of Molecular Medicine and Department of Medicine, University of Texas Health Sciences Center at Houston, and Texas Heart Institute at St. Luke’s Episcopal Hospital, Houston, TX 77030

* Current address: The University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216, email: ABell2@umc.edu

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Address for Correspondence:
AJ Marian, M.D.
Institute of Molecular Medicine
University of Texas Health Sciences Center
Texas Heart Institute at St. Luke’s Episcopal Hospital
6770 Bertner Street
Suite C900A
Houston, TX 77030
713 500 2350 (Direct line)
713 7500 2325 (Fax)
Ali.J.Marian@uth.tmc.edu
Material and Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by the Institutional Animal Care and Use Committee.

Genetically modified mice: PGWT and PGTR (FVB background) were generated per conventional methods under the transcriptional regulation of a 5.5-kbp α-myosin heavy chain (α-MyHC) promoter (a generous gift from Dr. Jeffrey Robbins, University of Cincinnati). To distinguish the transgene PGWT from the endogenous PG (PGEndo), we placed 3 sequential Flag epitopes at the N-terminal domain. To generate the deletion construct (PGTR), we induced a 2 bases deletion (PG23654del2) in the full-length mouse PG cDNA by site-directed mutagenesis. The deletion mutation causes a frameshift at the 3´ of the JUP (PG) gene, which introduces 11 new amino acids and leads to premature termination of the protein in humans. We also introduced a stop codon by site direct mutagenesis after 11 new amino acids to reproduce the human ARVC genotype in the transgenic mouse (Figure 1A). The presence of 11 new amino acids afforded the opportunity to develop a custom-made PGTR-specific antibody. The specificity of the antibody was tested in vitro by transfecting COS7 cells with the PGTR construct in a pcDNA3.0 plasmid downstream a CMV promoter (Online Figure I).

PG+/- mice (C57BL/6 background) were purchased from the Jackson Laboratory (Bar Harbor, Maine 04609 USA). Homozygous deficiency of PG (PG-/-) is embryonically lethal because of disrupted cardiogenesis. Thus, heterozygous (PG+/-) mice were maintained and crossed to generate PG-/- embryo for isolation of cardiac progenitor cells. In addition, PG+/- mice were crossed to PGTR transgenic mice to generate mice expressing the mutant PGTR in the background of deficiency of endogenous PG. Expression of PGTR did not rescue homozygous deficiency of the endogenous PG, as no viable PG+/-:PGTR mouse was born. However, PG-/-:PGTR mice expressing the mutant PGTR in the background of heterozygous deficiency of the endogenous PG were viable and characterized.
Genetically modified mice and embryos were genotyped by PCR of tail DNA. Sequences of the oligonucleotide primers used in PCR reactions are provided in Supplementary Table 1.

**Survival, gross cardiac morphology and histology in PG$^{WT}$ and PG$^{TR}$ mice:** We compared survival among non-transgenic (NTG) and transgenic mice over a period of 20 months by constructing Kaplan-Meier survival plots. We determined ventricular weight/body weight ratios in age- and sex-matched mice. We performed echocardiography in 23 to 29 mice per group under sodium pentobarbital-induced anesthesia, as previously described. We examined myocardial histology by H&E and Masson Trichrome staining of thin myocardial sections, as described previously and detected adipocytes by Oil Red O staining of optimal cutting temperature (OCT) frozen thin myocardial sections, as described previously. Likewise, cardiac progenitor cells isolated from transgenic mouse hearts or embryos were stained with Oil Red O to detect adipocytes. In brief, thin myocardial sections or isolated cells were washed one time with PBS and then fixed in 10% formalin in PBS for 15 min at room temperature. Following washing for 10 min in water, samples were stained at room temperature for 2 min in modified Mayer’s hematoxylin (Richard-Allan Scientific, Kalamazoo, MI; cat #72804). Samples were then washed with water for 10 min and placed in 100% propylene glycol (Poly Scientific, Bay Shore, NY; cat #s264) for 2 minutes and then placed in Oil Red O 0.5% solution in propylene glycol (Poly Scientific, Bay Shore, NY; cat #s1848) for 5 hour at room temperature. The Oil Red O solution was removed and the samples were dipped 2 times in 85% propylene glycol (Poly Scientific, Bay Shore, NY; cat #s264A). After washing thoroughly in water for 20 minutes the slides were mounted with aqueous mounting medium.

**Electrocardiography (EKG).** 7 months old mice (6 NTG, 6 PG$^{WT}$ and 8 PG$^{TR}$) were anesthetized by intraperitoneal injection of sodium pentobarbital (0.062mg/g) and placed on a heating pad with body temperature monitoring set at 37°C. Subcutaneous needle electrodes were placed over the upper precordial area in all mice in similar positions to obtain consistent recordings across mice. EKGs were recorded for about 15 min at a sampling rate of 2,000 Hz.
employing a PowerLab 4/30 System and ML136 Animal Bio Amp module (ADInstruments, Colorado Springs, CO, USA). The data recorded were submitted to a 60-Hz notch filter with an automatic setting determined by the software and manually confirmed (LabChart Pro V7). EKG intervals were measured by averaging 100 beats using the LabChart software package (ADInstruments). Measurements and identification of rhythm disturbances were performed by an investigator who was blinded to the genotypes of the mice.

