β-Catenin downregulation attenuates ischemic cardiac remodeling through enhanced resident precursor cell differentiation

Laura C. Zelarayán1, Claudia Noacka,1, Belaid Sekkalib,1, Jana Kmeova1,2, Christina Gehrkek, Anke Rengera, Maria-Patapia Zafirioua, Roel van der Nage, Rainer Dietzc, Leon J. de Windtd, Jean-Luc Balligandb,2, and Martin W. Bergmanna,c,2

1Department of Cardiology, Campus-Buch and Campus Virchow-Klinikum, Charité–Universitätsmedizin Berlin, Franz Volhard Klinik, Germany; 2Max Delbrück Center for Molecular Medicine, 13125 Berlin, Germany; 3Unit of Pharmacology and Therapeutics, University of Louvain Medical School, 1200 Brussels, Belgium; and 4Department of Medical Physiology, University Medical Center Utrecht, Royal Netherlands Academy of Sciences, 3524, Utrecht, The Netherlands

We analyzed the effect of conditional, αMHC-dependent genetic β-catenin depletion and stabilization on cardiac remodeling following experimental infarct. β-Catenin depletion significantly improved 4-week survival and left ventricular (LV) function (fractional shortening: CTex3–6: 24 ± 1.9%; β-catex3–6: 30.2 ± 1.6%, P < 0.001). β-Catenin stabilization had opposite effects. No significant changes in adult cardiomyocyte survival or hypertrophy were observed in either transgenic line. Associated with the functional improvement, LV scar cellularity was altered: β-catenin-depleted mice showed a marked subendocardial and subepicardial layer of small cTnTpos cardiomyocytes associated with increased expression of cardiac lineage markers Tbx5 and GATA4. Using a Cre-dependent lacZ reporter gene, we identified a noncardiomyocyte cell population affected by αMHC-driven gene recombination localized to these tissue compartments at baseline. These cells were found to be cardiac progenitor cells since they coexpressed markers of proliferation (Ki67) and the cardiomyocyte lineage (αMHC, GATA4, Tbx5) but not cardiac Troponin T (cTnT). The cell population overlaps in part with both the previously described c-kitpos and stem cell antigen-1 (Sca-1)pos precursor cell population but not with the Isl1pos precursor cell pool. An in vitro coculture assay of highly enriched (>95%) Sca-1pos cardiac precursor cells from β-catenin-depleted mice compared to cells isolated from control littermate demonstrated increased differentiation toward α-actinpos and cTnTpos cardiomyocytes after 10 days (CTex3–6: 38.0 ± 1.0% α-actinpos; β-catex3–6: 49.9 ± 2.4% α-actinpos, P < 0.001). We conclude that β-catenin depletion attenuates postinfarct LV remodeling in part through increased differentiation of GATA4pos/Sca-1pos resident cardiac progenitor cells.

Despite adaptive mechanisms including activation of cardiomyocyte survival pathways and hypertrophy, left ventricular (LV) remodeling often progresses to cardiac dilation and heart failure (1). Recently, the quantitative contribution of endogenous cardiac regeneration was found to account for at least 25% of cardiomyocytes in the infarct border zone (2). However, essential characteristics of this cardiac progenitor cell pool, like signaling pathways directing differentiation and/or proliferation, are largely unknown.

Transcription factors essential for embryonic cardiac development also affect adult cardiac remodeling in mice (3). Regulation of the Wnt/β-catenin pathway differentially regulates embryonic cardiac progenitor cells prespecification, renewal, and differentiation in the cardiac mesoderm (4–7). Activation of the Wnt/β-catenin pathway specifically stimulates Isl1-cardiac progenitor cells proliferation while delaying differentiation. Conversely, increased expression of Wnt signaling inhibitors in αMHCpos cardiac progenitor cells isolated from embryoid bodies lead to increased cardiomyocyte differentiation (8).

