Abstract—The phenotypic hallmark of arrhythmogenic right ventricular cardiomyopathy, a genetic disease of desmosomal proteins, is fibroadipocytic replacement of the right ventricle. Cellular origin of excess adipocytes, the responsible mechanism(s) and the basis for predominant involvement of the right ventricle are unknown. We generated 3 sets of lineage tracer mice regulated by cardiac lineage promoters α-myosin heavy chain (αMyHC), Nkx2.5, or Mef2C. We conditionally expressed the reporter enhanced yellow fluorescent protein while concomitantly deleting the desmosomal protein desmoplakin in cardiac myocyte lineages using the Cre-LoxP technique. Lineage tracer mice showed excess fibroadiposis and increased numbers of adipocytes in the hearts. Few adipocytes in the hearts of αMyHC-regulated lineage tracer mice, but the majority of adipocytes in the hearts of Nkx2.5- and Mef2C-regulated lineage tracer mice, expressed enhanced yellow fluorescent protein. In addition, rare cells coexpressed adipogenic transcription factors and the second heart field markers Isl1 and Mef2C in the lineage tracer mouse hearts and in human myocardium from patients with arrhythmogenic right ventricular cardiomyopathy. To delineate the responsible mechanism, we generated transgenic mice expressing desmosomal protein plakoglobin in myocyte lineages. Transgene plakoglobin translocated to nucleus, detected by immunoblotting and immunofluorescence staining and coimmunoprecipitated with Tcf7l2, a canonical Wnt signaling transcription factor. Expression levels of canonical Wnt/Tcf7l2 targets bone morphogenetic protein 7 and Wnt5b, which promote adipogenesis, were increased and expression level of connective tissue growth factor, an inhibitor of adipogenesis, was decreased. We conclude adipocytes in arrhythmogenic right ventricular cardiomyopathy originate from the second heart field cardiac progenitors, which switch to an adipogenic fate because of suppressed canonical Wnt signaling by nuclear plakoglobin. (Circ Res. 2009;104:1076-1084.)

Key Words: adipocytes • progenitor cells • Wnt signaling • desmosomes • heart failure

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a genetic disease characterized by the unique phenotype of fibroadipocytic replacement of cardiac myocytes, predominantly in the right ventricle.1-3 Clinical manifestations of ARVC include ventricular arrhythmias, typically originating from the right ventricle; sudden cardiac death, which often is its first manifestation; and right ventricular aneurysmal dilatation and failure.1,4 The left ventricle is also commonly involved in the advanced stages.1,5

ARVC is typically an autosomal dominant disease. Recessive forms in conjunction with palmoplantar keratoderma and woolly hair (Naxos disease) or predominant involvement of the left ventricle (Carvajal syndrome) are referred to as “cardiocutaneous syndromes.”6,7 Recently, mutations in 5 genes for ARVC, namely, DSP, JUP, PKP2, DSC2, and DSG2, encoding desmosomal proteins desmoplakin (Dsp), plakoglobin (PG), plakophilin 2, desmocollin 2, and desmoglein 2, respectively, have been identified.6-12 Hence, ARVC, at least in a subset, is a disease of desmosomes, intercellular junction structure responsible for cell-cell adhesion in epidermal cells and cardiac myocytes.

Pathogenesis of ARVC is not fully understood. Impaired myocyte to myocyte attachment because of defective desmosomes may explain cardiac dysfunction.13 It does not, however, explain the pathogenesis of the unique phenotype of fibro-fatty replacement of the myocardium and the cellular origin of excess adipocytes. Heart is a heterogeneous organ. It contains myocytes, fibroblasts, adipocytes, smooth muscle cells, endothelial cells, and pericytes, as well as circulating cells that implant in the myocardium. We posit the cell type that gives rise to adipocytes in ARVC must either express the mutant desmosomal protein or differentiate into adipocytes.
through a paracrine mechanism(s) emanating from cells expressing the mutant desmosomal protein. In the heart, the only cell type known to express desmosomal proteins is cardiac myocyte lineage. Adult cardiac myocytes are terminally differentiated and, hence, not plausible candidates to dedifferentiate to adipocytes. In contrast, cardiac progenitor cells expressing the desmosomal proteins might have the potential to differentiate to adipocytes. To test this hypothesis, we performed genetic fate-mapping experiments using the LoxP-Cre technology regulated by 3 cardiac lineage promoters. We extended the results of lineage tracing experiments to hearts of humans with autopsy-proven ARVC. We show adipocytes in the lineage tracer mice and in human hearts originate from the second heart field progenitor cells, which switch to an adipogenic fate because of suppressed canonical Wnt signaling by nuclear PG.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health and was approved by the Institutional Animal Care and Use Committee. Human heart samples, stripped from the identifiers, were used in the study. The Institutional Review Board approved the study protocol. The results of genetic fate-mapping experiments showing rare cardiac cells that coexpress adipogenic and the second heart field marker. The secondary antibodies were FITC-labeled donkey anti-rabbit IgG and anti-goat IgG, FITC-labeled goat anti-rabbit IgG, Texas red–labeled goat anti-rat IgG, and Texas red–labeled donkey anti-goat IgG. The sections were mounted in DAPI-containing hard setting medium (Vector Laboratories Inc, Burlingame, Calif) and examined under fluorescence microscopy.

Immunoblotting
We electrophoresed 30-μg aliquots of cardiac protein extracts on 12% SDS polyacrylamide gels, transferred the proteins to membranes and probed them with the specific antibodies. Following stripping, we reprobed the membranes with an anti-α-tubulin antibody.

Coimmunoprecipitation
We performed coimmunoprecipitation as published previously. In brief, we mixed 6-μg aliquots of anti-Tcl712 monoclonal antibody (Millipore–Upstate, Billerica, Mass) with 500-μg aliquots of total protein extracts. We precipitated the antibody–protein complex by adding 20-μL aliquots of Protein A/G PLUS-Agarose beads. The primary antibodies used in immunoblotting were monoclonal anti–β-catenin (Santa Cruz Biotechnology Inc, Santa Cruz, Calif), anti-FLAG (Sigma, St Louis, Mo), and anti–total (transgene + endogenous) PG (Invitrogen–Zymed, Carlsbad, Calif). The secondary antibody was donkey anti-mouse IgG horseradish peroxidase–conjugated (Santa Cruz Biotechnology Inc).