**Immunoblotting:** Aliquots of 50 mg of ventricular tissue were homogenized using a Covaris Sonicator in RIPA buffer [1X formulation: 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 1% Sodium deoxycholate and 0.1 % Sodium Dodecyl Sulphate (SDS); Pierce Biotechnology, Rockford, IL; cat #89901] and complete protease inhibitor cocktail, (Roche Diagnostics, GmbH, Mannheim, Germany; cat # 11-697-498-001). Following determination of protein concentration by Lowry assay (Bio-Rad Laboratories, Life Science Group, Hercules, California, DC Protein Assay Kit II; cat #500-0112), protein extracts were heated in Laemmli loading buffer at 95-100°C for 5 min. 20 µg aliquots of each protein extracts were loaded onto SDS-polyacrylamide gels (PAGE) and, following electrophoresis, transferred to nitrocellulose membranes.

Expression levels of the transgenic proteins were detected by probing the membranes with an anti FLAG (PG\textsuperscript{WT}) antibody (Sigma, St Louis, MO, monoclonal mouse IgG1, clone M2, isotype; cat # F3165, 1:10000 dilution) and a custom-made PG\textsuperscript{TR} –specific antibody (Pacific Immunology Corp., Ramona, CA, rabbit polyclonal IgG, 1:500 dilution). The latter antibody specifically recognizes the unique 11 amino acids at the C-terminal domain of the mutant truncated protein. We detected expression levels of transgenic (PG\textsuperscript{WT} or PG\textsuperscript{TR}) and PG\textsuperscript{Endo} using a pan-specific anti-PG antibody (Invitrogen – Zymed; mouse monoclonal IgG1-kappa, clone: 11E4, cat #13-8500, 1:500 dilution). The secondary antibodies used were: goat anti mouse IgG horseradish peroxidase (HRP) conjugated (Santa Cruz Biotechnology Inc., Santa
Cruz, CA; cat #sc 2005, 1:10000 dilution) and goat anti-rabbit IgG-HRP (Cell Signaling Technology, Inc. MA 01923; cat # 7074, 1:2000 dilution).

Membranes were stripped by incubation in Restore PLUS Western Blot Stripping Buffer (Thermo Scientific, Hudson, New Hampshire, cat #46430) for 10 min at room temperature and washed in TBS for 3 times. Membranes were then probed with an anti α- tubulin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA; polyclonal, goat IgG, cat# sc- 12462, 1:600 dilution). The secondary antibody was a polyclonal donkey anti goat IgG horseradish peroxidase (HRP) conjugate antibody (Santa Cruz Biotechnology cat #sc-2020, 1:2000 dilution).

**Immunofluorescence:** Thin myocardial sections were immunostained using transgene-specific and pan PG antibodies, as previously described \(^1\). In brief, freshly harvested thick cardiac cross-sections were placed in OCT compound (Sakura-Finetek U.S.A. Inc., Torrace, CA, cat#4583) and frozen in isopentane (2-Methyl Butane, Sigma-Aldrich St Louis, MO, cat # 320404) cooled at -155°C in a liquid nitrogen bath. The tissue was cut in 5 µm thin myocardial sections for the immunofluorescence staining.

Likewise for immunostaining of isolated cells grown on glass coverslips, cells were fixed in 4% paraformaldehyde for 5 min at RT. Cells or tissue were washed 3 times in PBS, blocked with 5% donkey or goat normal serum (Santa Cruz Biotechnology, Inc., cat # sc-2044 and sc-2043, respectively) in PBS for 1 hour, and incubated with the primary antibodies at 4 °C overnight. After 3 rounds of washing in PBS, fluorescence-labeled secondary antibodies were added for 1 hour at room temperature. After 3 more washes in PBS, the samples were mounted in DAPI-containing Hard SetTM mounting medium (Vector Laboratories, Inc, Burlingame, CA, cat # H-1500) and examined under fluorescence microscopy.

The primary antibodies used for immunofluorescence were rabbit polyclonal anti pan PG (Santa Cruz Biotechnology, Inc., cat # sc-7900, 1:500 dilution), rabbit polyclonal anti-Flag (Cell Signaling Technology, Inc. cat #2368, 1:1000 dilution), rabbit polyclonal anti PG\(^\text{TR}\) (Pacific Immunology Corp., Ramona, CA, custom made; 1:500 dilution), and goat polyclonal anti
C/EBPα (Santa Cruz Biotechnology, Inc., cat # sc-9314, 1:500 dilution). In addition, we co-stained the cardiac progenitor cells with pan PG specific and transgenic specific PG antibodies and anti mouse c-kit antibody (Fitzgerald, monoclonal Rat IgG 2b, clone 2B8, cat # 10R-CD117BMS; 1:50 dilution). The secondary antibodies were: donkey anti rabbit IgG-FITC conjugate (Chemicon Int., Danvers, MA, cat #AP182-F; 1:1000 dilution), goat anti rabbit IgG-FITC conjugate (Santa Cruz Biotechnology, cat# sc-2012; 1:1000 dilution), goat anti rat IgG-Texas Red conjugate (Abcam, Cambridge, MA cat # ab6843-1; 1:400 dilution), and donkey anti goat IgG-FITC conjugate (Chemicon Int., Danvers, MA, cat #AP180-F, 1:1000 dilution). To define cellular localization of PGEndo as well as PGWT and PGTG in isolated cardiac progenitor cells, we treated the cells with 0.1% of Triton-X for 10 min at room temperature and probed with anti-Flag (PGWT), anti PGTG, and anti pan PG antibodies.

