Online Supplementary Material

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 Istitute at St. Luke's Epsicopal 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: PG^{WT} and PG^{TR} (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) [1]. To distinguish the transgene PG^{WT} from the endogenous PG (PG^{Endo}), we placed 3 sequential Flag epitopes at the N-terminal domain. To generate the deletion construct (PG^{TR}), we induced a 2 bases deletion ($PG^{23654del2}$) 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 [2]. 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 PG^{TR} –specific antibody. The specificity of the antibody was tested in vitro by transfecting COS7 cells with the PG^{TR} 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 [3]. 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 PG^{TR} transgenic mice to generate mice expressing the mutant PG^{TR} in the background of deficiency of endogenous PG. Expression of PG^{TR} did not rescue homozygous deficiency of the endogenous PG, as no viable $PG^{-/-}$:PG^{TR} mouse was born. However, $PG^{-/-}$:PG^{TR} mice expressing the mutant PG^{TR} 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 [1, 4]. 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 [1, 4]. Likewise, cardiac progenitor cells isolated from transgenic mouse hearts or embryos were strained 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^{WT}) antibody (Sigma, St Louis, MO, monoclonal mouse IgG1, clone M2, isotype; cat # F3165, 1:10000 dilution) and a custom-made PG^{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^{WT} or PG^{TR}) and PG^{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^{o}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 ^{o}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^{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 PG^{Endo} as well as PG^{WT} and PG^{TR} 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 (PG^{WT}), anti PG^{TR}, 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, 4]. 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 PG^{TR} specific (Pacific Immunology Corp., custom made, 1:600 dilution), and anti Flag (PG^{WT}) (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 ⇞ 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 (PG^{WT}) antibody (Sigma, St Louis, MO, mouse monoclonal IgG1, clone M2; cat # F3165, 1:10000 dilution) and anti PG^{TR}-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, PG^{WT} and PG^{TR} mouse hearts: Hearts from 2-3 months old NTG, PG^{WT}, and PG^{TR} 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 10^6 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 [3], $PG^{+/-}$ 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 $PG^{+/+}$ and $PG^{-/-}$ 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 $PG^{+/+}$ and $PG^{-/-}$ 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 $c\text{-}kit^{+}$, $Sca1^{+}$, and $c\text{-}kit^{+}/Sca1^{+}$ 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 #

79254 and New England Biolabs, cat# M0303S). 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^{Endo}, PG^{WT} and PG^{TR}, α-MHC and Gapdh mRNAs. Two different sets of primers were designed at the 3` of the cDNA of PG^{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^{WT} and PG^{TR} mouse hearts and from $PG^{+/+}$ and $PG^{-/-}$ 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/EBPα 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/EBPα. 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 PG^{TR} 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 [5]. 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.

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Online Supplementary Material

TABLE I

Sequence of Oligonucleotide primers and probes used in this study

A. PCR Oligonucletide primers for mice genotyping:

PG^{WT} and PG^{tr} mice:

Flag Sense	CCATGGACTACAAAGACCATGACGG
α-MyHC-Sense	GGTGGTGTAGGAAAGTCAGGACTTC
PG-Antisense	CTTGAGCAACTGGGATGGTTT

$PG^{-/-}$ mice:

Intron-Sense	CTCCTGTAGCTCAGTCCTATG
Neo-Sense	CTTCTATCGCCTTCTTGACG
Common-Antisense	CCTCCTTCTTGGACAGCTGG

B. PCR Oligonucletide primers for RT-PCR:

α-MyHC:

α-MyHC Sense	GCCATCACAGATGCCGCC
α-MyHC Antisense	CTTCTTGTCTTCCTCTGTCTGG

Endogenous PG (PG^{Endo}):

Ex-1 Sense	GCGCCGAGCT CAGTTCGCTG
Ex2-Ex3 Antisense	GGTATTCCAGGTCACCTTGGTTC

Flag tagged PG^{WT} transgene:

Flag Sense	CCATGGACTACAAAGACCATGACGG
Ex2-Ex3 Antisense	GGTATTCCAGGTCACCTTGGTTC

PG^{TR} transgene:

Ex9-Ex10 Sense	CCCTGTTTGTCCAGCTCCTG
TG deletion Antisense	TCTGGGCAGCCTCCGGC
Mutant STOP Antisense	CCATGTCGTCTGCATAGTTATCG

