

SUPPLEMENTAL MATERIAL

β -hydroxybutyrate facilitates postinfarction cardiac repair via targeting PHD2

Expanded Methods

Experimental Animals and Study Design

All animal experiments were conducted in compliance with the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology. Animals were randomly assigned to the experimental groups, and the person performing the surgery and echocardiography was blinded for the genotype.

Animal model for myocardial infarction

All experiments were approved by the Huazhong University of Science and Technology Committee on Animal Care. Twelve-week-old C57BL/6 male mice were provided by the experimental animal center of Tongji Medical College, Huazhong University of Science and Technology. All mice were housed at 22°C with a 12-hour light, 12-hour dark cycle with free access to water and food. Mice were allocated to each group randomly using a random number generator. The sample size was determined empirically based on anticipated fold change from results obtained from similar experimental measurements or previous studies. No samples, whether intentionally or inadvertently, have been omitted from the in vivo study. The mouse model of permanent MI has been described previously⁵². Briefly, mice were anesthetized with 2% isoflurane. The heart was exposed by left thoracotomy after fixation, and the left anterior descending branch of the coronary artery was then ligated with 6-0 silk sutures. Anterior wall blanching suggested that ligation of the coronary artery was a success. For macrophage depletion, Cx3cr1^{Cre} mice were crossed with R26^{DTR} mice from Model Organisms Center Inc. (Shanghai, China). The Cx3cr1^{Cre} × R26^{DTR} mice exhibited selective depletion of macrophages following administration of

DT. Because previous studies demonstrated a protective effect of estrogen against ischemic cardiac injury and therefore suggested the male sex as an important risk factor for cardiovascular disease⁵³, only male mice were used in this study. Transthoracic echocardiography was performed with a Vevo3100 Imaging system equipped with a 40 MHz transducer (VisualSonics). To assess cardiac function, a parasternal short-axis view was utilized to capture M-mode images for the analysis of ventricular dimensions and functional indices reported. Mice in the β -OHB- or AcAc-treated group (1.6 mmol/kg/d) received subcutaneous administration (0.5 μ L/h) using the Alzet mini-osmotic pump.), which were replaced every two weeks throughout the study period. For all the in vivo animal studies, the samples were collected and analyzed by different investigators, ensuring that investigators who analyzed the data were blinded to the treatment groups.

CR mouse model

Twelve-week-old C57BL/6 male mice were housed individually and randomly allocated into different groups. AL-Con mice and AL-LAD mice were fed with AL food for 16 weeks. CR-Con mice and CR-LAD mice were fed a 10%-restricted diet for the first week and a 25%-restricted diet for the remaining experimental period of the CR diet. The AL and CR foods were made according to the composition of AIN-93 M, as previously described⁵⁴. During the last 4 weeks, AL-LAD and CR-LAD mice received LAD artery ligation surgery. In the last week of the experiment, the mice underwent indirect calorimetry, IPGTT, and blood biochemical analysis.

Generation of PHD2 K239R and K385R knock-in mouse

PHD2 K239R and K385R knock-in mouse lines were constructed as follows: The gRNA to mouse EglN1 gene, the donor vector containing "loxP-mouse EglN1 CDS-3*SV40 pA-loxPmutant mouse EglN1 CDS-p.K239R(AAA to CGT)-p.K385R(AAA to CGT)-rBG pA" cassette, and Cas9 mRNA were co-injected into fertilized mouse eggs to generate targeted conditional knock-in offspring. F0 founder animals were identified by PCR followed by sequence analysis, which were bred to wildtype mice to test

germline transmission and F1 animal generation. Breed F1 targeted mouse with Cx3cr1-Cre or Cdh5-Cre mouse to generate mice that are heterozygous for a targeted allele and a hemizygous/heterozygous for the Cre transgene.

Indirect calorimetry

Whole-body metabolic states of the mice were tested by indirect calorimetry in a comprehensive animal metabolic monitoring system (Comprehensive Lab Animal Monitoring System; Columbus Instruments) for 2 days after 1 day of habituation according to the manufacturer's instructions. Light and feeding conditions were kept the same as in the home cages. EE and RER were calculated using the equations $EE \text{ (kcal/h)} = (3.818 \times VO_2) + (1.232 \times VCO_2)$ and $RER = VO_2/VCO_2$. VO_2 represents the volume of oxygen consumed per hour, and VCO_2 represents the volume of carbon dioxide produced per hour. Given the significant difference in BW between the AL and CR mice, we compared their metabolic rates by normalizing to the metabolic size, as reflected by the $BW^{0.75}$ of each mouse.

Intraperitoneal glucose tolerance test (IPGTT)

Before the IPGTT, the mice were fasted overnight. Fasting blood glucose levels were measured with blood that was collected via tail tip cutting using a portable glucose meter. Mice were injected intraperitoneally with D-glucose dissolved in saline at 2g/kg body weight, and blood glucose levels were measured at 15, 30, 60, and 120 min after glucose injection using a portable Freestyle blood glucose meter (Abbott Laboratories, North Chicago, IL) .

Mouse serum collection

Mice were fasted overnight and anesthetized before blood collection. Blood was collected from the abdominal vena cava before euthanasia, allowed to clot for 20–30 min, and centrifuged at 3,000 g for 20 min. Serum was collected from the centrifuged samples and stored at -70°C until use. .