**Cell protein subfractionation:** Nuclear, cytosolic and membrane proteins were extracted using a commercial kit (Chemicon Int., Danvers, MA; cat #2145) as previously described 1. All the steps were conducted at 4 °C. Whole hearts were minced and homogenized in a dounce glass homogenizer in 5 volumes of a cold buffer containing HEPES (pH7.9), MgCl2, KCl, EDTA, Sucrose, Glycerol, Sodium OrthoVanadate plus protease inhibitors cocktail (Roche Diagnostics, GmbH, Mannheim, Germany; cat # 11-697-498-001). After incubation for 20 min with gentle rocking, each homogenate was centrifuged at 18,000g for 20 min to pellet membrane and nuclear fractions and to collect the supernatant containing the cytosolic proteins. After two washes, the remaining pellet was resuspended in 100 µL of an ice cold buffer containing HEPES (pH7.9), MgCl2, NaCl, EDTA, Glycerol, Sodium OrthoVanadate, plus proteinase inhibitors cocktail. After gentle mixing for 20 min and centrifugation at 18,000g for 20 min, the supernatant containing nuclear proteins was collected. The final pellet was resuspended in 100 µL of a cold buffer containing HEPES (pH7.9), MgCl2, KCl, EDTA, Sucrose, Glycerol, Sodium deoxycholate, NP-40, Sodium OrthoVanadate plus protease inhibitors cocktail.
and, following incubation for 20 min with gentle rocking and centrifugation at 18,000 g for 20 min, the supernatant, containing the membrane proteins was collected.

Aliquots of 30 µg of proteins from cytosolic, membrane and nuclear fractions, respectively, were electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with the following antibodies: anti pan-PG (Invitrogen - Zymed cat#13-8500, 1:600 dilution), anti PGTR specific (Pacific Immunology Corp., custom made, 1:600 dilution), and anti Flag (PGWT) (Sigma; cat # F3165, 1:1000 dilution) antibodies. The secondary antibodies were goat anti mouse IgG HRP conjugated (Santa Cruz Biotechnology Inc.; cat #sc 2005; 1:10000 dilution) and goat anti-rabbit IgG-HRP (Cell Signaling Technology; cat # 7074; 1:3000 dilution). Following each immunoblotting the membranes were stripped in Restore Plus stripping buffer for 10 min at room temperature and reprobed with anti α-tubulin (goat polyclonal IgG, Santa Cruz cat# sc-12462, 1:600 dilution), anti Connexin 43 (rabbit polyclonal IgG, Sigma cat # c6219; 1:5000 dilution) and anti Lamin A (rabbit polyclonal IgG, Santa Cruz, cat# sc20680; 1:500 dilution) antibodies, to test purity of the separation of cell protein fractions. The secondary antibodies used were: donkey anti-goat IgG-HRP (Santa Cruz, cat #sc2020; 1:5000 dilution) and donkey anti-rabbit IgG-HRP (Cell Signaling, cat # 7074; 1:2000 dilution).

Co-immunoprecipitation: Co-immunoprecipitation was performed, as described previously 1, 4. 50 mg aliquots of ventricular myocardium were minced into small pieces and homogenized by using a Covaris Sonicator in NP-40 lysis buffer [0.5% Nonidet P-40, 120mM, sodium chloride, 50mM Tris-HCl pH 7.4, 5% glycerol and a proteinase inhibitor cocktail (Roche, Germany)]. The cell debris was separated by centrifugation at 10,000 rpm for 2 min and the protein concentration in the supernatant was measured by the Lowry assay (Biorad cat #500-0112). To co-immunoprecipitate the proteins, 4 µg of the primary antibody were added to each 500 µg aliquot of total protein extracts. The solution was gently mixed and incubated it on a
rocker platform at 4°C overnight. The antibodies used for immunoprecipitation were anti Dsp1/2 (Fitzgerald, North Acton, MA; mouse monoclonal IgG1, clone: DP1/2-2.15, cat #10R-D108ax), anti Dsc2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA; rabbit polyclonal IgG, cat# sc66863), anti Dsg2 (Progen Biotechnik GmbH, Heidelberg, Germany; mouse monoclonal, clone DG 3.10; cat #61002) and anti Tcf-4 antibody (Millipore – Upstate, Billerica, MA; mouse monoclonal IgG2a, clone 6H5-3, cat # 05-511). As a control, 4 µg of normal mouse or rabbit IgG (Santa Cruz Biotechnology, cat# sc 2025 and sc 2027,respectively) were added to each 500 µg aliquot of total protein from the NTG heart protein lysate. After the overnight incubation with the respective antibody, 20 µl of Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology Inc. cat# sc 2003) were mixed into the solutions and the reactions were incubated on a rocker platform at 4°C overnight. The mixtures were centrifuged for 5 min at 4°C at 10,000 g and the precipitates were washed thrice by resuspension in ice-cold PBS and re-centrifugation. The final pellets were resuspended in 70 µl of loading buffer, consisting of Laemmli Buffer (63 mM Tris-HCl, pH:6.8, 25% glycerol, 2% SDS, 0.01% brome phenol blue) and 2.5% DTT and boiled for 5 minutes. 20 µl aliquots of each suspension were loaded onto SDS-polyacrylamide gels. Following electrophoresis and transfer to nitrocellulose membranes, the membranes were incubated with the following antibodies: anti pan PG (Invitrogen – Zymed, mouse monoclonal IgG1-kappa, clone: 11E4, cat# 13-8500, 1:600 dilution) anti Flag (PGWT) antibody (Sigma, St Louis, MO, mouse monoclonal IgG1, clone M2; cat # F3165, 1:10000 dilution) and anti PGTR-specific antibody (Pacific Immunology Corp., custom made; rabbit polyclonal IgG, 1:600 dilution). The secondary antibodies used were goat anti mouse IgG HRP conjugated (sc 2005, 1:10000 dilution) and goat anti-rabbit IgG HRP conjugate (Cell Signaling Technology cat # 7074, 1:3000 dilution).