We previously reported that downregulation of β-catenin in adult heart is required for adaptive hypertrophy upon chronic angiotensin II challenge (9). Here, we describe the effect of β-catenin depletion on ischemic LV-remodeling. We used two transgenic mouse models to study the effect of conditional depletion (β-catex3–6) or stabilization (β-catex3–6) of β-catenin upon αMHC-driven gene recombination in the adult heart (9). To monitor recombined cells we used the ROSA26 (lacZ) reporter mice (10). Our analysis revealed a specific population of cardiac progenitor cells including Sca-1pos and c-kitpos cells to be affected by αMHC-dependent gene recombination. In association with the functional improvement after infarct in β-catenin-depleted mice, isolated Sca-1pos cardiac precursor cells exhibited enhanced cell differentiation toward mature cardiomyocytes. In Vivo, early cardiac transcription factors GATA4 and Tbx5 were upregulated upon β-catenin depletion in infarcted mice. These data suggest β-catenin depletion to be beneficial in postinfarct LV remodeling in part through enhanced differentiation of αMHCpos cardiac resident progenitor cells.

Results

αMHC-Restricted β-Catenin Depletion or Stabilization Plus lacZ Reporter Gene Expression in Mice. Mice with conditional β-catenin depletion (β-catex3–6) or stabilization (β-catex3–6) depending on αMHC-CrePRI recombination in adult myocardium were generated as described before (9), CreERt2 littermate were used as controls and termed CTex3–6 or CTex3–6, respectively.

To monitor cells affected by αMHC-directed recombination, ROSA26 reporter mice were bred to β-catex3–6 mice expressing the lacZ gene in recombined cells. Cre-mediated conditional recombination was induced by mifepristone (RU-486) injection [Fig. S1A and see supporting information (SI Text)]. Using the indicated primers (blue arrows in S1A), successful genomic recombination was confirmed (Fig. S1B). Depletion of the full-length β-catenin protein and expression of the β-galactosidase (β-gal) reporter were detected by immunofluorescence and Western blot in adult cardiomyocytes (Fig. S1C and S2A). Decreased expression of β-catenin (*, P < 0.05) and its target genes LEFI (**, P < 0.01) and TCF4 (***, P < 0.01) was confirmed by quantitative real-time-PCR 3 weeks after Cre induction (CTex3–6 n = 6; β-catex3–6 n = 15; Fig. S1D).

The same strategy was used to obtain mice with αMHC-restricted β-catenin stabilization (9). Excision of exon 3 containing the
GSK3β phosphorylation site blocks proteasome-mediated degradation and results in cytoplasmic accumulation of β-catenin (11). These truncated β-catenin products were detected by PCR and Western blot (Fig. S1E and Fig. S2B), indicating successful recombination. The β-catenin target genes Lef1, Tcf4, and Axin2 were upregulated as demonstrated by real-time PCR (data not shown) (9).

**β-Catenin Depletion Attenuates Left Ventricular Remodeling After Myocardial Infarction.** To determine whether β-catenin modulates ischemic cardiac remodeling, β-catenin-depleted, stabilized, and respective control littermates were subjected to chronic ligation of the left anterior descending coronary artery (LAD) (LAD) (Fig. S3A). Mice who died within 2 days of the surgery or had no clear infarct scar in echocardiography and histology were excluded from further analysis (Fig. S3B and S4). At 4 weeks, β-catenin-depleted animals exhibited improved fractional shortening (Fig. 1 A, CTΔex3–6 n = 7; 24.2 ± 1.9% vs. β-catΔex3–6 n = 13; 30.2 ± 1.6% *** P < 0.001) and reduced heart weight/body weight ratio (HW/BW) (CTΔex3–6: 6.1 ± 0.42 vs. β-catΔex3–6: 5.3 ± 0.21 * P < 0.05) (Table S1). In addition, infarct size was significantly reduced in β-catenin-depleted mice vs. controls (CTΔex3–6 n = 6; 47.2 ± 3.7% vs. β-catΔex3–6 n = 6; 37.2 ± 2.1% * P < 0.05) (Fig. 1A and Fig. S3C). β-Catenin depletion also led to reduced mRNA expression of heart failure markers ANP and BNP at two weeks (CTΔex3–6 n = 5; β-catΔex3–6 n = 15, Fig. S6A).