GAPDH	
Gapdh-Sense	GGTGAAGGTCGGTGTGAACG
Gapdh-Antisense	CCGTGAGTGGAGTCATACTGGAAC
qPCR Probes	
c-myc	CAGCAGCGACTCTGAAGAAGAGCAA
Cyclin D1	TGCCACAGATGTGAAGTTCATTTCC
Cyclin E1	AGGATAGCAGTCAGCCCTGGGATGA
PCNA	CAACTTGGAATCCCAGAACAGGAGT
Sox2	GCTGGGCTACCCGCAGCACCCGGGC
KLF15	CGGCTGGAGGTTTTCCCGCTCAGA
IGFBP-5	AGAGAAAGCAGTGTAAGCCCTCCCG
CTGF	GGAGGAAAACATTAAGAAGGGCAAA
Wnt 5b	GCTGGCCGCCGGGCCGTGTATAAGA
Gapdh	GTGAACGGATTTGGCCGTATTGGGC

ONLINE SUPPLEMENTARY TABLE II

Electrocardiographic Findings in NTG, PG^{WT} and PG^{TR} Mice

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

Abbreviations: bpm: Beats per minutes; AV: Atrioventricular

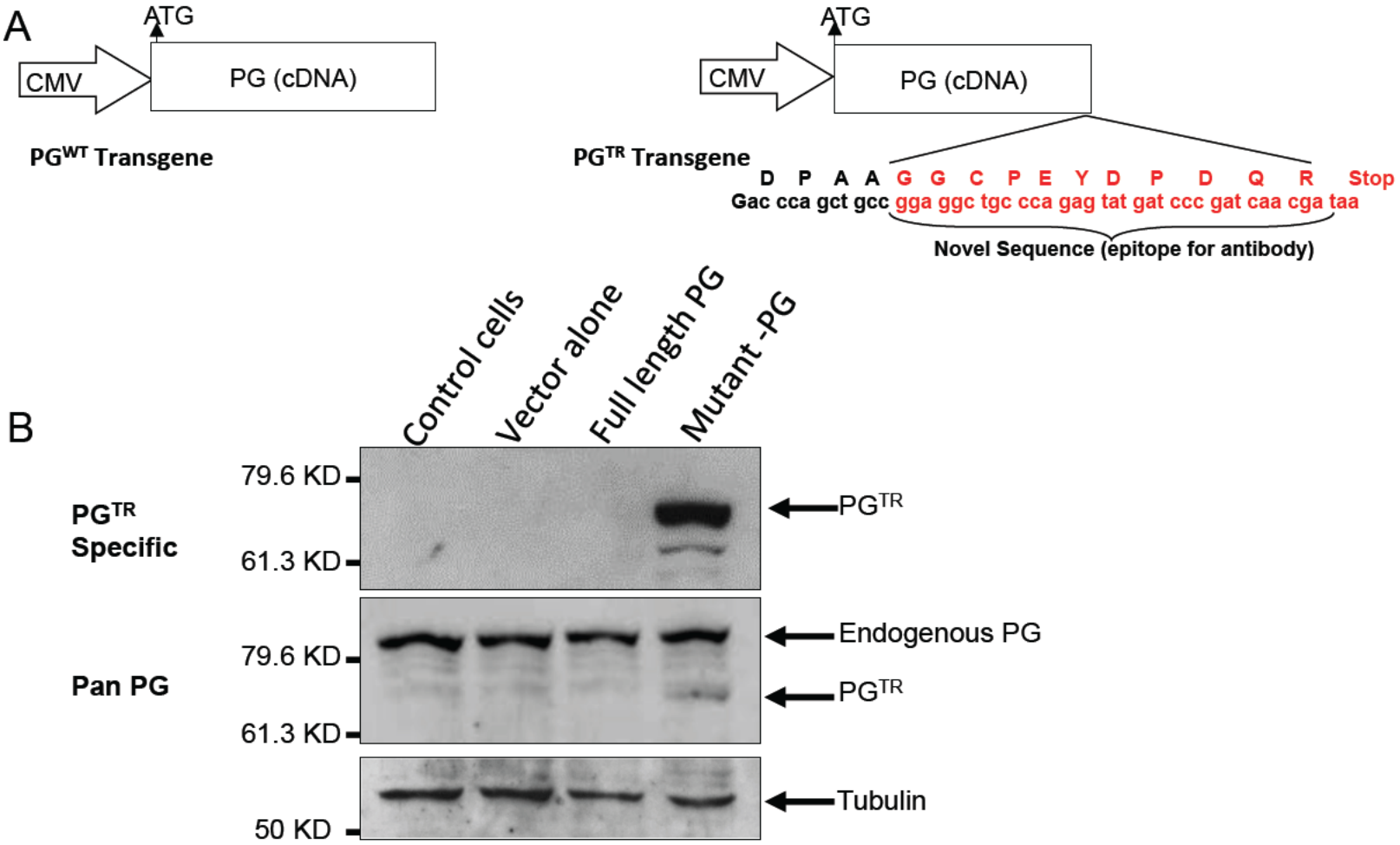
ONLINE SUPPLEMENTARY TABLE III

Echocardiographic Findings in NTG, $PG^{+/-}$ and $PG^{+/-}$:PG^{TR} Mice

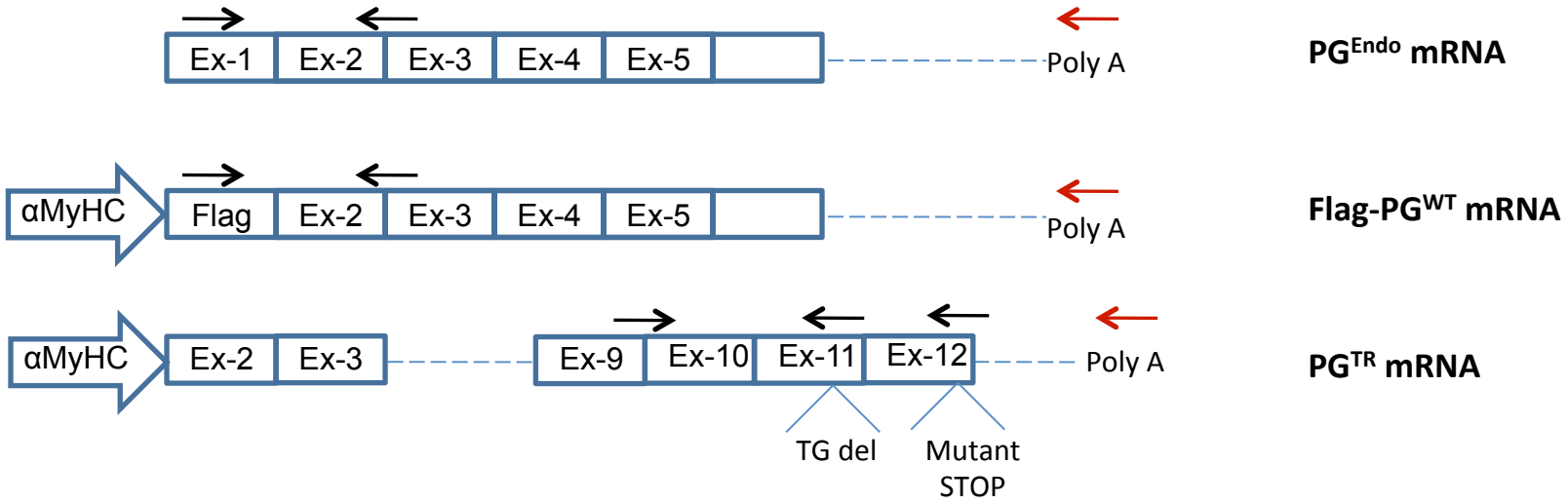
	NTG	$PG^{+/-}$	$PG^{+/-}$:PG^{TR}	p (ANOVA)
N	17	8	8	NA
Sex (males/females)	8/9	4/4	4/4	0.564
Age (months)	9.2±3.1	8.5±2.2	8.1±1.9	0.611
Body weight (g)	30.3±2.7	34.8±5.3*	34.9±3.6*	0.006
Heart rate (bpm)	561.5±83.1	536.3±99.4	492.5±73.2	0.184
IVST (mm)	0.97±0.08	0.84±0.07*	0.83±0.11*	<0.001
PWT (mm)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

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;