Isolation and culture of cardiac progenitor cells from adult NTG, PGWT and PGTR mouse hearts: Hearts from 2-3 months old NTG, PGWT, and PGTR mice were excised and extensively washed with cold sterile PBS to remove contaminating debris and red blood cells. A
myocyte-depleted population of cells was prepared by incubating minced hearts in 3 mL of 0.22 µm filter-sterilized 0.1% type 2 collagenase (Worthington Biochemical Corp; Lakewood, NJ 08701; cat# LS004176) in α-MEM medium (Hyclone; cat# SH30265.01) without serum for 20 min at 37°C with gentle agitation. These conditions are expected to be lethal to mature myocytes. The collagenase was then diluted with an equal volume of α-MEM supplemented with 10% stem cells certified FBS (Millipore, Billerica, MA; cat #ES-011-B) and 1% Antibiotic-Antimycotic (Gibco; cat # 15240).

To sort progenitor cells, cellular suspensions were passed through 70µm mesh cell strainer (BD Bioscience) to remove debris followed by centrifugation at 300 g for 5 min at room temperature; the supernatant was removed and the cell pellet was washed twice in 2 mL of MACS Buffer (Miltenyi Biotec, Bergisch Gladbach, Germany; cat # 130-091-221). After multiple washes, cells were resuspended in 1 mL of MACS buffer and passed through a 40µm mesh strainer (BD Bioscience cat# 352340). Primary labeled antibody were then added at the concentration of one µg per 10⁶ cells and cell preparations incubated at 4 degrees for 20 min in the dark. Cells were labeled with the following monoclonal antibodies: anti-Sca1 (PE-Rat Anti-Mouse Sca1, clone E13-161.7; BD Pharmingen, San Diego, CA; cat#553336) and anti c-kit (APC Rat Anti-Mouse c-kit, clone 2B8; BD Pharmingen, San Diego, CA; cat#553356). Unbound antibody was removed by two washes in MACS buffer. We also labeled the cells with the appropriate isotype IgG controls (BD Pharmingen: PE Rat IgG2a, κ Isotype Control, cat# 553930 and APC Rat IgG2b, κ Isotype Control, cat # 553991). Soon before sorting the cell suspensions were passed through a 35 µm mesh strainer (BD Bioscience cat# 352235).

The FACS-Aria flow cytometer (BD Pharmingen, San Diego, CA) was used for sorting of c-kit⁺, Sca1⁺, and c-kit⁺/Sca1⁺ cells from adult mouse hearts. The c-kit⁺ cells were negative for markers of the myeloid, lymphoid, and erythroid lineages. Gating strategy used for sorting is shown in Online Figure IV.
After sorting, cells were seeded into 0.1% gelatin coated 24 well-plate in growth medium (MEM α Modification, containing L-Glutamine and Ribo and Deoxyribonucleosides; Hyclone Laboratories-ThermoScientific, SouthLogan, UT; cat # SH30265.01), supplemented with 10% embryonic stem cell certified FBS (Millipore cat# ES-011-B), 10 ng/mL mouse basic Fibroblast Growth Factor (bFGF) (R&D, Minneapolis, MN; cat #3139-FB), 1000 U/mL of mouse Leukemia Inhibitory Factor (mLIF) (Millipore; cat # ESG1106) and 1% Antibiotic-Antimycotic (Invitrogen-Gibco; cat # 15240). After cell seeding, the medium was partially removed during the first 2 days and fresh medium was added. After 48h in culture, when adherent cells were visible under light microscopy, the medium was completely changed to remove non-adherent cells. Cultures were expanded by serial passages as follow: when 70% confluent, the cells were detached by Trypsin digestion and transferred from 24 well to a 6 well plate (first passage, P1), from 6 well plate to 10 mm-Petri dishes (second passage, P2). Subsequent passages were maintained in 10 mm-Petri dishes. All the experiments were conducted on cells yielded from the third to fifth passage.