Histological analyses

Animals were euthanized before necropsy, and hearts were arrested in diastole during the harvest process by an intravenous bolus of 0.08mg/g KCl. Mouse hearts were fixed in 4% paraformaldehyde and embedded in paraffin. Tissues were cut into 5- μ m sequential slices and subjected to hematoxylin and eosin staining (H&E staining) and Masson's trichrome staining. For H&E staining, hematoxylin (Fisher Scientific, Cat# 22-110-639) and eosin Y (Fisher Scientific, Cat# SE23-500D) were utilized. Trichrome Stain (Masson) Kit (statlab, Cat#: KTTRBPT) was used to measure fibrosis in the heart tissue following the kit protocol. Collagen fibers were stained differentially when treated with Biebrich Scarlet-Acid Fuchsin, phosphomolybdic/phosphotungstic acid (PTA/PMA) and Aniline Blue. The collagen fibers were stained blue, the nuclei were stained black, and the background was red. The infarct size was calculated as total infarct circumference divided by total LV circumference \times 100. The wall thickness of the scars at the papillary and apical levels was measured. These data were analyzed using ImageJ software. We selected illustrative images that best represent the average results for each group as representative images from the entire series of experiments.

Immunofluorescence staining

For immunofluorescence staining experiments, after deparaffinization and rehydration, high-pressure antigen retrieval process with citrate buffer (pH 6.0) (S1699, Agilent Dako) was performed for 20 minutes at 120 °C. The sections were blocked in PBS with 10% goat serum for 1 hour, incubated overnight with the indicated primary antibodies for CD31 (1:100, Cell Signaling Technology, 15585, Invitrogen, MA1-26196), α -SMA (1:100, Cell Signaling Technology, 19245), CD68 (1:100, Cell Signaling Technology, 97778), Ki67 (1:100, Cell Signaling Technology, 9129), then washed with PBST and incubated with the appropriate fluorescence-conjugated secondary antibodies (1:200, Thermo Fisher Scientific) for 1 hour. To validate antibody specificity, isotype controls were conducted with rabbit IgG (1:100, Cell Signaling technology, 3900) or mouse IgG (1:100, Abcam, ab280974) at the same concentrations as the primary antibodies. Secondary antibody only controls were performed to distinguish genuine target staining from background. The nuclei were marked with DAPI (Sigma-Aldrich,

28718-90-3). The images were obtained using a fluorescence microscope. TUNEL staining was performed with an In Situ Cell Death Detection Kit-Fluorescein (Roche, 11684795 910) according to the manufacturer's instructions. Sections were counterstained with DAPI. Images were acquired with an Olympus BX61 microscope and analyzed using ImageJ. We selected illustrative images that best represent the average results for each group as representative images from the entire series of experiments. Immunostaining staining was quantified by a researcher blinded to the genotype.

Primary bone marrow-derived macrophage (BMDM) culture

BMDMs were collected by flushing the tibia and femur of mice. Cleaned bones were cut, sterilized in 70% alcohol, and flushed with DMEM to collect bone marrow cells. The collected bone marrow suspension was washed with PBS and plated in DMEM supplemented with 10 ng ml⁻¹ M-CSF (Peprotech Inc, 315-02). After 5 days of culture the cells were detached from the dishes with TBS with 5 mM EDTA, resuspended in fresh DMEM for further study.

Western blot assay

Proteins were determined using the BCA protein assay kit (Thermo Scientific, 23225). The samples were loaded on 4-20% acrylamide/bis-acrylamide gels (MULTIGEL II mini 4/20, DCB-414879, CosmoBio), electrophoretically separated by SDS-PAGE and blotted onto PVDF transfer membranes (Millipore, IPVH07850), which were incubated with 5% nonfat milk in TBST (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.2% Tween 20) and immunoblotted at 4°C overnight with agitation following primary antibodies (1:1000 in 2% BSA buffer): anti-VEGF (Invitrogen, MA5-13182, MA1-16629), anti-FGFβ (Antibodies.com, A40635), anti-PDGF (Invitrogen, PA5-19524), anti-Ang 1 (Abcam, ab8451), anti-β-hydroxybutyrylation (PTM BIO, PTM-1201) anti-HIF-1α (Cell Signaling Technology, 4835), anti-PHD2 (Cell Signaling Technology, 4835), anti-HA (Cell Signaling Technology, 3724), anti-His (Invitrogen, MA1-21315), and anti-β-Actin (Cell Signaling Technology, 8457). Membranes were then rinsed three times for 10 minutes

each with TBST and incubated with HRP conjugated secondary antibodies (Cell Signaling Technology, USA) diluted 1:20000. After three times rinsing for 10 minutes with TBST, membranes were incubated with Chemiluminescence signals were detected by Image Lab software (Bio-Rad, CA, USA). Western blot images were selected to best represent the average results per group.

Coimmunoprecipitation (Co-IP)

Briefly, 300 µg of protein extracts was incubated with the indicated antibodies or nonspecific IgG at 4 °C overnight, and protein-A/G agarose (Thermo Scientific, 20421) was added for an additional 2-3 hours at 4 °C. The immunoprecipitates were pelleted by centrifugation at 5000 rpm for 4 minutes and washed 3 times with RIPA lysis buffer. The pellets were suspended in SDS gel loading buffer and subjected to western blot assays.