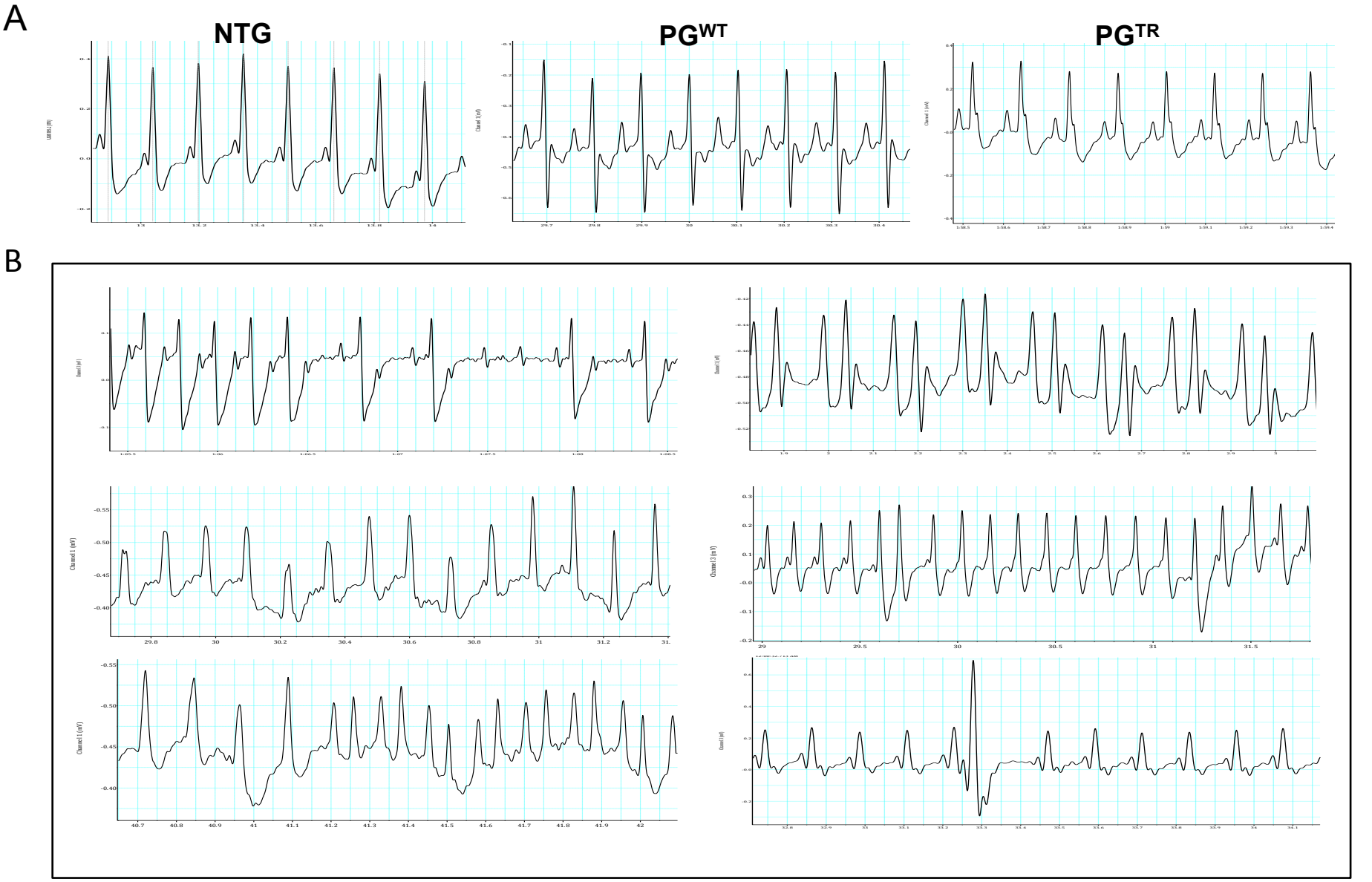
*p-value ≤ 0.05 vs NTG, # p-value ≤ 0.001, by Bonferroni pairwise comparison with non-transgenic mice.



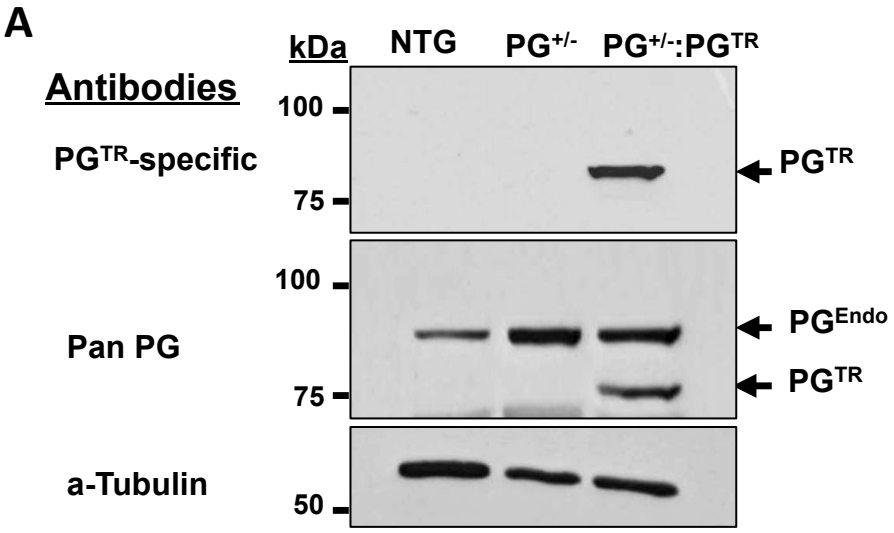
Online Supplementary Figure I. (A) Full lengh mouse PG cDNA was placed, downstream to a CMV promoter, in pcDNA-3.0 plasmid. Truncated mutant PG (PG^{TR}) construct was generated by site directed mutagenesis. The plasmids were used to transfect COS7 cells in order to assess the effectiveness of transcription and translation of the PG^{TR} construct, and the specificity of the customer made PG^{TR} antibody. (B) Immunoblots showing expression of transgene and endogenous PG proteins detected using PG^{TR} specific and pan PG [endogenous PG (PG^{Endo}) + PG^{TR}] antibodies in COS 7 cells.