Separation of Cardiac Protein Subfractions
We extracted nuclear, cytosolic and membrane proteins using a commercial kit (Chemicon International, Danvers, Mass). In brief, we homogenized the hearts in 5 volumes of a cold buffer containing HEPES (pH 7.9), MgCl2, KCl, EDTA, sucrose, glycerol, sodium orthovanadate, and protease inhibitors. We collected the supernatant containing the cytosolic proteins after centrifugation at 18 000g. We resuspended the pellets in 100 μL of a cold buffer containing HEPES, MgCl2, NaCl, EDTA, glycerol, sodium orthovanadate, and protease inhibitors and centrifuged and collected the supernatant containing the nuclear proteins. We resuspended the residual pellets in 100 μL of a cold buffer containing HEPES, MgCl2, KCl, EDTA, sucrose, glycerol, sodium deoxycholate, NP-40, sodium orthovanadate, and protease inhibitors and centrifuged to collect the supernatant containing the membrane proteins.

To detect expression of Flag-tagged PG (transgene), we probed the membranes with a rabbit anti–DYKDDDDK-Tag antibody (Cell Signaling Technology Inc, Danvers, Mass). To detect the endogenous and the Flag-tagged PG simultaneously, we probed the membranes with a rabbit anti-PG antibody. The secondary antibody was donkey anti-rabbit IgG horseradish peroxidase–conjugated (Santa Cruz Biotechnology Inc).

Immunofluorescence
We embedded thin myocardial sections from mid-ventricle in optimal cutting temperature compound (Sakura Finetek Inc, Torrance, Calif) and flash-froze them in an isopentane–liquid nitrogen bath. We stained the sections with the primary antibodies in 5% donkey or goat normal serum (Santa Cruz Biotechnology Inc). The primary antibodies (Santa Cruz Biotechnology Inc, unless otherwise specified) were goat polyclonal anti-Islet1 (Is11), rabbit polyclonal anti-Mef2C (Aviva System Biology LLC, San Diego, Calif), rabbit polyclonal anti–enhanced yellow fluorescent protein (EYFP), rabbit polyclonal anti–peroxisome proliferator-activated receptor (PPAR)-γ, goat polyclonal anti–C/EBP-α, rabbit polyclonal anti–C/EBP-β, rabbit polyclonal anti–PPAR-γ, rabbit anti-Flag (Cell Signaling Technology Inc, Beverly, Mass), and rat monoclonal anti–F4/80 (Abcam, Cambridge, Mass), the latter as a macrophage-specific marker. The secondary antibodies were FITC-labeled donkey anti-rabbit IgG and anti-goat IgG, FITC-labeled goat anti-rabbit IgG, Texas red–labeled goat anti-rat IgG, and Texas red–labeled donkey anti-goat IgG.

Human Myocardial Sections
The results of genetic fate-mapping experiments showing rare cardiac cells that coexpressed adipogenic and the second heart field markers and given that fibroadiposis in humans is an evolving and progressive phenotype, we postulated that human hearts with ARVC might contain rare cells in transition from a myogenic to an adipogenic fate. Therefore, we costained paraffin-embedded thin myocardial sections from 3 human patients with autopsy-proven ARVC who died of sudden cardiac death (ages 28 to 38 years) for coexpression of Is11 or Mef2C with C/EBP-α or PPAR-γ. As a control, we stained thin sections of a normal heart for the above markers.
Statistical Analysis
Statistical calculations (STATA-Intercooled version 10.1, StataCorp LP, College Station, Tex) were as published.22 We compared the differences for normally distributed continuous variables among the 3 groups by ANOVA, followed by pairwise comparisons by Bonferroni method. We analyzed variables that violated the normality assumption and the nonparametric variables by Kruskal–Wallis test.

Results
Generation of Dsp-Deficient Lineage Tracer Mice
Using the LoxP-Cre technology, we generated 3 sets of lineage tracer mice regulated by cardiac lineage promoters Nkx2.5, an early and pan-specific cardiogenic marker; Mef2C, a second heart field-specific marker; and αMyHC, a relatively late marker of cardiogenesis (Figure 1). We screened the offspring for the presence of floxed alleles and the Cre recombinase by PCR (oligonucleotide primers are shown in Online Table I). Distribution of the genotypes of the offspring for Dsp-deficient mice deviated from the expected Mendelian inheritance, because no liveborn homozygous offspring for Dsp-deficient mice deviated from the expected ratio (Online Table I). The latter findings reflect specificity of the Mef2C-Cre:DspW/F:R26-EYFPF/F mice.

Cardiac Phenotype in Control and Lineage Tracer Mice
We did not detect a discernible cardiac phenotype in mice expressing EYFP or Cre recombinase alone. Cardiac histology, analyzed by hematoxylin/eosin, Masson trichrome and oil red O staining of thin myocardial sections, was normal in the αMyHC-Cre, Nkx2.5-Cre, Mef2C-Cre, R26-EYFPF/F, and αMyHC- or Nkx2.5- or Mef2C-Cre:R26-EYFPF/F mice. Cardiac size and function, determined by M-mode, 2D, and Doppler echocardiography, were also normal in the above mice. Likewise, expression of EYFP in the background of Dsp deficiency did not influence cardiac phenotype because there were no significant differences in cardiac phenotype between Nkx2.5-Cre:DspW/F and Nkx2.5-Cre:DspW/F:R26-EYFPF/F or Mef2C-Cre:DspW/F and Mef2C-Cre:DspW/F:R26-EYFPF/F or αMyHC-Cre:DspW/F and αMyHC-Cre:DspW/F:R26-EYFPF/F mice. Therefore, for brevity, data on WT (control) and 3 sets of lineage tracer mice (αMyHC- or Nkx2.5- or Mef2C-Cre:DspW/F:R26-EYFPF/F) is presented along with relevant data on Nkx2.5-Cre:DspW/F and Mef2C-Cre:DspW/F, whenever appropriate.