Isolation and culture of PG^{+/-} and PG^{-/-} cardiac progenitor cells: Because of homozygous deficiency of PG is embryonically lethal around E12-E13, PG^{+/-} mice were crossed to generate PG^{-/-} embryos. Pregnant females were euthanized at day E11 by cervical dislocation, uterine horns were dissected out, washed extensively with PBS, and placed into petri dishes containing PBS. Each embryo was separated from its placenta and surrounding membranes under a dissecting microscope. After each dissection, dissecting instruments were carefully cleaned with 70% alcohol pads and each embryo was washed at least 2 times with PBS to avoid any cross contamination. A piece of tail from each embryo was cut and used for genotyping by direct PCR (Phire Animal Tissue Direct PCR Kit; Finnzymes-New England Biolabs Inc, Ipswich, MA cat # F-140). PG^{+/-} and PG^{-/-} null embryos were used to isolate cardiac progenitor cells. A representative PCR screening of 11-day embryos is shown in Online Figure VII.
Five to six PG<sup>+/+</sup> and PG<sup>-/-</sup> 11-day old embryos were incubated in trypsin-EDTA (Gibco. Cat. # 25300-096) with gentle shaking at 37°C for 10 min. Cell suspensions were obtained by gentle pipetting up and down and α-MEM media supplemented with 10% of FBS was added to inhibit trypsin-digestion. To isolate mouse embryonic fibroblasts (MEFs), cell suspensions from PG<sup>+/+</sup> and PG<sup>-/-</sup> embryos were passed through a 70µm mesh and then 40 µm cell strainer (BD Bioscience, cat # 352350), resuspended in warm α-MEM (Hyclone), supplemented with 10% stem cells certified FBS (Millipore) and 1% Antibiotic-Antimycotic (Gibco). Cells were then plated at 1 embryo equivalent per 10 cm dish. The medium was changed on the following day to remove floating cells. Adherent cell (fibroblastic) cultures were fed every 2-3 days and allow growing to 70% confluence.

To isolate cardiac progenitor cells, embryonal cells were incubated with mouse lineage antibodies cocktail (BD Pharmingen, V450 Mouse Lineage Antibodies Cocktail, cat #561301) and anti Flk1 (BD Pharmingen, PE-Cy™7 Rat Anti-Mouse Flk-1, cat #561259), anti Sca1 (PE-Rat Anti-Mouse Sca1, clone E13-161.7; BD Pharmingen, San Diego, CA; cat#553336) and anti c-kit (APC Rat Anti-Mouse c-Kit, clone 2B8; BD Pharmingen, San Diego, CA; cat#553356) antibodies. The FACS-Aria flow cytometer (BD Pharmingen, San Diego, CA) was used to identify and sort c-kit<sup>+</sup>, Sca1<sup>+</sup>, and c-kit<sup>+</sup>/Sca1<sup>+</sup> cells from the population negative for markers of the myeloid, lymphoid, and erythroid lineages (CD34, CD45, CD20, CD45RO, CD8, and TER-119) as well as the endothelial marker Flk1. Sorted cells were processed and cultured as described above.

**Reverse-transcription polymerase chain reaction:** To eliminate any residual mature myocytes or myocytes debris, isolated cells were cultured and passaged three times and then were collected for RT-PCR reactions. Total RNA was extracted from cardiac progenitor cells using Qiagen RNeasy Mini Kit (QIAGEN Inc., 27220 Turnberry Lane, Valencia CA 91355; cat # 74104). To eliminate genomic DNA, the extracts were treated with DNase 1 sequentially with two different DNAse reagents (QIAGEN Inc., 27220 Turnberry Lane, Valencia CA 91355; cat #
Aliquots of 2 µg of total RNA extracts were reverse transcribed into cDNA with SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA 92008; cat # 18080-051) using oligo dT primers, following the manufacturer’s instructions. The reverse transcription products were then amplified by PCR using primers designed to specifically amplify the PG\textsuperscript{Endo}, PG\textsuperscript{WT} and PG\textsuperscript{TR}, α-MHC and Gapdh mRNAs. Two different sets of primers were designed at the 3’ of the cDNA of PG\textsuperscript{TR} to specifically amplify the mutant truncated PG. Primer design strategy is shown in online Supplementary Figure II and a complete list of primers is in online Table I.

**Induction of adipogenesis:** C-kit+ progenitor cells from NTG, PG\textsuperscript{WT} and PG\textsuperscript{TR} mouse hearts and from PG\textsuperscript{+/+} and PG\textsuperscript{−/−} embryos were plated at a density of 60,000 cells per well in a 24-well culture plate on 0.1% gelatin coated cover glass with 1 mL volume of proliferating media per well. After incubation overnight at 37°C in a 5% CO2 humidified incubator, the medium was carefully aspirated from each well and 1 mL of Adipogenesis Induction Medium [α-MEM supplemented with 10% FBS, 1% Antibiotic-Antimycotic, 10 µg/mL insulin (Sigma-Aldrich, 3050 Spruce St.; St. Louis, MO 63103; cat # I-0516), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, cat # I-7018), and 1 µM dexamethasone (DXM; Sigma-Aldrich, cat# D-8893)] was added. This medium change corresponded to differentiation day 1. The media was changed with fresh Adipogenesis Induction Medium every other days for 5 days. The media was then changed to Maintenance Medium containing 10 µg/mL insulin. After 2 days in Maintenance Medium the cells were stained with Oil Red O to visualize accumulated fat droplets and with C/EBP\textgreekalpha to visualize the adipogenic cells. Control groups of cell not treated with Adipogenesis Induction and Maintenance Media were also grown side by side, in regular α-MEM media with 10 % FBS and 1% antibiotics.