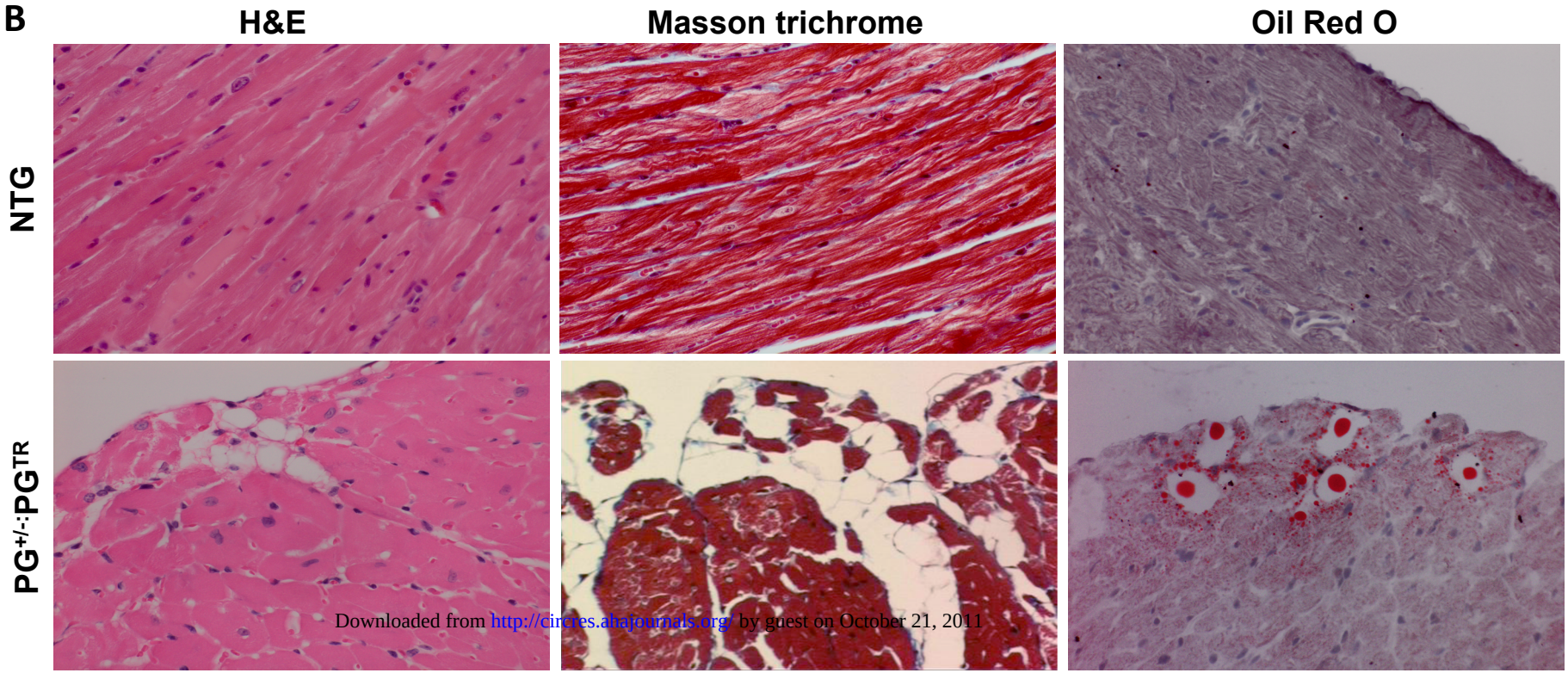
Online Supplementary Figure II. Design of primers for specific detection of PG^{Endo}, PG^{WT} and PG^{TR} mRNA by RT-PCR. RNA was double digested with DNase 1 to eliminate genomic DNA contamination. RNA was reverse transcribed to cDNA by using oligo T primers. RT-PCR primers were designed to span introns. To specifically detect PG^{WT} the sense primer was designed on the Flag sequence. To specifically amplify the deletion mutant PG (PG^{TR}), two sets of primers were designed at the 3` of the cDNA of PG^{TR} in the region where the TG deletion and the Stop codon were introduced in the transgene construct.