Quantitative real-time PCR assay

Total RNA was isolated using TRIzol reagent (Invitrogen, 15596026). For mRNA quantification, 2 µg of the RNA sample was reverse-transcribed using QuantiTect reverse transcription kit (Qiagen, 205311). Genomic DNA in RNA samples was eliminated using the gDNA Wipeout Buffer provided in the kit. The RNA sample was mixed with the gDNA wiping buffer and incubated for 2 minutes at 42°C, followed by incubation with the reverse-transcription master mix containing all components for cDNA synthesis for 15 minutes at 42°C. The PCR thermocycling condition was as follows: an initial denaturation at 95°C for 10 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension was performed at 72°C for 5 minutes, followed by an indefinite hold at 4°C. The primers used for RT-qPCR are listed in Table 2. The relative level of mRNA was calculated using the $\Delta\Delta C_q$ method with GAPDH as an internal control.

Chromatin immunoprecipitation (ChIP)

BMDMs were treated according to the procedure and then cross-linked with 1% formaldehyde for 10 min. The reaction was stopped by adding glycine to a final concentration of 125 mM. Then, cells were harvested and sonicated to generate DNA fragments of 0.25-1 kb. The lysates were centrifuged, and an aliquot of supernatant was saved as input DNA. Supernatants were then immunoprecipitated with the indicated antibodies or IgG as a negative control. Finally, DNA was purified using the QIAquick PCR purification kit. Purified DNA was analyzed by real-time PCR with specific primers for the VEGF-A promoter.

Flow cytometry analysis and sorting

Cardiac tissues were meticulously minced into 1 mm³ fragments and digested in a buffer containing collagenase (450 U/ml, Sigma–Aldrich, #C5138), hyaluronidase type I-S (60 U/ml Sigma–Aldrich, #H3506), and DNase (60 U/ml, Sigma–Aldrich, #AMPD1) at 37 °C for 1 hour. Following digestion, the cell suspensions derived from the heart were strained through a 40µm cell strainer and subsequently followed by flow cytometry analysis or macrophages sorting. For flow cytometry analysis, Cell suspensions were incubated with an array of antibodies for 30 minutes, including 7-AAD (BD Pharmingen, #559925), CD45-APC-Cy7 (BD Pharmingen, #557659), CD11b-FITC (BD Pharmingen, #557396), F4/80-BV421 (BD Pharmingen, #565411), DDR2-APC (SantaCruz, #sc-81707AF647), CD31-PE-Cy7 (BD Pharmingen, #561410), CCR2-BV786 (Biolegend, #150621), and FOLR2-APC (Biolegend, #153306). Flow cytometric analysis was conducted using the BD LSRFortessa™ Flow cytometer, with subsequent data analysis performed via FlowJo software. For macrophages isolation, the enzyme-digested cardiac cell suspensions were then labeled with antibodies targeting CD45, CD11b, and F4/80. The CD45+ CD11b+ F4/80+ cells, identified as macrophages, were sorted using the BD FACSAria II cell sorter under low pressure and collected. The purity of the sorted macrophages was evaluated by flow cytometry using 10,000-20,000 cells, while the remaining sorted cells were reserved for further study. For endothelial cells isolation, the enzyme-digested cardiac cell suspensions were labeled with antibodies targeting CD45 and

CD31. For fibroblasts isolation, the cell suspensions were labeled with CD45 and DDR2 antibodies. The CD45- CD31+ cells were identified as endothelial cells, while the CD45- DDR2+ cells were identified as fibroblasts. The subsequent isolation procedures for these cell types followed the same methodology as that employed for the isolation of macrophages.

Conditioned medium

After FACS sorting (BD FACSAria III), cardiac macrophages, endothelial cells and fibroblasts were seeded on a 48-well plate for 24 h. The conditioned medium of each cardiac cell population was collected and analyzed for the release of proangiogenic factors and its functional effects on endothelial cell activity.

Endothelial cell assays

Mouse primary endothelial cells (passages 5–10) cultured as previously⁵⁵, were grown in high-glucose DMEM. In transwell assays, ECs were added to the top wells of transwell-modified Boyden chambers of a 24-well transwell dish and then exposed to conditioned media derived from cultured cardiac macrophages post-MI in the lower chamber. Six hours later, the cells that migrated to the bottom of the membranes were stained with 0.1% crystal violet, photographed and counted. To study tube formation, 96-well plates were filled with 70 μ l Matrigel (Corning, #354230), and ECs were seeded on top of the gel. Subsequently, endothelial cells were incubated with conditioned media derived from cultured cardiac macrophages post-MI. After 12 h, the network structure images were obtained and quantified. When indicated, the supernatants were supplemented with a VEGF neutralizing antibody (R&D systems, #AF-493-NA) at a concentration of 10 ng/ml.

Measurement of ketones

Blood samples were taken from a tail vein for determination of ketone body (acetoacetate+ β -OHB) concentrations measured photometrically on an Olympus AU 400 analyzer (Beckman Coulter) using Autokit Total Ketone Bodies. Concentrations of β -OHB and AcAc in the heart were assessed as previously described. β -OHB was

determined by adding β -hydroxybutyrate dehydrogenase (Sigma, USA), NAD^+ and Tris buffer (pH 8.5) to the final supernatant; NADH was measured using a spectrophotometer. In a similar manner, the concentration of AcAc was measured by adding NADH, Tris buffer (pH 7.0) and the appropriate enzyme to the final supernatant and by measuring the decrease in fluorescence as the NADH was oxidized to NAD^+ .