Ventricular weight/body weight ratio was increased in the αMyHC-Cre:DspW/F:R26-EYFPF/F and Nkx2.5-Cre:DspW/F:R26-EYFPF/F mice but not in Mef2C-Cre:DspW/F:R26-EYFPF/F mice, as compared to WT mice (Figure I in the online data supplement). Likewise, ventricular weight/body weight ratio was also increased in the Nkx2.5-Cre:DspW/F (without EYFP) mice as was reported in the αMyHC-Cre:DspW/F mice.13 The αMyHC and Nkx2.5 regulated lineage tracer mice as well as the Nkx2.5-Cre:DspW/F mice exhibited left ventricular enlargement and dysfunction (Online Figure II and Online Table II). In contrast, left ventricular size and function were normal in Mef2C-Cre:DspW/F:R26-EYFPF/F (and Mef2C-Cre:DspW/F) mice (Online Figure II and Online Table II). The latter findings reflect specificity of the Mef2C-Cre in deleting Dsp in the second heart field, which gives rise to the right but not the left ventricle.

Myocardial histology in the lineage tracer mice was remarkable for excess fibroadiposis (Figure 2). Adipocytes, scored from oil red O–stained sections, comprised 0.16±0.02% of the total cells examined in the hearts of 3- to 6-month-old WT mice (N=5 mice, 10 800±1643 total cells per mouse). The corresponding percentages in the hearts of αMyHC-Cre–, Nkx2.5-Cre–, and Mef2C-Cre–regulated lineage tracer mice were 0.28±0.14% (N=7 mice, 10 000±4898 total cells per mouse), 0.44±0.26% (N=9 mice, 10 132±1286 total cells per mouse), and 0.54±0.20% (N=9 mice, 10 000±4898 total cells per mouse) respectively.
mice, 11 625±3739 total cells per mouse), and 0.63±0.32% (N=5 mice, 21 000±6363 total cells per mouse; all pair wise probability values versus WT mice were <0.05). As observed in human ARVC, fibroadiposis was more prominent at the epicardium in the lineage tracer mice (Figure 2). In accordance with the morphological data on cardiac size, cardiac myocyte cross-sectional area was also increased in the αMyHC-Cre:Dsp<sub>W/F</sub>:R26-EYFP<sub>F/F</sub> and Nkx2.5-Cre:Dsp<sub>W/F</sub>:R26-EYFP<sub>F/F</sub> but not in the Mef2C-Cre:Dsp<sub>W/F</sub>:R26-EYFP<sub>F/F</sub> mice, as compared with WT mice (Figure 2).

**Lineage Tracing of Excess Adipocytes**

To determine the origin of excess adipocytes in the heart of lineage tracer mice, we communostained myocardial sections with antibodies against EYFP and C/EBP-α, an adipogenic transcription factor. EYFP and C/EBP-α were coexpressed in epicardial adipocytes in the hearts of Nkx-2.5– and Mef2C-regulated lineage tracer mice (Figure 3 and Online Figure III, A). The number of adipocytes, identified by C/EBPα stained cells, that stained positive for EYFP varied. On average, 78% of adipocytes in the hearts of Mef2C-
Cre::Dsp<sup>W/F</sup>:EYFP<sup>F/F</sup> costained positive for EYFP (N=6 mice, 100±57/128±73 adipocytes per mouse). A similar percentage of adipocytes in the hearts of Nkx2.5-Cre::Dsp<sup>W/F</sup>:R26-EYFP<sup>F/F</sup> and Mef2C-Cre::Dsp<sup>W/F</sup>:R26-EYFP<sup>F/F</sup> lineage tracer mice were detected. B, Individual and merged images showing coexpression of second heart field marker Mef2C and adipogenic marker C/EBP-α are presented along with oil red O-stained image. As shown, Mef2C and C/EBP-α are coexpressed in a subset of adipocytes in the hearts of Nkx2.5::Dsp<sup>W/F</sup>:R26-EYFP<sup>F/F</sup> and Mef2C::Dsp<sup>W/F</sup>:R26-EYFP<sup>F/F</sup> lineage tracer mice.

Figure 4. Coexpression of second heart field and adipogenic transcription factors in the hearts of lineage tracer mice. A, Oil red O-stained and communostained thin myocardial sections for DNA (DAPI), Isl-1 (Isl1), a second heart field marker; and PPAR-γ, an adipogenic transcription factor, are shown. Rare cells showing coexpression of Isl1 and PPAR-γ in the hearts of Nkx2.5::Dsp<sup>W/F</sup>:R26-EYFP<sup>F/F</sup> and Mef2C-Cre::Dsp<sup>W/F</sup>:R26-EYFP<sup>F/F</sup> lineage tracer mice were detected. B, Individual and merged images showing coexpression of second heart field marker Mef2C and adipogenic marker C/EBP-α are presented along with oil red O-stained image. As shown, Mef2C and C/EBP-α are coexpressed in a subset of adipocytes in the hearts of Nkx2.5::Dsp<sup>W/F</sup>:R26-EYFP<sup>F/F</sup> and Mef2C::Dsp<sup>W/F</sup>:R26-EYFP<sup>F/F</sup> lineage tracer mice.

(4/279, 1.4%) in the αMyHC-Cre::Dsp<sup>W/F</sup>:EYFP<sup>F/F</sup> lineage tracer mice hearts stained positive for EYFP expression.

To establish the specificity of antibodies in detecting EYFP and C/EBPα, we stained myocardial sections with the IgG isotypes of the primary antibodies and the secondary antibod-
ies. The isotypes and secondary antibodies did not react with EYFP and C/EBPα (Online Figure III, B). To further substantiate the specificity of the findings, we stained visceral and subcutaneous adipose tissues with the same antibodies against EYFP and C/EBPα. Adipocytes from visceral and subcutaneous adipose tissues did not express EYFP, further indicating cardiac specificity (Online Figure IV).