The number of adipocytes was quantified by 2 different methods: ORO staining and IF-staining with the adipogenic marker C/EBP\textgreekalpha. After adipogenesis induction, 2 sets of cells per
each group were stained with Oil Red O and C/EBPα. The number of Oil Red O stained positive cells was counted in 25 fields (4x- magnification) per group. The number of C/EBPα positive-stained cells and the total number of nuclei was counted in 20 (63x) fields and the percentage of positive-stained cells on the total number of cells per each field was calculated. Each experiment was repeated at least 3 times. The mean and standard deviation of the number of ORO stained cells and the percentage of C/EBP-α positive-stained cells per field per each group was calculated.

**Activation of Wnt signaling by pharmacological inhibition of GSK-3β:** Cardiac progenitor cells from the heart of PGTR mice were plated on 0.1% gelatin coated cover glass in proliferation media, supplemented with different concentrations of 6-bromoindirubin-3'-oxime (BIO; EMD Chemicals-Calbiochem, Gibbstown, NJ 08027; cat # 361550), a known activator of the canonical Wnt signaling. The cells were treated with three different doses of BIO at 2 µM, 5 µM, and 10 µM. A group of untreated cells were also included as control. After 24 hours of incubation, adipogenesis was induced for 7 days, as described above. In addition, control groups of cells treated with BIO but not subjected to adipogenesis were also cultured side by side in α-MEM media supplemented with 10% FBS and 1% antibiotics but without mLIF and bFGF. At the end of the treatment the cells were stained with Oil Red O and C/EBP-α side by side and the number of ORO and C/EBPα positive cells was quantified.

**Real-time polymerase chain reaction:** Expression levels of mRNAs for selected molecular markers were determined by quantitative real-time polymerase chain reaction (qPCR), using specific TaqMan Gene expression assays. The expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA level. The marker used and their sequences are listed in Online supplementary Table I.

**Statistical analysis.** Statistical analysis was performed using STATA-Intercooled version 10.1 software (StataCorp LP, College Station, Tex). Data are expressed as mean±1
SD. Normal distribution of all continuous variables was tested using the 1-sample Kolmogorov-Smirnov test. All variables were normally distributed. Differences in continuous variables were compared by ANOVA or unpaired Student t test when appropriate. Pairwise comparisons were performed by Bonferroni multiple comparisons test. Kaplan-Meier survival curves were constructed and the total survival rates were compared among the 3 study groups (NTG, PG$^{WT}$, and PG$^{TR}$) by log-rank test.
REFERENCES


Table I: Sequence of Oligonucleotide primers and probes used in this study

### A. PCR Oligonucleotide primers for mice genotyping:

#### PG\textsuperscript{WT} and PG\textsuperscript{tr} mice:

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flag Sense</td>
<td>CCATGGACTACAAAGACCATGACGG</td>
</tr>
<tr>
<td>α-MyHC-Sense</td>
<td>GGTGGTGTAGGAAAGTCAGGACTTC</td>
</tr>
<tr>
<td>PG-Antisense</td>
<td>CTTGAGCAACTGGGATGGTTT</td>
</tr>
</tbody>
</table>

#### PG\textsuperscript{−/−} mice:

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intron-Sense</td>
<td>CTCCTGTAGCTCAGTCTATG</td>
</tr>
<tr>
<td>Neo-Sense</td>
<td>CTTCTATCGCCTTCTTGACG</td>
</tr>
<tr>
<td>Common-Antisense</td>
<td>CCTCCTTCTTGAGACGCTGG</td>
</tr>
</tbody>
</table>

### B. PCR Oligonucleotide primers for RT-PCR:

#### α-MyHC:

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-MyHC Sense</td>
<td>GCCATCACAGATGCCGCC</td>
</tr>
<tr>
<td>α-MyHC Antisense</td>
<td>CTTTTGTCTTCTCTGTCTGG</td>
</tr>
</tbody>
</table>

#### Endogenous PG (PG\textsuperscript{Endo}):

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex-1 Sense</td>
<td>GCAGCGGAGCT CAGTTGCTTG</td>
</tr>
<tr>
<td>Ex2-Ex3 Antisense</td>
<td>GGTATTCCAGGTACCTTGGTTC</td>
</tr>
</tbody>
</table>

#### Flag tagged PG\textsuperscript{WT} transgene:

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flag Sense</td>
<td>CCATGGACTACAAAGACCATGACGG</td>
</tr>
<tr>
<td>Ex2-Ex3 Antisense</td>
<td>GGTATTCCAGGTACCTTGGTTC</td>
</tr>
</tbody>
</table>

#### PG\textsuperscript{TR} transgene:

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex9-Ex10 Sense</td>
<td>CCCTGTTTGTCCAGCTCCTTG</td>
</tr>
<tr>
<td>TG deletion Antisense</td>
<td>TCTGGGCAGCCTCCGGC</td>
</tr>
<tr>
<td>Mutant STOP Antisense</td>
<td>CCATGTCGTCTGCATAGTTATCG</td>
</tr>
</tbody>
</table>
GAPDH