In vitro β -hydroxybutyrylation assay

The following reactions were prepared in buffer (20 mM HEPES, pH 8.0; 1 mM DTT; 1 mM PMSF): 1 μg of PHD2 and 100 ng of CBP in 0.1 mM β -hydroxybutyrylation-CoA (β -OHB-CoA) or 1 μg of p53, 100 ng of CBP and 0.1 mM β -OHB-CoA. After incubation for 1 h at 30 °C, the reaction products were analyzed by western blot assays with antibodies target β -hydroxybutyrylation.

Mass spectrometry assay

Protein complexes were separated by SDS-PAGE and stained with silver stain reagent (Pierce, 24600). The visible band was cut and digested with trypsin and then subjected to liquid chromatography-mass spectrometry (LC-MS/MS) analysis.

Cellular thermal shift assay

BMDMs (10^6 cells) were harvested, and cell lysates were incubated with either vehicle or β -OHB. For every volume, 50 μl of the mixture was transiently heated to different temperatures ranging from 42 °C to 75 °C for 5 min using a TaKaRa PCR Thermal Cycler Dice® Gradient (TaKaRa), followed by cooling at room temperature for 5 min. After the heating step, the plate was centrifuged at 12,000g for 10 min at 4 °C to separate the soluble proteins from the aggregates, and the supernatant samples were analyzed by SDS-PAGE followed by western blotting.

SPR binding experiment.

The binding kinetics of β -OHB were assayed via SPR using a Biacore T200 instrument and manufacturer provided software (GE Healthcare, America). All measurements were performed at 25 °C. PHD2 were diluted and then covalently immobilized on CM5

chips. The proteins coupling solution was injected over the activated chip surface to achieve an immobilization level of 5,000 ~ 10,000 resonance units (RU). A blank surface was similarly treated but without any protein solution and used as a reference surface. β -OHB was assayed using single cycle kinetics mode as provided by the Biacore T200 control software. β -OHB was diluted in the running buffer (10 mM Hepes (pH 7.4), 150 mM NaCl and 0.005% surfactant P20) and injected in a series of increasing concentrations. Sensor grams were processed and analyzed using Biacore T200 evaluation software and the binding curves were fitted to determine the equilibrium dissociation constant (K_d).

Molecular docking.

Coordinates of the crystal structure of PHD2 (EGLN1) were downloaded from the AlphaFold 2 software. The structure of β -OHB molecule was generated with the Chem3D tool in Chemoffice software. The AutoDock program (version 4.2) was employed to generate an ensemble of docked conformations for the β -OHB molecule bound to PHD2. The objective docking method was utilized, in which a distinct large-sized cavity on the protein was chosen as the possible molecule binding area. We used the genetic algorithm for conformational search, and all the C-C bonds of β -OHB were set to be rotatable. The docking box was centered at the mass center of the binding area and was large enough to enclose the whole binding area. The protein structure was kept fixed during molecular docking.

In vitro hydroxylation assay

An in vitro hydroxylation assay was performed as previously described⁵⁶. Briefly, the resin-bound bacterially expressed oxygen-dependent degradation domain of HIF1 α (aa 401-603) or its P564A mutant was mixed with the indicated cell lysate in NETN buffer. After mild agitation, the reaction mixtures were centrifuged and washed. The final resin-bound proteins were dissolved in SDS buffer followed by western blotting with the indicated antibodies.

Statistical analysis

All data are expressed as the mean \pm SD, and statistical analysis was performed with Prism Software. Normality of data distribution was assessed by the Shapiro–Wilk test prior to the application of parametric tests. For non-normally distributed data, nonparametric tests were used to analyze statistical differences. Data with a sample size ≥ 6 that passed the normality test were analyzed by parametric tests. For nonnormally distributed data or when the sample size was <6 , data were analyzed using nonparametric tests. For comparisons between two groups, significance was determined using Student's t-test or nonparametric Mann–Whitney test. For comparisons among multiple groups, ANOVA followed by post hoc Bonferroni test or nonparametric Kruskal–Wallis test followed by the Dunn's post hoc test. An F test (two groups) or Brown–Forsythe test (multiple groups) was used to determine difference in variances for t-test and ANOVA, respectively. Survival curves were assessed with the Kaplan-Meier method and compared by log-rank tests. The experimenters were blinded to animal genotype and grouping information and all data were derived from biological replicates as indicated. The number of samples is determined based on previous publications, estimated with pilot experiments to achieve a power of 0.8 and a type 1 error of 5% for these studies. Exact *P* values of statistically significant changes are indicated in each figure. Values of $P < 0.05$ were considered statistically significant.