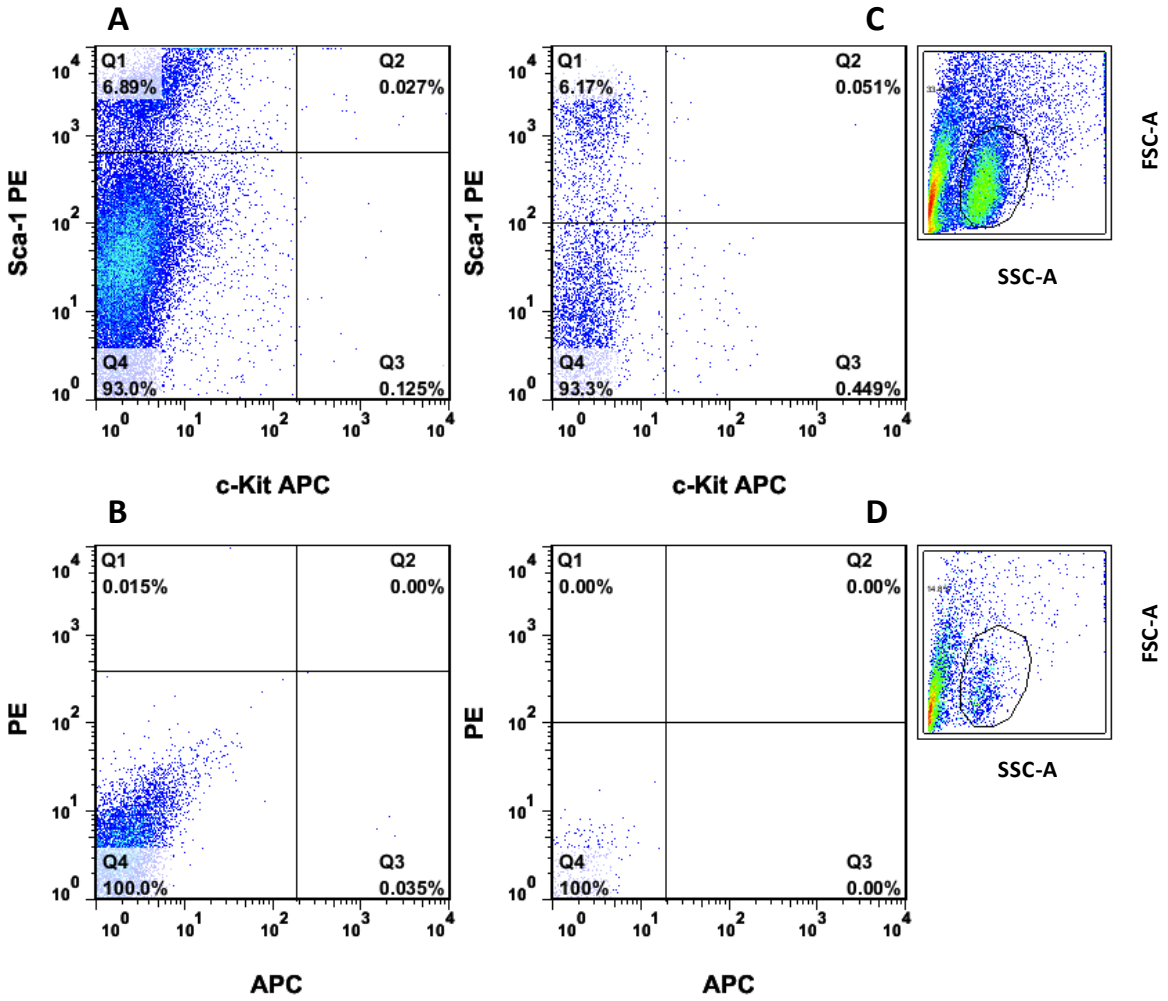


Online Supplementary Figure III (A) Single-lead surface EKGs in 7 months old NTG, PG^{WT} and PG^{TR} animals, showing sinus rhythm with normal P, PR and QRS intervals. (B) Episodes of atrial and ventricular arrhythmias and AV blocks, detected in PG^{WT} and PG^{TR} transgenic mice. No conduction defect or arrhythmias was detected in NTG mice.