Detection of expression of EYFP in adipocytes from the Mef2C-regulated lineage tracer mice indicated an origin from the second heart field progenitors. We surmised that the hearts in the ARVC mice might contain cells in transition from a myogenic to an adipogenic fate. To test this hypothesis and further validate the finding in Mef2C-regulated lineage tracer mice, we costained myocardial sections with antibodies against Isl1 or Mef2C and C/EBPα or PPARγ. We detected rare cells (0 to 6 cells per heart) in the hearts of Nkx2.5- and Mef2C-regulated lineage tracer mice that coexpressed Isl1 and PPARγ or Mef2C and C/EBPα (Figure 4A and 4B). We did not detect expression of second heart field markers in the few EYFP-expressing adipocytes in the hearts of MyHC-regulated lineage tracer mice.

Because Isl1 is not an exclusive cardiac lineage marker, to exclude the possibility that macrophages were the rare cells that expressed the second field markers, we costained thin myocardial sections with an antibody against F4/80, a macrophage-specific marker and EYFP. We included the corresponding oil red O-stained section and thin sections from spleen as controls. The results (Online Figure V) showed adipocytes in the heart of lineage tracer mice expressed EYFP but not F4/80 macrophage marker.

Detection of Expression of Isl1 and Mef2C in Adipocytes in Human Hearts With ARVC

To corroborate the results of lineage tracing studies in mice in human patients, we costained thin right ventricular sections from 3 human patients with autopsy-proven ARVC for cardiac progenitor markers and adipogenic transcription factors. In accordance with our findings in the genetic fate-mapping studies in mice, we detected rare cells (1 to 3 cells per heart) in the fibroadiposis area in the myocardium that coexpressed Isl1 or Mef2C and the adipogenic transcription factor C/EBPα (Figure 5 and Online Figure VI).

Nuclear PG Promotes Adipogenesis Through Suppression of the Canonical Wnt Signaling

We have implicated PG, an armadillo member of desmosomal protein with signaling function,26–28 in the pathogenesis of ARVC.13 Likewise, Wnt signaling is implicated in development of the second heart field (right ventricle), the predominant site of involvement in ARVC.29,30 To elucidate a mechanism for differentiation of second heart field progenitors to adipocytes in ARVC, we expressed Flag-tagged PG in cardiac myocytes through transgenesis under transcriptional regulation of the αMyHC-promoter. Expression level of the Flag-tagged transgene PG comprised approximately 25% to 35% of the total PG levels in the heart (Figure 6). The PG transgenic mice showed excess adipocytes in the heart along with patchy areas of fibrosis, an intact desmosome structure and normal left ventricular function (Figure 6 and Online Figure VII).

Immunostaining of thin myocardial sections from the Flag-tagged PG transgenic mice as well as immunoblotting of
cell protein subfractions showed nuclear localization of the transgene PG in cardiac myocytes (Figure 7A and 7B). To determine whether nuclear PG (also known as γ-catenin) interacted with protein constituents of the canonical Wnt signaling, we analyzed binding of PG with Tcf7l2 transcription factor by coimmunoprecipitation. Immunoblotting of the immunoprecipitates using transgene-specific anti-Flag and pan-PG antibodies showed binding of the transgene PG to Tcf7l2 (Figure 7C). In contrast, binding of β-catenin to Tcf7l2 in the PG transgenic hearts was reduced, implying competitive interactions between PG and β-catenin for binding to Tcf7l2.27

To determine the biological effects of nuclear localization and binding of PG to Tcf7l2 on canonical Wnt signaling, we determined expression levels of selected canonical Wnt/β-catenin signaling target genes by quantitative PCR (N=4 per group, probes and primers sequence are provided in Online Table I and Online Figure VIII). Concordant with reduced binding of β-catenin to Tcf7l2 in the PG transgenic mice, expression of c-Myc, a known target of activation of the canonical Wnt signaling, was suppressed (Figure 7D). In addition, expression levels of adipogenic factors Wnt5b and BMP7, which are normally inhibited by the canonical Wnt signaling, were increased by 3- to 4-fold (Figure 7E and 7F).31,32 Recent data implicate BMP7 as a major regulator of switch from myogenesis to adipogenesis.32,33 In contrast, expression level of connective tissue growth factor (CTGF), which is known to inhibit adipogenesis,34 was reduced by ~5-fold (Figure 7G).

**Discussion**

Through a series of genetic fate-mapping experiments in mice, we show adipocytes in ARVC originate from the second heart field cardiac progenitor cells. We corroborate the findings in autopsy-proven human hearts with ARVC by showing coexpression of second heart field markers, along with adipogenic transcription factors in rare cells in fibroadipocytic areas in the heart. At a mechanistic level, we show nuclear localization of desmosomal protein PG is associated with suppression of the canonical Wnt/β-catenin signaling pathway.
and a transcriptional switch to adipogenesis through activation of BMP7 and Wnt5b and suppression of CTGF. The paucity of EYFP-expressing adipocytes in the hearts of αMyHC-regulated lineage tracer mice and a significantly higher percentage of EYFP-expressing adipocytes in the hearts of Mef2C- or Nkx2.5-regulated lineage tracer mice indicate that the adipogenic switch occurs before commitment of the progenitors to a myocyte lineage. The results indicate that adipocytes in ARVC originate from the second heart field progenitor cells, which switch to adipogenesis because of suppressed canonical Wnt/β-catenin signaling imparted by the nuclear PG (Online Figure IX).