Mice with β-catenin stabilization displayed no major differences compared to controls in infarct size or LV function (Fig. S5). β-Catenin depletion significantly decreased mortality over the first 4 weeks after experimental myocardial infarction (CTΔex3–6 n = 11; β-catΔex3–6 n = 15 *P < 0.05), while β-catenin-stabilized mice showed a nonsignificant increase of mortality (Fig. 1B). A prominent subendo- and subepicardial layer of cTnT^pos cardiomyocytes was found in the scar upon β-catenin depletion, while controls showed a fibrotic scar with only few cTnT^pos cells (right panel, Fig. 1A). A similar fibrotic scar was observed in β-catΔex3 mice and their respective controls (right panel, Fig. S5). In summary, depletion of β-catenin in the adult heart attenuates ischemic LV remodeling and postinfarct mortality.

**Improved Cardiac Function Upon β-Catenin Depletion Is Not Associated With Changes in Adult Cardiomyocyte Hypertrophy or Apoptosis.** As a molecular and cellular mechanism of attenuated LV remodeling upon β-catenin depletion, we hypothesized adult cardiomyocyte hypertrophy and/or apoptosis to be altered. We investigated hypotrophy and apoptosis 2 and 4 weeks after LAD ligation. We found no significant difference concerning the hypertrophy markers α-skeletal-actin and β-MHC between β-catΔex3–6 mice and their controls 2 weeks after LAD (Fig. S6A). At 4 weeks, neither gene markers of cardiomyocyte hypertrophy nor echocardiographic septal or free wall thickness as a measure of global LV hypertrophy showed any significant differences (Table S1 and Fig. S4). Because hypertrophy of the noninfarcted myocardium contributes to LV remodeling (1), myocardial area was measured in the remote zone. No significant change was detected in β-catΔex3–6 mice (Fig. S6B) or β-catΔex3 mice (data not shown) compared to their control littermates both at 2 and 4 weeks after infarct. In addition, we found no evidence for an altered rate of cardiomyocyte apoptosis as detected by TUNEL assay (Fig. S6C). Therefore, we conclude that the attenuation of cardiac remodeling after ischemia upon β-catenin depletion is not mediated by inhibition of cardiac hypertrophy and/or decreased apoptosis of adult cardiomyocytes.

**Resident Cardiac Progenitor Cells Are Targeted by αMHC-Dependent Gene Recombination.** Since neither adult cardiomyocyte apoptosis nor hypertrophy explained the observed phenotype, we asked whether other cardiac cell types, apart from mature cardiomyocytes, were affected by αMHC-dependent gene recombination. The ROSA26 reporter mice allowed for the identification of cells targeted for Cre recombinase through detection of β-gal expression. We aimed to identify β-gal^pos cells using flow cytometry in a cardiomyocyte-depleted cell fraction from adult heart and found ~10% β-gal^pos cells. Next, we tested whether the β-gal^pos cells have stem cell characteristics and analyzed the coexpression of the cardiac progenitor cell marker Sca-1. Of the noncardiomyocyte cells, 8.3 ± 0.4% were detected to be Sca-1^pos (Fig. S7A–C). More than 90% of these Sca-1^pos cells (6.6 ± 0.8% cells of the total noncardiomyocyte cell population) were β-gal^pos in both CTΔex3–6lacZ and β-catΔex3–6lacZ mice (data not shown) used as negative controls for β-gal detection (Fig. 2A and data not shown). Similarly, flow cytometry analysis of another heart-specific inducible Cre line, the αMHC–MerCreMer mice (12) mated to the ROSA26 reporter mice showed >60% of the Sca-1^pos cells to coexpress β-gal following Cre-induction (Fig. S8A). Immunofluorescence analysis of the noncardiomyocyte cell fraction proved coexpression of the Sca-1 and c-kit epitope in a subpopulation of β-gal^pos cells. Moreover, αMHC protein expression was observed in

---

**Fig. 1.** αMHC-dependent β-catenin depletion attenuates postinfarct LV remodeling. (A) β-Catenin depletion results in improved fractional shortening and reduced infarct size 4 weeks after infarct associated with a prominent subendocardial and subepicardial layer of cardiomyocytes as identified by cTnT staining. (B) Kaplan-Meier survival curve demonstrated significantly enhanced survival in β-catΔex3–6 mice compared to CTΔex3–6 mice 4 weeks following experimental infarct. In contrast, increased mortality was observed in β-catΔex3 mice compared to CTΔex3 mice. CTΔex3–6 n = 11; β-catΔex3–6 n = 15; CTΔex3 n = 8; β-catΔex3 n = 9; MI, myocardial infarct; LV, left ventricle; cTnT, cardiac Troponin T; *, P < 0.05.
a subpopulation of Sca-1pos cells confirming the activation of the endogenous αMHC promoter and protein expression in the identified cell population (Fig. 2B).