Figures S1-S18

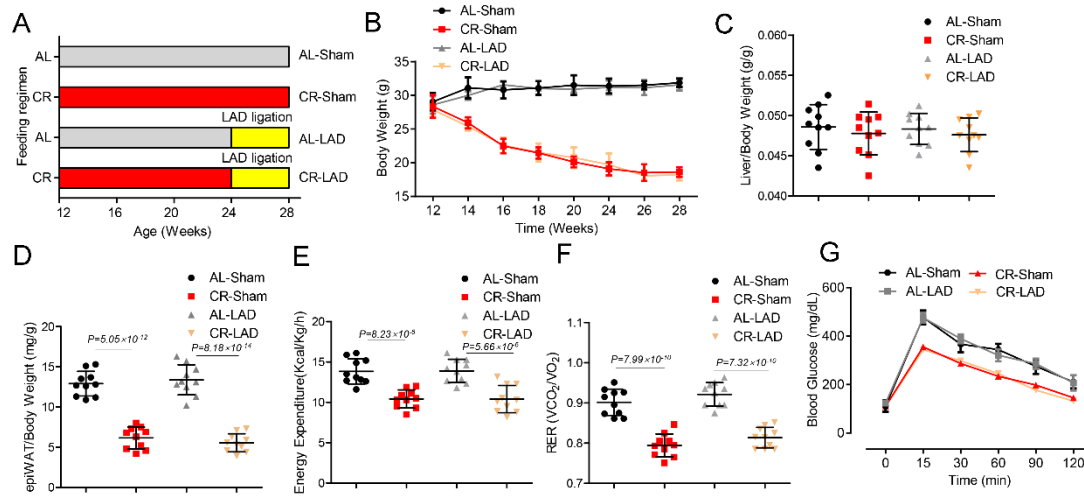


Figure S1. Systemic metabolic indices of mice after CR and LAD artery ligation. **(A)** Schematic outlines of the feeding and LAD artery ligation regimens for the four groups. **(B)** Body weight curve. **(C and D)** Liver weight-to-body weight ratio (C) and eWAT weight-to-body weight ratio (D) of mice after CR and LAD artery ligation. (n=10) **(E and F)** EE (E) and RER (F) as measured by indirect calorimetry. (n=10) **(G)** Blood glucose levels during the IPGTT (2 g/kg). (n=10) Data depicted in **B** and **G** were analyzed via repeated measures 2-way ANOVA. Data presented in **C, D, E** and **F** were analyzed via 2-way ANOVA followed by the Bonferroni post hoc test.