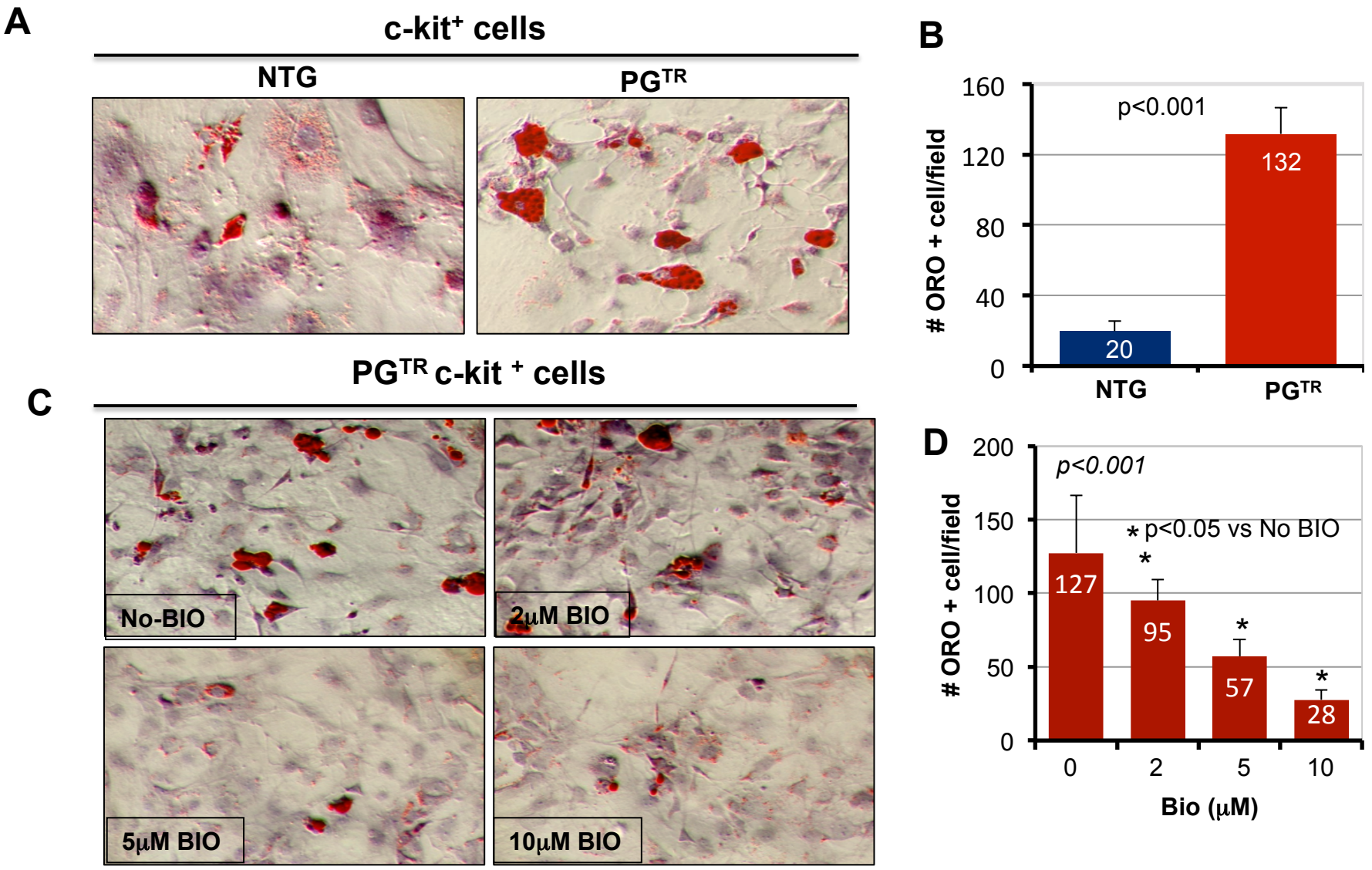


Online Figure IV. Cardiac phenotype in $PG^{+/-}$:PG^{TR} bigenic mice: **A.** Immunoblots showing expressing of the mutant truncated in the heart, detected using a mutant PG-specific antibody (upper blot) and in the presence of heterozygous endogenous PG genotype (middle panel), detected using a pan PG antibody. The lower panel represent tubulin, as a control for loading conditions.
B. Myocardial histology in control non-transgenic (NTG) and in $PG^{+/-}$:PG^{TR} mice, showing presence of adipocytes in the bigenic mice.