Using magnetic cell sorting (MACS) technology we enriched cardiac Sca-1pos cells from β-catenin depleted, stabilized and their respective control littermates to >95% for further characterization. Independent from the genotypes, mRNA quantification showed coexpression of c-kit. Other known precursor cell markers such as Islet-1 or Oct3/4 were not coexpressed, suggesting different subsets of cardiac precursor cells to be present in the adult heart. Sca-1pos cells from β-catΔex3-6 and β-catΔex3-6 showed both Cre recombinase and αMHC gene expression consistent with activation of the αMHC promoter in these cells (Fig. 2C). Gene expression of the cardiac transcription factors GATA4, Tbx2, Tbx5, and Tbx20 was detected, confirming that these Sca-1pos/β-galpos cells in the noncardiomyocyte fraction are progenitor cells of the cardiac lineage (Fig. 2C, Fig. S8 D and E).

Aiming to visualize the identified cardiac progenitor cells in *vivo*, we analyzed heart sections from β-catΔex3-6/αlacz and CTΔex3-6/αlacz mice at baseline. Small intramyocardial β-gal-expressing cells were detected by enzymatic αlacz reaction. In addition, immunoperoxidase detection identified endo- and epicardial β-galpos cells (arrows, Fig. 2D). Heart sections contained with β-gal and cTnT showed a subendo- and subepicardial layer of β-galpos/cTnTpos cells with a large nucleus to cytoplasmic ratio as expected for cardiac precursor cells (Fig. 2E, see Fig. S9 for controls). Double stainings of consecutive slides confirmed β-galpos/Sca-1pos cells to be cTnTneg, GATA4pos, and Tbx5pos (arrows and numbers in Fig. S8 D and E, see S7 for Sca-1 control staining). In conclusion, we identified a population of αMHCpos/Sca-1pos/c-kitpos/GATA4pos/cTnTneg cardiac progenitor cells in an endocardial and epicardial compartment.

**Cardiac Progenitor Cell Proliferation and Distribution Following Experimental Infarct.** We demonstrated αMHCpos/Sca-1pos/cTnTneg cardiac progenitor cells to be targeted for β-catenin depletion. As a hallmark of cardiac progenitor cells, a BrdU/Sca-1 and Sca-1pos/β-galpos double staining identified proliferating Sca-1pos cells. β-Catenin depletion did not affect the number of proliferating Sca-1pos cells (Fig. 3A and Fig. S10B). While these data provide evidence for self-renewal of cardiac resident Sca-1pos precursor cells no evidence was found that differences in proliferation rate explains the observed functional phenotype upon β-catenin depletion.

Next, we asked whether altered migration might contribute to LV remodeling upon β-catenin depletion. We investigated the distribution of Sca-1pos/GATA4pos/cTnTneg cardiac progenitor cells using confocal microscopy in heart sections 2 and 4 weeks after ischemia. At 2 weeks the distribution of β-galpos/Sca-1pos/GATA4pos/cTnTneg progenitor cells and a semiquantification of the Sca-1 cells in the scar vs. remote zone showed no major difference between β-catΔex3-6 and controls (representative pictures in Fig. S10B and data not shown). A prominent layer of GATA4pos/cTnTneg cells was detected along the scar at 4 weeks (white arrows in Fig. 3 B and C). Additionally, a few small GATA4pos/cTnTneg cells were localized in the ischemic region of β-catΔex3-6 and control littermates in the proximity (subendo- and subepicardium) of the compartment where the Sca-1pos/β-galpos cardiac precursor cells were observed initially (endoand epicardium) (red arrows in Fig. 3 B and C). In summary, we found no evidence that altered migration of cardiac progenitor cells would explain the observed functional phenotype or the more prominent layer of cTnTpos cells in the scar of β-catΔex3-6 mice.