In human patients with ARVC, fibroadiposis predominately involves the right ventricle, a feature that has also been an enigma. The right ventricle primarily originates from the second (anterior) heart field as opposed to the left ventricle, which originates from the primary heart field. The 2 heart fields are distinguished by expression of different sets of transcriptional factors and signaling molecules. Accordingly, Nkx2.5 is common to both heart fields; however, Tbx5 and Hand1 mark the primary heart field, whereas Is11, Mef2C, and Hand2 characterize the second heart field. Identification of the second heart field progenitors as the cell source of adipocytes in ARVC offers a plausible explanation for the predominant involvement of the right ventricle in ARVC. Our findings are also in accordance with the recent data emphasizing the significance of the canonical Wnt signaling in the formation of the right ventricle from the second heart field. It is also noteworthy that the canonical Wnt signaling is considered a major regulator of a switch between adipogenesis and myogenesis. Furthermore, changes in expression levels of BMP7, Wnt5b, and CTGF, which are targets of the canonical Wnt signaling, favored adipogenesis. However, the characteristics of the subset of the second heart field progenitors and complete molecular mechanisms that govern their differentiation to an adipogenic fate in ARVC remain unknown.

Our focus on cardiac progenitor cells as the cell source of adipocytes is based on the premise that in ARVC caused by desmosomal mutations the stimulus for adipocytic differentiation has to originate from cells that express the desmosomal proteins. There are other potential sources that could be categorized into 2 sets. First, cells other than the myogenic lineage in the heart may express the mutant desmosomal proteins and, hence, could directly differentiate into adipocytes. This seems unlikely, because the only cell type in the heart known to express desmosomal proteins is the myocyte lineage. Second, cells that do not express the desmosomal proteins could differentiate into adipocytes through paracrine mechanisms emanating from desmosome-defective myocytes. In preliminary studies, we have cocultured myocytes isolated from the hearts of Dsp-deficient mice with cardiac fibroblasts and have not detected enhanced differentiation of the fibroblasts to adipocytes. Nevertheless, cellularly, the heart is a heterogeneous organ, and, hence, the possible differentiation of cells such as pericytes, fibrocytes, or circulating cells that seed at the myocardium and differentiate to adipocytes cannot be completely dismissed.

In conclusions, we have traced the origin of excess adipocytes in desmosomal ARVC to second heart field progenitor cells, a finding that was also corroborated in human hearts with ARVC. We implicate suppression of the canonical Wnt signaling by nuclear PG as a mechanism for switching differentiation of cardiac progenitor cells to adipocytes. The findings also explain the predominant involvement of the right ventricle in ARVC.

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Disclosures

None.

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Supplementary Material

Genetic Fate Mapping Identifies Second Heart Field Progenitor Cells as a Source of Adipocytes in Arrhythmogenic Right Ventricular Cardiomyopathy

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Short Title: Origin of Adipocytes in ARVC

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Online Methods

Genetically modified mice: Dsp-floxed (courtesy of Dr. Elaine Fuchs), Mef2C-Cre (courtesy of Dr. Brian Black), α-MyHC-Cre (courtesy of Dr. Michael D Schneider) and Nkx2.5-Cre mice have been published 1-4. R26-EYFP mouse was a generous gift from Dr. F. Costantini 5. EYFP is an enhanced yellow fluorescent protein variant of the Aequorea victoria GFP, which is cloned into the ROSA26 locus, preceded by a loxP-flanked stop sequence. Cre recombinase-mediated excision of the loxP-flanked transcriptional "stop" sequence results in expression of EYFP, which functions as a faithful monitor of Cre activity.

We generated the PG transgenic mice by the conventional method. The transgene was comprised of a 5′ end Flag-tagged wild type full-length PG which was placed downstream to a 5.5 Kbp cardiac-specific α-MyHC promoter.

M mode, 2-Dimensional, and Doppler Echocardiography: We performed echocardiography using a HP Sonos 5500 System, equipped with a 15 MHz linear transducer using sodium pentobarbital for anesthesia 1, 6. We calculated ventricular fractional shortening, ejection time, left ventricular dimensions and mass and Doppler indices without knowledge of the genotype 1, 6.

Gross and histological cardiac phenotype: We determined ventricular weight/body weight ratio in sex-matched mice at 6 months of age. We analyzed myocardial histology by H&E and Masson Trichrome and Oil Red O staining, the latter to detect fat droplets. An investigator without knowledge of the genotypes performed and analyzed histology in age and sex-matched mice and in a random order 1, 6, 7. We scored the number of adipocytes on Oil Red O stained thin myocardial sections in approximately 10,000 -12,000 cells per mouse and in 5 to 6 mice per group.

We calculated myocyte cross sectional area (CSA) by semi-automated planimetry in age- and sex- and body weight-matched non-transgenic and lineage tracer mice. Freshly
harvested thick cardiac cross-sections were placed in optimal cutting temperature (OCT) compound (Sakura-Finetek U.S.A. Inc., Torrace, CA, cat#4583) and frozen in isopentane (2-Methyl Butane) cooled at -155°C in a liquid nitrogen bath. Thin myocardial sections were cut, washed in PBS for 3 times and stained with Wheat Germ Agglutinin (WGA) conjugated with Texas Red (Molecular Probes Inc., Eugene, OR; cat # W21405). The working concentration was 2 µg/mL and incubation was at room temperature for 2 hours. Sections were then washed 3 times in PBS and mounted in Fluorescence Mounting Medium (Biomed Corp., Foster City, CA; cat # 17983-20). To quantify myocyte CSA, 6 thin sections per heart in 3 mice per each group (wild type, α-MyHC-, Nkx2.5-, and Mef2C-lineage tracer mice) were analyzed. Each thin section was divided into 12 approximately equal sectors and 5 high magnification (x400) microscopic fields per sector were sampled (approximately 18,000 myocytes per mouse).