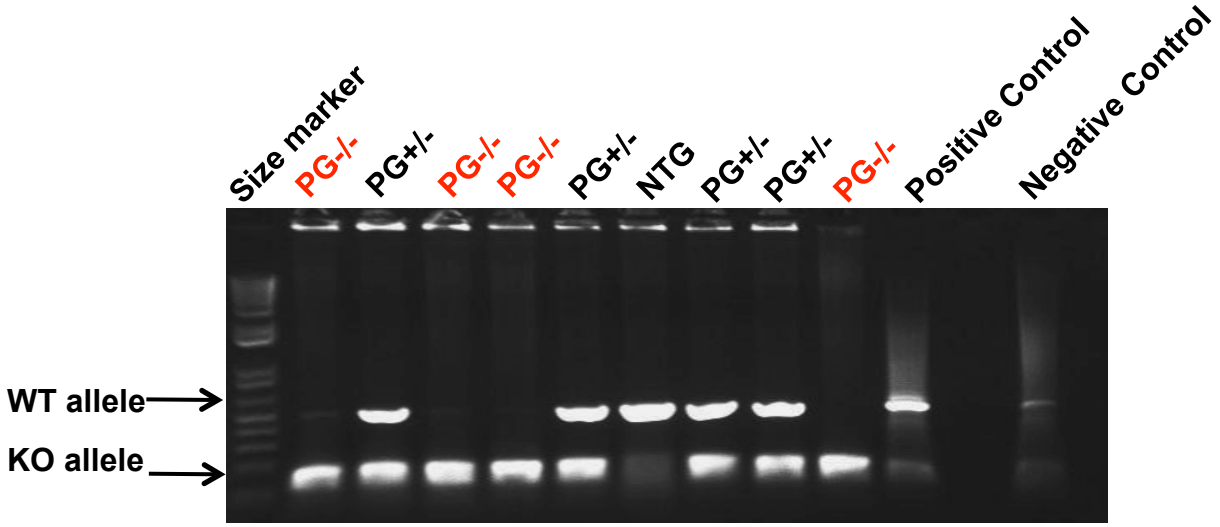




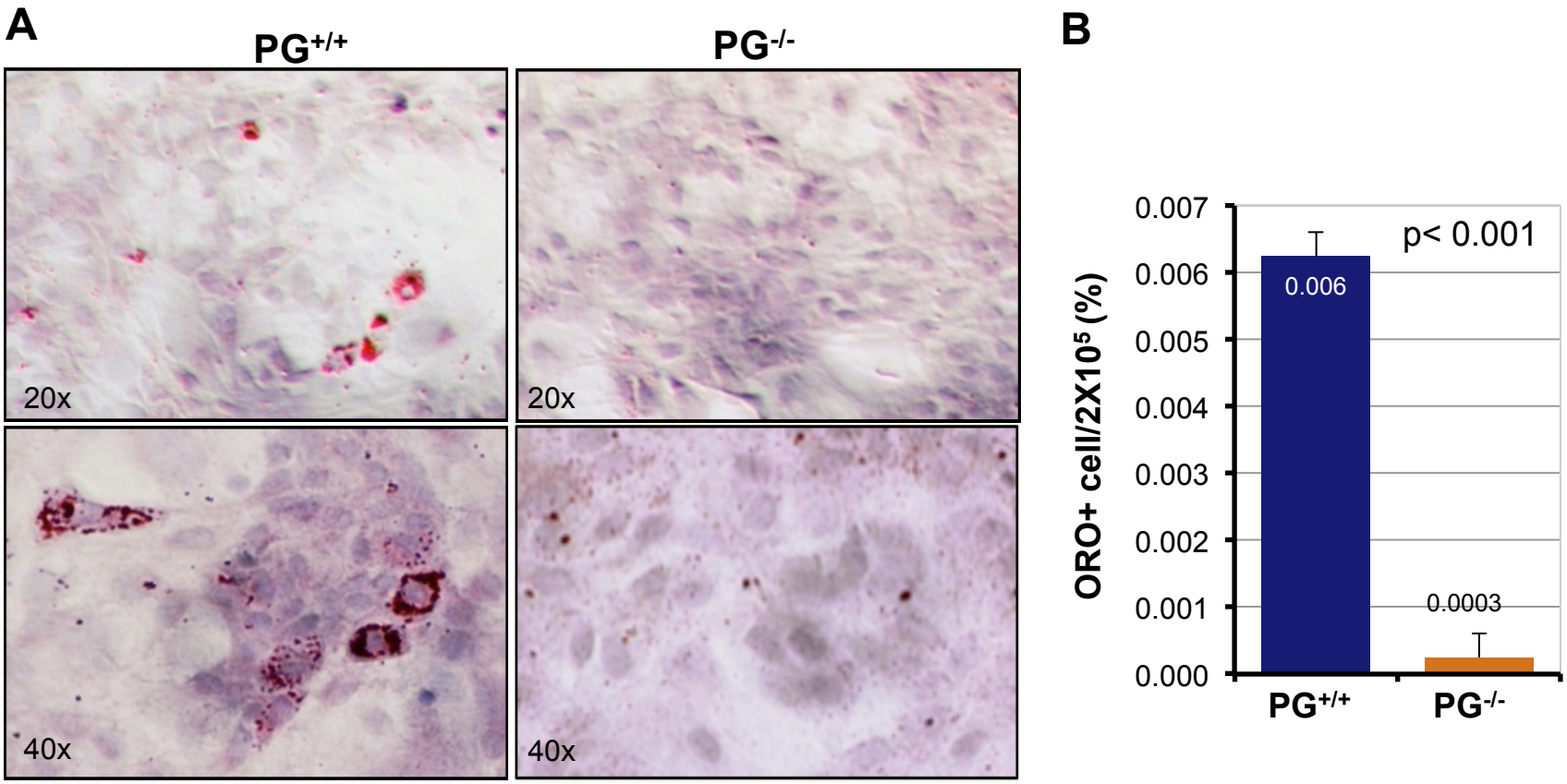
Online Supplementary Figure V. Representative dot plots from one PG ^{TR} mouse, showing the gating strategy used to sort c-Kit+/Sca1+ cells from the heart of adult mice. Preceding the acquisition of events, small debris and aggregates were gated out based on SSC, FCS and singlets distribution. The plot 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 (2μM, 5μM and 10μM). 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 $2X10^5$ 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.