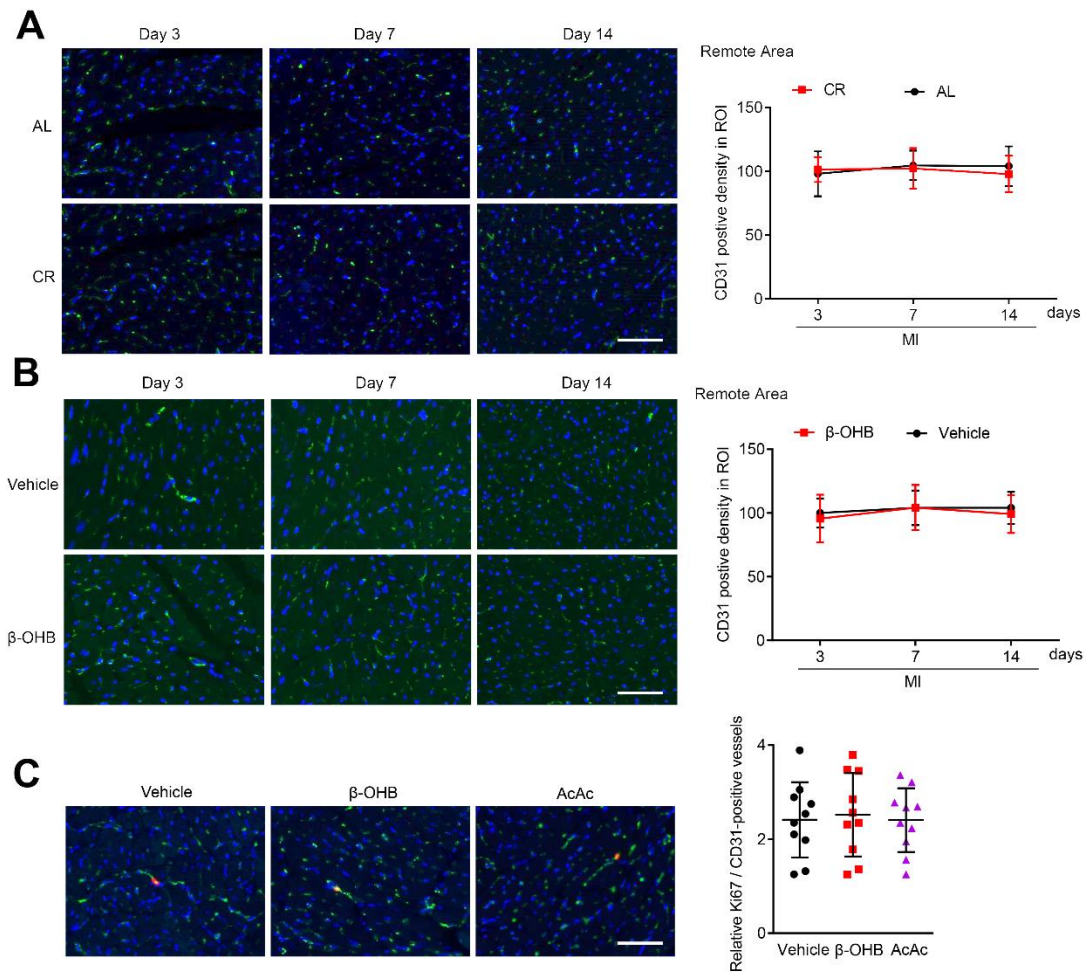


Figure S2. (A) Representative CD31 staining of the heart tissue sections obtained from AL and CR mice in remote area at Day 3, 7 and 14 post-MI. (n=10) (B) Representative CD31 staining of the heart tissue sections obtained from Veh- and β -OHB- treated mice in remote area at Day 3, 7 and 14 post-MI. (n=10) (C) Immunofluorescence of the Ki67(Red) /CD31 (Green)-positive cells in the heart from Veh-, β -OHB- and AcAc- treated mice prior of MI. (n=10) scale bars 20 μ m. Data presented in **A** and **B** were analyzed via 2-way ANOVA followed by the Bonferroni post hoc test. Data in **C** were analyzed by one-way ANOVA followed by the Bonferroni post hoc test.

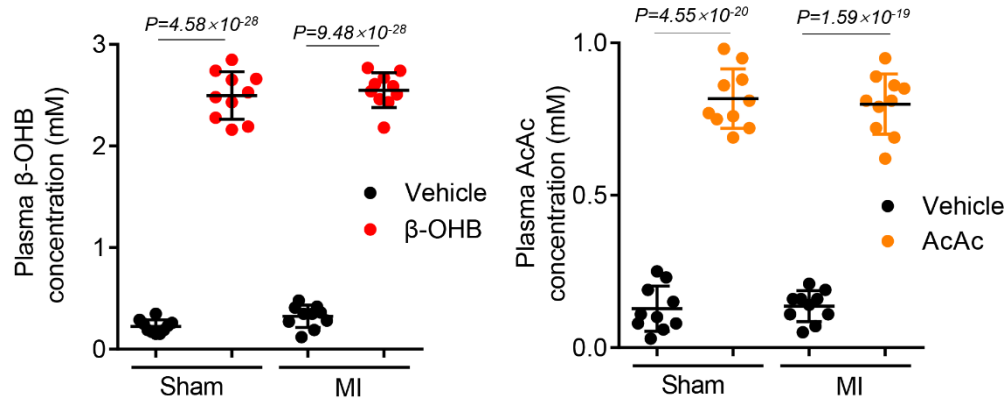


Figure S3. Blood ketone levels in Veh-, β-OHB- and AcAc-treated mice at Day 28 after MI or sham operation. (n=10) Data were analyzed by 2-way ANOVA followed by the Bonferroni post hoc test.

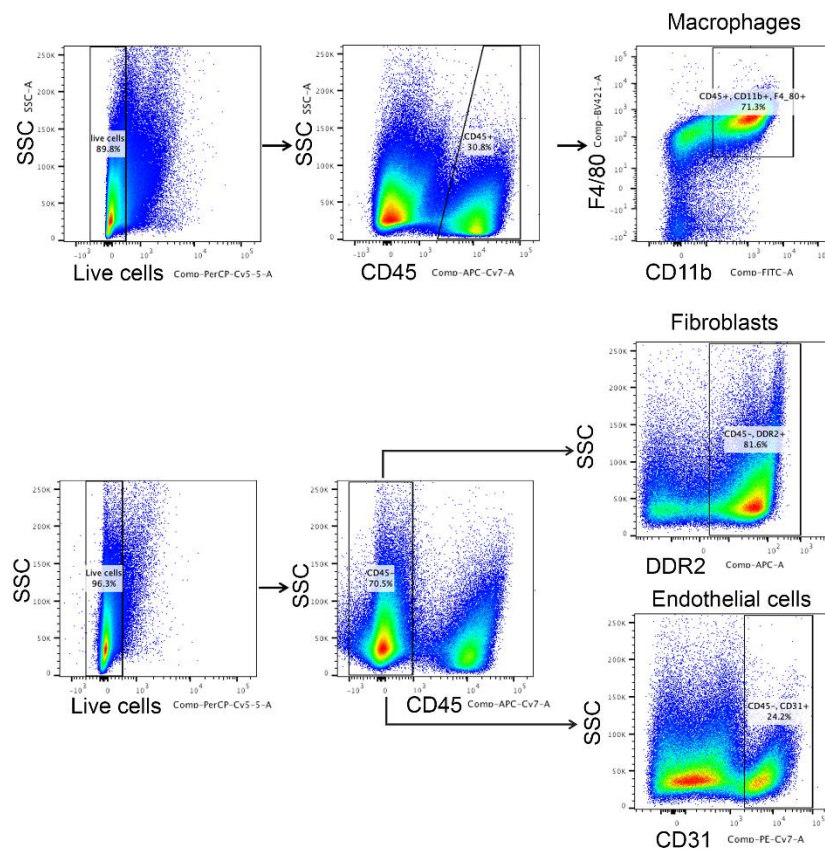


Figure S4. Gating strategy to sort cardiac macrophages, fibroblasts and endothelial cells from heart homogenates. Macrophages were identified as CD45+ CD11b+ F4/80+; fibroblasts as CD45- DDR2+ and endothelial cells as CD45- CD31+.