**Immunoblotting (IB):** We detected expression levels of the target proteins by IB. In brief, we homogenized myocardial tissues in a relaxing lysis buffer (0.5% Nonidet P-40, 120[mM] sodium chloride, 50[mM] Tris-HCl pH: 7.4, 5% glycerol) containing proteinase inhibitors (Roche Diagnostics, GmbH, Mannheim, Germany; cat# 11-697-498-001) and determined the protein concentration by Bradford assay (Coomassie Protein Reagent; Pierce Biotechnology, Rockford, IL; cat # 23200). We loaded 30 µg aliquots of total protein extracts onto 12% SDS polyacrylamide gels, subjected to electrophoresis and transferred to cellulose membranes. The primary antibody against EYFP was polyclonal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA. #sc-8334, 1:200 dilution). The secondary antibody was anti rabbit IgG-Horseradish Peroxidase (polyclonal, donkey IgG, General Electric Health-care, cat #NA934V, 1:2000 dilution). We detected the signals by chemiluminescence (ECL detection reagents and Hyperfilm by Amersham Bioscience, Piscataway, NJ, cat #RPN-2106 and 28-9068-37, respectively).
We stripped the membranes in Restore PLUS Western Blot Stripping Buffer (Thermo Scientific, Hudson, New Hampshire, cat #46430,) and reprobed the membranes with an anti α-tubulin antibody (polyclonal, goat IgG, Santa Cruz Biotechnology Inc., Santa Cruz, CA, cat# sc-12462, 1:600 dilution). The secondary antibody was polyclonal donkey anti goat IgG-Horseradish Peroxidase (Santa Cruz Biotechnology cat #sc-2020, 1:2000 dilution).

**Co-immunoprecipitation (Co-IP):** We performed Co-IP as published 6. In brief, we homogenized 50 mg aliquots of ventricular myocardium and prepared the protein extracts as described above for IB. To co-immunoprecipitate, we gently mixed 6 µg aliquots of anti Tcf7l2 antibody (monoclonal, clone 6H5-3, mouse IgG2a isotype, Millipore – Upstate, Billerica, MA; cat # 05-511) to 500 µg aliquots of total protein extracts and incubated the reaction at 4°C overnight on a rocker platform. We added 20 µl of Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology Inc. cat# sc 2003) to the solutions and incubated overnight on a rocker platform at 4°C. We precipitated the proteins and resuspended the final pellets in Laemmli loading buffer (63 mM Tris-HCl, pH:6.8, 25% glycerol, 2% SDS, 0.01% brome phenol blue) and 2.5% DTT for IB. The primary antibodies for IB were anti β-catenin (monoclonal, clone BDI-480, mouse IgG1, Santa Cruz Biotechnology, cat # sc-59893), anti FLAG (monoclonal, clone M2, mouse IgG1 isotype, Sigma, St Louis, MO, cat # F3165,) and anti pan-specific PG (N-terminal, aa45-114, monoclonal, clone: 11E4, mouse IgG1-kappa isotype Invitrogen – Zymed, Carlsbad, CA, cat #13-8500).

**Separation of cardiac protein subfractions:** We extracted nuclear, cytosolic and membrane proteins using a commercial kit (Chemicon Int., Danvers, MA; cat #2145) as described 8. In brief, we homogenized the whole hearts in 5 volumes of a cold buffer containing HEPES (pH7.9), MgCl2, KCl, EDTA, Sucrose, Glycerol, Sodium OrthoVanadate and protease inhibitors cocktail. We centrifuged the homogenates at 18,000g for 20 min to pellet membrane and nuclear fractions and collected the supernatant containing the cytosolic proteins. We
resuspended the pellet in 100 µl of an ice cold buffer containing HEPES (pH7.9), MgCl₂, NaCl, EDTA, Glycerol, Sodium OrthoVanadate and proteinase inhibitors cocktail. After gentle mixing and centrifugation at 18,000g, we collected the supernatant containing nuclear proteins. We resuspended the residual pellet in 100 µl of a cold buffer containing HEPES (pH7.9), MgCl₂, KCl, EDTA, Sucrose, Glycerol, Sodium deoxycholate, NP-40, Sodium OrthoVanadate and protease inhibitors, incubated for 20 min with gentle rocking and centrifuged at 18,000 g for 20 min to collect the supernatant containing membrane proteins fraction.

We probed the membranes with a rabbit anti DYKDDDDK-Tag antibody (Cell Signaling Technology, Inc. Danvers, MA; cat #2368, 1:500 dilution) to detect expression of the Flag-tagged PG (transgene) and rabbit anti PG antibody (Santa Cruz Biotechnology Inc., cat #sc 7900, 1:500 dilution) to detect expression of the endogenous and Flag-tagged PG (pan-PG antibody). The secondary antibody was donkey anti rabbit IgG HRP-conjugated (Santa Cruz Biotechnology Inc., cat # sc 2313, 1:3000 dilution).

**Immunofluorescence (IF):** We removed the hearts immediately from CO₂-euthanized mice and washed them in cold PBS to remove blood. We cut 1 mm thick sections at the level of mid ventricle and embedded the thick sections in optimal cutting temperature compound (Sakura-Finetek U.S.A. Inc., Torrance, CA), flash-froze in an isopentane-liquid nitrogen bath and kept at −80 °C until use. We prepared and washed thin sections (5 µm) in PBS (x3) and blocked the sections with 5% donkey or goat normal serum in PBS for 60 min at room temperature (Santa Cruz Biotechnology, Inc., cat # sc-2044 and sc-2043, respectively). We incubated the sections overnight at 4 °C with the primary antibodies at 1:200 -300 dilutions in 5% blocking solution. Antibodies were from Santa Cruz Biotechnology, Inc., unless specified. The primary antibodies were goat polyclonal anti Islet1 (sc-23590), rabbit polyclonal anti Mef2C (Aviva System Biology, San Diego, CA, cat #ARP37342-T100), rabbit polyclonal anti EYFP (sc-32897), rabbit polyclonal anti PPAR-γ (sc-7196), goat polyclonal anti C/EBP-α (sc-9314), rabbit
polyclonal anti C/EBP-α (sc-61), rabbit polyclonal anti PG (sc-7900), and rabbit polyclonal anti-
Flag (Cell Signaling Technology, Inc. cat #2368). We treated the sections with the secondary
antibodies FITC labeled donkey anti rabbit IgG (Chemicon Int., Danvers, MA, cat #AP182-F)
and Texas red labeled donkey anti goat IgG (sc-2783) at dilutions of 1:1,000 -2,000 for 1 h at
room temperature. After the final PBS-wash, we mounted the sections in DAPI-containing Hard
Set™ mounting medium (Vector Laboratories, Inc, Burlingame, CA, cat # H-1500) and
examined under fluorescence microscopy.