**β-Catenin Depletion Enhances Cardiac Progenitor Cell Differentiation After Ischemia.** We next asked whether depleting β-catenin alters differentiation of cardiac-resident progenitor cells toward cTnTpos
cardiomyocytes. Flow cytometry analysis of the noncardiomyocyte cell fraction revealed the fraction of Sca-1pos cells coexpressing GATA4 to significantly increase in β-cat-depleted mice 4 weeks after infarct compared to β-cat-depleted mice at baseline and control mice after infarct (Fig. 4A and B). This increased expression of cardiac differentiation markers was accompanied by a decrease of total Sca-1pos cells in β-cat-depleted mice vs. baseline. Control animals did not decrease the Sca-1pos cell population after infarct (Fig. 4B).

If progenitor cell differentiation contributes to stabilize the scar, early TnTpos-expressing cells with smaller cell size than mature cardiomyocytes should be detectable. Therefore, we analyzed the scar cellularity 4 weeks after ischemia. β-Catenin-depleted mice showed a significant increase of GATA4pos/TnTpos cells (white circle in Fig. 4C) with <16 μm² surface area (matched to DAPIpos cell number). In contrast, mice with stabilized β-catenin did not show any significant difference in comparison to their respective controls (Fig. 4C).

To confirm the hypothesis of enhanced precursor cell differentiation upon β-catenin depletion, we performed an in vitro differentiation assay. Isolated Sca-1 cells from β-catenin depleted, stabilized, and respective control littersmates were enriched by MACS to >95% purity and labeled with the cell tracer CM-Dil. The cells were co-cultured with neonatal mouse (FVB WT strain) cardiomyocytes for 10 days and the number of double acpos/CM-Dilpos cells. Sca-1pos cells isolated from neonatal mouse (FVB WT strain) cardiomyocytes for 10 days and the number of double acpos/CM-Dilpos cells was calculated in relation to the total CM-Dilpos cells. Sca-1pos cells isolated from β-cat-depleted mice showed significantly increased differentiation capacity in comparison to cells isolated from controls (CTex3-6 n = 10; 38 ± 1.0% of α-sr acpos + CM-Dilpos/total CM-Dilpos, β-cat-depleted n = 10; 49.9 ± 2.4% **, P < 0.001, Fig. 4D). Similar to the in vivo situation, we detected an increased percentage of differentiated GATA4pos/TnTpos cells in β-catenin-depleted cells compared to control cells (CTex3-6, 21.2 ± 1.5% vs. β-cat-depleted, 34.0 ± 1.6% **, P < 0.001, Fig. 4E). In contrast, Sca-1pos cells isolated from β-catenin-mobilized mice exhibited decreased differentiation capacity (CTex3 n = 4; 49.1% ± 4.8 of α-sr acpos/CM-Dilpos/total CM-Dilpos, β-cat-depleted n = 6: 38.3 ± 2.1% *, P < 0.05, Fig. 4D) and decreased differentiated GATA4pos/TnTpos cells (CTex3, 26.7 ± 0.9% vs. β-cat-depleted, 18.3 ± 0.7% **, P < 0.001, Fig. 4E). Collectively, these data indicate that following chronic LAD ligation, β-catenin depletion enhances resident endogenous Sca-1pos cardiac progenitor cell differentiation toward GATA4pos/TnTpos/α-sr acpos-expressing cells (scheme in Fig. 4G). Enhancing endogenous repair mechanisms contributes to global LV remodeling including infarct size extension (1).

Reexpression of Cardiac Developmental Transcription Factors in Ischemic Adult Heart. During embryogenesis, the cardiac mesoderm activates several transcriptional regulators of the cardiac program including GATA4 and members of the T-box family necessary for ventricular cardiac differentiation in response to inductive signals (13). Tbx5 is specifically expressed in the first heart field, which gives rise to left ventricular cardiomyocytes (14). Therefore, we studied Tbx5 and GATA4 expression following infarct via quantitative real-time PCR using heart samples obtained from the apex containing scar tissue, border zone, and remote area. Four weeks after infarct, expression of Tbx2, Tbx5, and GATA4 was significantly upregulated in β-cat-depleted mice in comparison to controls and Tbx20 was downregulated. Gene regulation in heart samples from mice with β-catenin stabilization showed the opposite results (Fig. 4F). These data suggest β-catenin depletion in the adult myocardium to induce cardiomyocyte differentiation similar to the embryonic formation of the left ventricle.