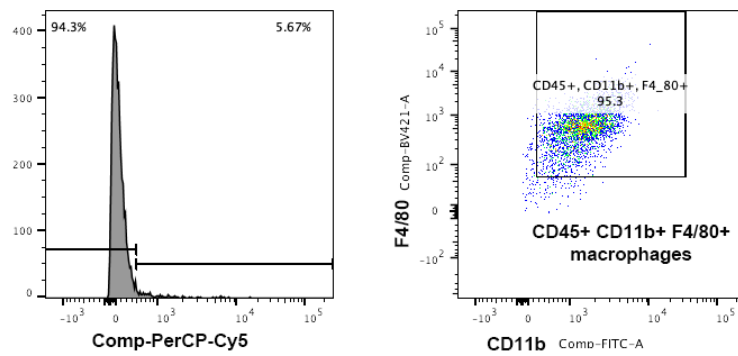


Figure S5. Representative flow cytometric analysis of the viability and purity of isolated cardiac macrophages from heart homogenates of scar areas. Macrophages were identified as CD45+ CD11b+ F4/80+.

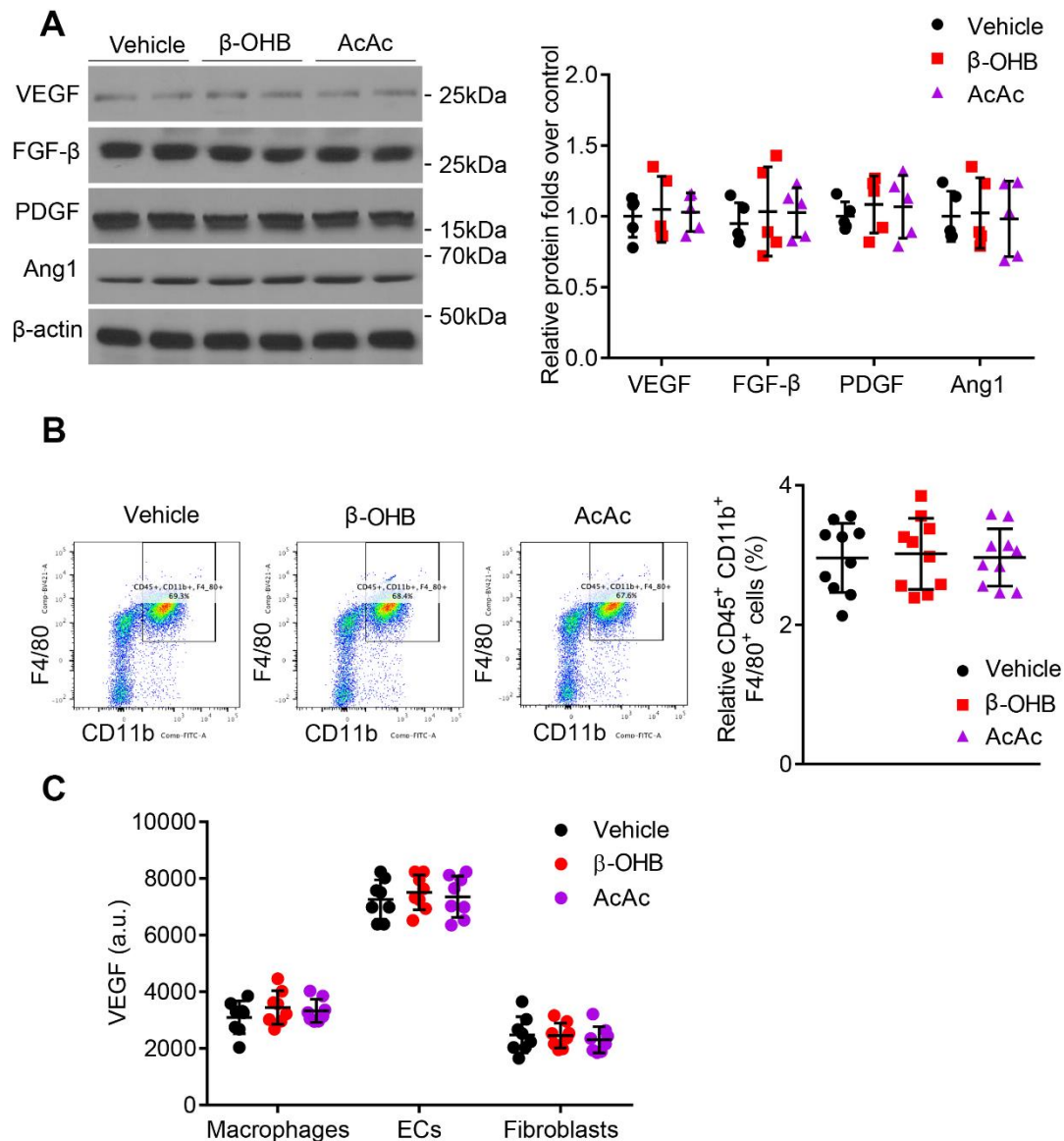


Figure S6. (A) VEGF-A, FGF β , PDGF and Ang1 protein expression in heart tissue isolated from Veh-, β -OHB- and AcAc-treated mice. (n=5) **(B)** Representative flow cytometric analysis of macrophage numbers on gated CD45⁺ CD11b⁺ F4/80⁺ macrophages in the heart from Veh, β -OHB- and AcAc-treated mice. (n=10) **(C)** Assessment of VEGF-A released in medium derived from cardiac macrophages, endothelial cells and fibroblasts that were sorted using fluorescence-activated cell sorting from hearts of Veh, β -OHB- and AcAc-treated mice. (n=8) Data presented in **A** was analyzed by Kruskal-Wallis test followed by a Dunn's multiple comparison tests. Data presented in **B** and **C** were analyzed by one-way ANOVA followed by the Bonferroni post hoc test.