To determine whether EYFP positive cells originated from a monocyte/macrophage
lineage, we co-stained heart tissues from wild type and lineage tracer mice for the expression of
EYFP and the monocyte/macrophage specific marker F4/80. The primary antibodies were rabbit
polyclonal anti EYFP (sc-32897) and rat monoclonal anti F4/80, (IgG 1, clone number: Cl:A3-1,
Abcam, Cambridge, MA, cat # ab6640) at 1:200 and 1:1000 dilutions, respectively. The
secondary antibodies were goat anti rabbit IgG-FITC conjugate (sc-2012) at a 1:1000 dilution
and goat anti rat IgG-Texas Red conjugate (Abcam cat # ab6843-1) at a 1:400 dilution.

To further demonstrate the specificity of staining of cardiac adipocytes for expression of
EYFP, we isolated visceral and subcutaneous fat tissues from the wild type and lineage tracer
mice and stained tissue sections, as controls, using the same antibodies as in staining of
cardiac tissues.

To further support the specificity of immunostaining for the antigen, heart tissues were
incubated with the corresponding IgG isotypes at the same concentrations as the primary
antibody. Primary IgG controls were normal goat IgG (Santa Cruz, cat# sc-2028; normal rabbit
IgG (Santa Cruz, cat# sc-2027) and normal rat IgG (Vector Laboratories, cat # I-4000). The
secondary conjugated antibodies were also used at the same concentration as in the
corresponding staining.
To quantify the number of adipocytes that stained positive for expression of EYFP reporter protein, we stained frozen thin myocardial sections from wild type, α-MyHC-, Nkx2.5-, and Mef2C-lineage tracer mice for Oil Red O and the adjacent frozen thin sections from the same mice for the expression of EYFP and C/EBPα. We divided each thin myocardial section into 60 high magnification (x400) microscopic fields and scored the number of cells that stained positive for Oil Red O (adipocytes), C/EBP-α and EYFP (the reporter protein) in each field in 14 to 44 sections and in 5 to 9 mice per group. We calculated the mean and SD of adipocytes that stained positive for EYFP per mouse.

**Human myocardial sections:** Based on the findings of rare cardiac cell expressing second heart field and adipogenic markers in the lineage tracer mice and given that fibro-adiposis in humans with ARVC evolves over time, we postulated that human hearts with ARVC may also contains cells expressing both lineage markers in the fibro-adiposis area. Therefore, we co-stained paraffin-embedded thin myocardial sections from 3 human patients with autopsy proven ARVC who were victims of SCD (ages between 28 to 38 years) for expression of markers of cardiac progenitor cells (Isl1 or Mef2C) and adipocytes (C/EBP-α). We deparaffinized the sections through treatment with xylene and retrieved the antigens by heating the sections in 10 mM sodium citrate buffer (pH 6.0) in a microwave. As a control, we stained thin sections of a normal heart for the above markers. The human heart samples were paraffin-embedded and could not be stained for Oil Red O.

**Statistical analysis:** Statistical calculations (STATA-Intercooled v.10.1, StataCorp LP College Station, Texas) were as published 6, 7, 9. We tested the variables for normality assumptions and compared the differences for normally distributed continuous variables among the three groups by ANOVA, followed by pairwise comparisons by Bonferroni test. We analyzed variables that violated the normality assumption and the non-parametric variables by Kurskal-Wallis test.
REFERENCES


### Online TABLE I

**Sequence of Oligonucleotide Primers for PCR and qPCR Probes**

#### A. PCR oligonucleotide primers

<table>
<thead>
<tr>
<th>Gene/Cre</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<tbody>
<tr>
<td>Nkx2.5-Cre</td>
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<td></td>
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<tr>
<td>Nkx2.5 Forward</td>
<td>GATTAGCTTAAGCGGAGCTGGGTGTCC</td>
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</tr>
<tr>
<td>Nkx2.5 Reverse</td>
<td>GTTCTGGAACCAGATCTTGACCTGCTGGGA</td>
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</tr>
<tr>
<td>Cre recombinase</td>
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<td></td>
</tr>
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<td></td>
<td>GCCGCATAACCAGTGAAACAGCATTGC</td>
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</tr>
<tr>
<td>Mef2c-Cre</td>
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<td></td>
</tr>
<tr>
<td>Mef2c AHF Cre-Forward</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>CCAGGCAAAAGGCAAGAATAA</td>
<td></td>
</tr>
<tr>
<td>Mef2c AHF Cre-Reverse</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>Desmoplakin</td>
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<td></td>
</tr>
<tr>
<td>Forward</td>
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<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>TTCTCTTTGTCTGTGGCCATGT</td>
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</tr>
<tr>
<td>Enhanced Yellow Fluorescent Protein (EYFP):</td>
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</tr>
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<td>Rosa26R-1</td>
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</tr>
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<td>Rosa26R-2</td>
<td>GCGAAGAGTTTGTCTCCTCAACC</td>
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<tr>
<td>Rosa26R-3</td>
<td>GGAGCGGGGAGAAATGGATATG</td>
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#### B. qPCR Probe Sequence:

<table>
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</thead>
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<tr>
<td>BMP-7</td>
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<td>Wnt 5b</td>
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<td>CTGF</td>
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<td>c-myc</td>
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<td>GAPDH</td>
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<tr>
<td></td>
<td>NTG</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td>12</td>
</tr>
<tr>
<td><strong>Sex (males/females)</strong></td>
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</tr>
<tr>
<td><strong>Age (months)</strong></td>
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</tr>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>29.7 ± 4.2</td>
</tr>
<tr>
<td><strong>Heart rate (bpm)</strong></td>
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</tr>
<tr>
<td><strong>IVST (mm)</strong></td>
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</tr>
<tr>
<td><strong>PWT (mm)</strong></td>
<td>0.96 ± 0.12</td>
</tr>
<tr>
<td><strong>LVEDD (mm)</strong></td>
<td>2.79 ± 0.30</td>
</tr>
<tr>
<td><strong>LVEDD/BW (mm/g)</strong></td>
<td>0.095 ± 0.009</td>
</tr>
<tr>
<td><strong>LVESD (mm)</strong></td>
<td>0.97 ± 0.20</td>
</tr>
<tr>
<td><strong>LVM (mg)</strong></td>
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<td><strong>LVM/BW (mg/g)</strong></td>
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</tr>
<tr>
<td><strong>FS (%)</strong></td>
<td>64.9 ± 6.4</td>
</tr>
<tr>
<td><strong>E (m/s)</strong></td>
<td>1.2 ± 0.5</td>
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<tr>
<td><strong>Ao Vmax (m/s)</strong></td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>ET (ms)</td>
<td>53 ± 11</td>
</tr>
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**Abbreviations**: bpm: Beats per minutes; IVST: Interventricular septal thickness; PWT: Posterior wall thickness; LVEDD: Left ventricular end diastolic diameter; LVESD: Left ventricular end systolic diameter; BW: Body weight; LVM: Left ventricular mass; FS: Fractional shortening; E: early mitral inflow velocities; Ao Vmax: Maximum aortic outflow velocity; m/s: meter per second; ET: Ejection time.

*p-value ≤ 0.05, # p-value ≤ 0.01, ξ p-value ≤ 0.001 by Bonferroni pairwise comparison with non-transgenics.*
Online Figure I. Box plots showing median, 25%, 75%, outliers (•) and adjacent values of ventricular weight/body weight (VW/BW) ratio. VW/BW ratio was increased significantly in the αMyHC- (N=12) and Nkx2.5-regulated (N=24) but not in Mef-2C-regulated (N=12) Dsp-deficient (with and without EYFP), as compared to wild type (N=26) mice. The pair wise p values are corrected for multiple comparisons by Bonferroni’ method.
Online Figure II. Echocardiographic phenotype. Representative M-mode echocardiograms of the left ventricle from wild type, \( \alpha \)-MyHC-Cre:Dsp\(^{WF}\):R26-EYFP\(^{F/F}\), Nkx2.5-Cre:Dsp\(^{WF}\):R26- EYFP\(^{F/F}\), and Mef2C-Cre:Dsp\(^{WF}\):R26-EYFP\(^{F/F}\) mice are shown. Compared with non-transgenic mice (Panel A), Left ventricle diastolic and systolic diameters were increased and systolic function was reduced in \( \alpha \)-MyHC-- Cre:Dsp\(^{WF}\):R26-EYFP\(^{F/F}\) and Nkx2.5-Cre:Dsp\(^{WF}\):R26-EYFP\(^{F/F}\) mice (Panels B and C). In contrast cardiac dimensions and function was preserved in Mef2C-Cre:Dsp\(^{WF}\):R26-EYFP\(^{F/F}\) mice (Panel D).
Online Figure III. Panel A. Oil Red O, DAPI, C/EBP-α and EYFP co-stained sections in wild type (non-transgenic) mice are shown as controls. As shown, immunostaining did not show expression of C/EBP-α and EYFP in the heart of wild type mice.

Panel B. Additional controls including stained sections with IgG isotypes from the corresponding host animals for the primary antibodies are shown to further substantiate the specificity of the findings.
Online Figure IV. Oil Red O, DAPI, C/EBP-α and EYFP co-stained sections in visceral fat tissues from Nkx-Cre:Dsp<sup>W/F:R26-EYFP<sup>F/F</sup> and Mef2C-Cre:Dsp<sup>W/F:R26-EYFP<sup>F/F</sup> lineage tracer mice are shown as controls. As shown, EYFP is not expressed in visceral fat tissues from the lineage tracer mice.
Online Figure V. Exclusion of macrophages as a cell source of excess adipocytes in ARVC. A. Absence of expression of F4/80, a specific marker of macrophage in adipocytes in Nkx2.5-Cre:Dsp<sup>W/F</sup>:R26- EYFP<sup>F/F</sup> mice hearts. The adipocytes show expression of EYFP. B. Sections of spleen from Nkx2.5-Cre:Dsp<sup>W/F</sup>:R26-EYFP<sup>F/F</sup> mice showing expression of F4/80, as a positive control for antibody. C. Myocardial sections stained for Oil Red O and specific IgG isotypes, as controls for the specificity of the antibodies tested.
Online Figure VI. Bright field and co-stained sections for C/EBP-α and DNA, Mef2C and DNA or Isl1 and DNA in a normal heart, as controls.
Online Figure VII. M-Mode echocardiograms from wild type and plakoglobin (PG) transgenic mice. The time scale represent 200 msec intervals. As shown cardiac size and function were normal in the PG transgenic mice and similar to that in the wild type (non-transgenic) mice.
Online Figure VIII. Quantitative PCR amplification plots showing expression levels of GAPDH as control and four targets of the canonical Wnt signaling in non-transgenic and wild type PG transgenic mice. Amplification plots for GAPDH were practically superimposed in the control and transgenic mice. In contrast, expression levels of Wnt5b and BMP7, both known to be inhibited by the canonical Wnt signaling and both involved in adipogenesis, were increased by 3 to 4 folds. In accord with a shift to adipogenesis, relative expression level of CTGF, an inhibitor of adipogenesis was decreased by 5-fold. Similarly, relative expression level of c-Myc, activated by the canonical Wnt signaling, was down regulated by approximately 2-fold.
Online Figure IX. Pathogenesis of excess adipocytes in ARVC. Mutations in desmosomal proteins by disrupting proper desmosome assembly free plakoglobin (PG) to translocate from the desmosome to the nucleus. In the nucleus PG, also known as γ-catenin because of structural and functional similarity to it, suppresses the canonical Wnt signaling through Lef/Tcf transcription factors. The net effect is removal of the inhibitory effects of the canonical Wnt signaling on expression of BMP7 and Wnt5b, known promoters of adipogenesis and suppression of expression of CTGF, known inhibitor of adipogenesis. Together they promote differentiation of cardiac progenitor cells to adipocytes.