Gapdh-Sense      GGTGAAGGTCGGTGTGAACG
Gapdh-Antisense  CCGTGAGTGGAGTCATGGAAC

qPCR Probes

c-myc          CAGCAGCGACTCTGAAGAAGAGCAA
Cyclin D1       TGCCACAGATGTGAAGTTCCATTTCC
Cyclin E1       AGGATAGCAGTCACCTGGGATGA
PCNA            CAACCTTGGGATCCAGCACACAGGAGT
Sox2            GCTGGGCTACCCGCAGCACACCGGGGC
KLF15           CGGCTGGAGGTTTTCCGCTCAGA
IGFBP-5         AGAGAAAGCAGTGAAGCCCTCCCG
CTGF            GGAGGAAAACATTAAGAGGGCAAA
Wnt 5b          GCTGGCCGCGCGGCGGCGTGTAAGAAG
Gapdh           GTGAACGGATTTGGCCGTATTGGGC
## ONLINE SUPPLEMENTARY TABLE II

**Electrocardiographic Findings in NTG, PG\textsuperscript{WT} and PG\textsuperscript{TR} Mice**

<table>
<thead>
<tr>
<th></th>
<th>NTG</th>
<th>PG\textsuperscript{WT}</th>
<th>PG\textsuperscript{TR}</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>6</td>
<td>6</td>
<td>8</td>
<td>NA</td>
</tr>
<tr>
<td>Sex (males/females)</td>
<td>4/2</td>
<td>4/2</td>
<td>6/2</td>
<td>0.075</td>
</tr>
<tr>
<td>Age (months)</td>
<td>7.7±0.2</td>
<td>7.4±0.4</td>
<td>7.6±0.1</td>
<td>0.060</td>
</tr>
<tr>
<td>Body weight (grams)</td>
<td>37±6</td>
<td>35±6</td>
<td>35±4</td>
<td>0.661</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>460±78</td>
<td>485±54</td>
<td>503±77</td>
<td>0.535</td>
</tr>
<tr>
<td>P duration (ms)</td>
<td>13.5±0.5</td>
<td>12.5±1.1</td>
<td>15.1±2.9</td>
<td>0.143</td>
</tr>
<tr>
<td>PR interval (ms)</td>
<td>32.3±4.0</td>
<td>37.2±8.4</td>
<td>32.3±5.5</td>
<td>0.337</td>
</tr>
<tr>
<td>QRS duration (ms)</td>
<td>15.6±4.2</td>
<td>17.4±6.0</td>
<td>18.8±6.9</td>
<td>0.609</td>
</tr>
<tr>
<td>QT interval (ms)</td>
<td>21.8±2.0</td>
<td>30.7±5.0</td>
<td>31.0±13.9</td>
<td>0.336</td>
</tr>
<tr>
<td>AV Blocks</td>
<td>0/6</td>
<td>1/6</td>
<td>0/8</td>
<td>0.293</td>
</tr>
<tr>
<td>Ventricular Conduction defects</td>
<td>0/6</td>
<td>2/4</td>
<td>1/7</td>
<td>0.016</td>
</tr>
<tr>
<td>Ventricular tachyarrhythmias</td>
<td>0/6</td>
<td>1/6</td>
<td>2/8</td>
<td>0.262</td>
</tr>
</tbody>
</table>