Discussion
Our study describes a resident cardiac progenitor cell population exhibiting aMHC-promoter activity and expression of cardiac transcription factors GATA4 and Tbx5 as specific markers for the first heart field (FHF) giving rise to the left ventricle during embryonic cardiac development. Enhancing signaling cascades orchestrating LV embryonic cardiomyocyte differentiation, namely depletion of β-catenin, positively affects global LV function and survival at 4 weeks. We suggest enhanced cardiomyocyte differentiation of endogenous cardiac resident stem cells to mediate this effect by limiting secondary infarct expansion.

β-Catenin Depletion Attenuates Postischemic Mortality. Infarct mortality was ameliorated by β-catenin depletion already during the first 2 weeks post infarct. Similarly, ubiquitous overexpression of the Wnt signaling antagonist frizzled4, which prevents β-catenin accumulation after infarct, resulted in reduced mortality between 2 and 5 days after permanent LAD ligation because of reduced cardiac rupture (15). Fatal arrhythmias resulting from “vulnerable” myocardium might be reduced through the effects of β-catenin on cellular scar composition. In association with the improved LV function following infarct, we found an increased number of cTnTpos-expressing cells with small cell size upon β-catenin depletion. These data suggest that despite the dramatic size difference between the small-identified cTnTpos cells in the scar and the adult cardiomyocytes, these cells might positively affect secondary scar expansion (16) and therefore affect ventricular wall stability early on.

Cardiomyocyte Hypertrophy and Apoptosis Upon β-Catenin Depletion. Four weeks after chronic LAD ligation mice with β-catenin depletion exhibited a complete lining of cTnTpos cells along the scar subendocardium and subepicardium, while control animals showed only scattered cTnTpos cells. One explanation is that these cells might have survived the initial hypoxia through
increased expression of survival genes. However, we have not observed significant differences in the TUNEL analysis. A second explanation is that β-catenin influences proliferation but β-catenin depletion did not alter proliferation as quantified by analysis of Sca-1pos/Ki67pos cells. Accordingly, β-catenin depletion in the early mesoderm decreased proliferation of cardiac precursor cells in the embryonic FHF (17). Upregulation of β-catenin enhances expansion of Islet-1 cardiac stem cells (7, 18). Lastly, hypertrophy of surviving cardiomyocytes was demonstrated to be unaffected upon β-catenin depletion. Thus, none of the cellular mechanisms involving αMHC-dependent gene recombination in adult cardiomyocytes sufficiently explains the phenotype observed here.

**Identification of αMHCpos Cardiac Resident Precursor Cells.** Employing the ROSA26 reporter mice in which the expression of the lacZ gene depends on Cre activation, we documented αMHC-dependent gene recombination, mRNA, and protein expression in a cTnTneg cell population. Cardiac stem cells were previously shown to express αMHC during early cardiac developmental stages in embryoid bodies in vitro (8). As the adult cardiac cell population under investigation here expresses markers of cell proliferation (Ki67,
Beta-Catenin Downregulation Improves Cardiac Function After Experimental Infarct. Previous studies suggest a negative role for Wnt/\beta-catenin during cardiac differentiation (7, 25, 26). Active \beta-catenin signaling supports stemness and delays differentiation of cardiac precursors in vivo and in vitro (27). Gene expression analysis from differentiating aMHC-expressing embryonic stem-derived cells in comparison to nondifferentiating cells showed upregulation of negative regulators of the Wnt signaling (8). We identified a subpopulation of cardiac resident progenitors committed to the cardiac lineage to be targeted for \beta-catenin depletion, which results in enhanced differentiation. Similar observations have been described for Islet-1-expressing cardiac precursors; differentiation decreased upon \beta-catenin stabilization while proliferation of the undifferentiated cells is enhanced (7). Moreover, upregulation of Tbx5 and GATA4 gene expression was observed after ischemia, suggesting a reactivation of the LV cell differentiation program in adult heart in adaptation to injury.

We suggest that endogenous cardiac regeneration contributes to LV remodeling following chronic ischemia through differentiation of resident precursor cells, amplified by \beta-catenin downregulation. Similarly, increased differentiation of Sca-1-expressing resident cardiac precursor cells was observed upon upregulation of FGF2. Depletion of FGF2 was found to worsen LV remodeling by enhancing secondary infarct expansion (7, 25, 26). We propose limited secondary infarct expansion to contribute to the improved cardiac function observed in our study.