**Abbreviations:** bpm: Beats per minutes; AV: Atrioventricular
### ONLINE SUPPLEMENTARY TABLE III

Echocardiographic Findings in NTG, PG^+/− and PG^+/−:PG^TR Mice

<table>
<thead>
<tr>
<th></th>
<th>NTG</th>
<th>PG^+/−</th>
<th>PG^+/−:PG^TR</th>
<th>p (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>17</td>
<td>8</td>
<td>8</td>
<td>NA</td>
</tr>
<tr>
<td>Sex (males/females)</td>
<td>8/9</td>
<td>4/4</td>
<td>4/4</td>
<td>0.564</td>
</tr>
<tr>
<td>Age (months)</td>
<td>9.2±3.1</td>
<td>8.5±2.2</td>
<td>8.1±1.9</td>
<td>0.611</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>30.3±2.7</td>
<td>34.8±5.3*</td>
<td>34.9±3.6*</td>
<td>0.006</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>561.5±83.1</td>
<td>536.3±99.4</td>
<td>492.5±73.2</td>
<td>0.184</td>
</tr>
<tr>
<td>IVST (mm)</td>
<td>0.97±0.08</td>
<td>0.84±0.07*</td>
<td>0.83±0.11*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PWT (mm)</td>
<td>0.98±0.13</td>
<td>0.80±0.11*</td>
<td>0.83±0.14*</td>
<td>0.005</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>2.89±0.21</td>
<td>3.50±0.25*</td>
<td>3.95±0.34*#</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>1.05±0.17</td>
<td>1.28±0.21*</td>
<td>1.94±0.25*#</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>90.4±18.4</td>
<td>92.8±8.0</td>
<td>115.5±26.3*#</td>
<td>0.013</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>63.9±4.8</td>
<td>63.4±4.8</td>
<td>51.0±3.5*#</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Abbreviations:** NTG: Non-transgenic mice; PG^+/−: Heterozygous for plakoglobin deficiency; PG^+/−:PG^TR: expression of mutant truncated plakoglobin in the background of heterozygous deficiency of plakoglobin; bpm: Beats per minutes; IVST: Interventricular septal thickness; PWT: Posterior wall thickness; LVEDD: Left ventricular end diastolic diameter; LVESD: Left ventricular end systolic diameter; LV Mass: Left ventricular mass; FS: Fractional shortening; *p-value ≤ 0.05 vs NTG, # p-value ≤ 0.001, by Bonferroni pairwise comparison with non-transgenic mice.
Online Supplementary Figure I. (A) Full length mouse PG cDNA was placed, downstream to a CMV promoter, in pcDNA-3.0 plasmid. Truncated mutant PG (PG\textsuperscript{TR}) construct was generated by site directed mutagenesis. The plasmids were used to transflect COS7 cells in order to assess the effectiveness of transcription and translation of the PG\textsuperscript{TR} construct, and the specificity of the customer made PG\textsuperscript{TR} antibody. (B) Immunoblot showing expression of transgene and endogenous PG proteins detected using PG\textsuperscript{TR} specific and pan PG [endogenous PG (PG\textsuperscript{Endo}) + PG\textsuperscript{TR}] antibodies in COS 7 cells.
Online Supplementary Figure II. Design of primers for specific detection of PG\textsuperscript{Endo}, PG\textsuperscript{WT} and PG\textsuperscript{TR} mRNA by RT-PCR. RNA was double digested with DNase 1 to eliminate genomic DNA contamination. RNA was reverse transcribed to cDNA by using oligo T primers. RT-PCR primers were designed to span introns. To specifically detect PG\textsuperscript{WT} the sense primer was designed on the Flag sequence. To specifically amplify the deletion mutant PG (PG\textsuperscript{TR}), two sets of primers were designed at the 3` of the cDNA of PG\textsuperscript{TR} in the region where the TG deletion and the Stop codon were introduced in the transgene construct.
Online Supplementary Figure III (A) Single-lead surface EKGs in 7 months old NTG, PG\textsuperscript{WT} and PG\textsuperscript{TR} animals, showing sinus rhythm with normal P, PR and QRS intervals. (B) Episodes of atrial and ventricular arrhythmias and AV blocks, detected in PG\textsuperscript{WT} and PG\textsuperscript{TR} transgenic mice. No conduction defect or arrhythmias was detected in NTG mice.
Online Figure IV. Cardiac phenotype in PG\textsuperscript{+/-}:PG\textsuperscript{TR} bigenic mice: A. Immunoblots showing expressing of the mutant truncated in the heart, detected using a mutant PG-specific antibody (upper blot) and in the presence of heterozygous endogenous PG genotype (middle panel), detected using a pan PG antibody. The lower panel represent tubulin, as a control for loading conditions.

B. Myocardial histology in control non-transgenic (NTG) and in PG\textsuperscript{+/-}:PG\textsuperscript{TR} mice, showing presence of adipocytes in the bigenic mice.
Online Supplementary Figure V. Representative dot plots from one PG TR mouse, showing the gating strategy used to sort c-Kit+/Sca1+ cells from the heart of adult mice. Preceding the acquisition of events, small debris and aggregates were gated out based on SSC, FCS and singlets distribution. The plot A shows the distribution of $10^5$ acquired events based on PE-Sca1 versus APC FITC intensity; plot B shows the respective control tube labeled with isotypic IgGs. Sorting of c-kit+/Sca-1+ cells was performed within the region corresponding to low-medium SSC and FSC (plot C) where a gate was created (small plot to the side); respective IgG control is shown in plot D.
Online Supplementary Figure VI. Enhanced adipogenesis in c-kit\(^+\) cells isolated from the heart of PG\(^{TR}\) mice. (A). Oil Red staining of c-kit\(^+\) cells isolated from the hearts of NTG and PG\(^{TR}\) mice, respectively, after 7 days of adipogenic induction. (B). Number of Oil Red O positive cells in each group. (C and D). Oil Red O stained panels show prevention of adipogenesis upon treatment of c-kit\(^+\) cells isolated from PG\(^{TR}\) mouse hearts with increasing dosages of BIO (2\(\mu\)M, 5\(\mu\)M and 10\(\mu\)M). Cells treated with BIO showed a dose dependent reduction in the number of Oil Red O positive cells as compared with non-treated cells.
Online Supplementary Figure VII. Representative PCR screening of 11 day embryos, showing the detection of wild type and deleted PG alleles.
Online Supplementary Figure VIII. Absence of Adipogenesis in mouse embryonic fibroblasts (MEFs) isolated from PG⁻/⁻ embryos: (A). Oil Red O staining of MEFs isolated from PG⁺/+ and PG⁻/⁻ embryos after one week of adipogenesis induction. (B). Approximately 2X10⁵ cells were counted per group. PG⁻/⁻ cells showed minimal Oil Red O positive cells (0.0003%) as compared with PG⁺/+ cells (0.006%), a 20-fold decrease in PG⁻/⁻ cells.