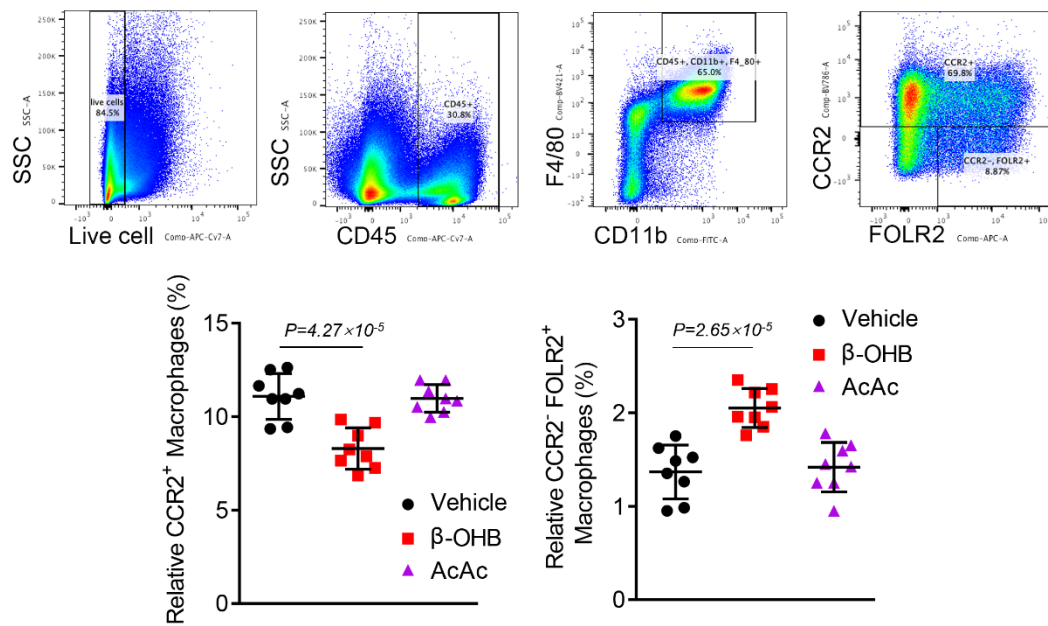


Figure S7. Representative flow cytometric analysis of number of infiltrating (CCR2+) and resident (CCR2-, FOLR2+) macrophages in the heart from Veh-, and β -OHB-treated mice at Day 7 post-MI. (n=8) Data were analyzed by one-way ANOVA followed by the Bonferroni post hoc test.

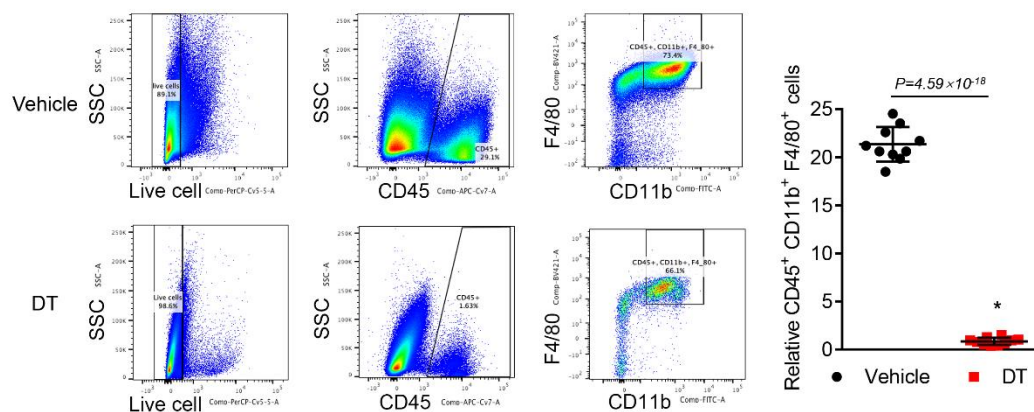


Figure S8. Representative flow cytometric plots of macrophages following the administration of DT to Cx3cr1^{Cre}×R26^{DTR} mice. (n=10) Data were analyzed by Student t test.

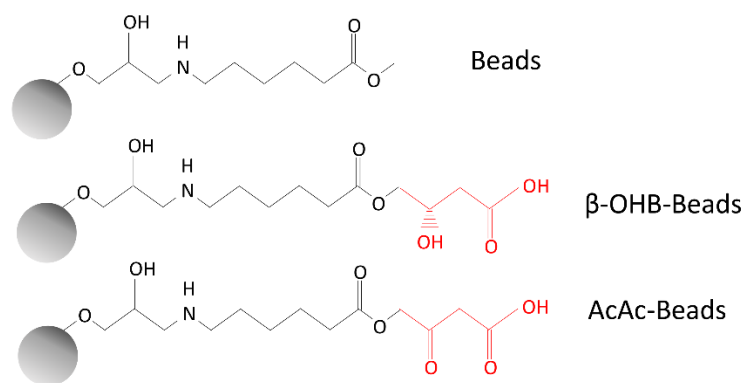
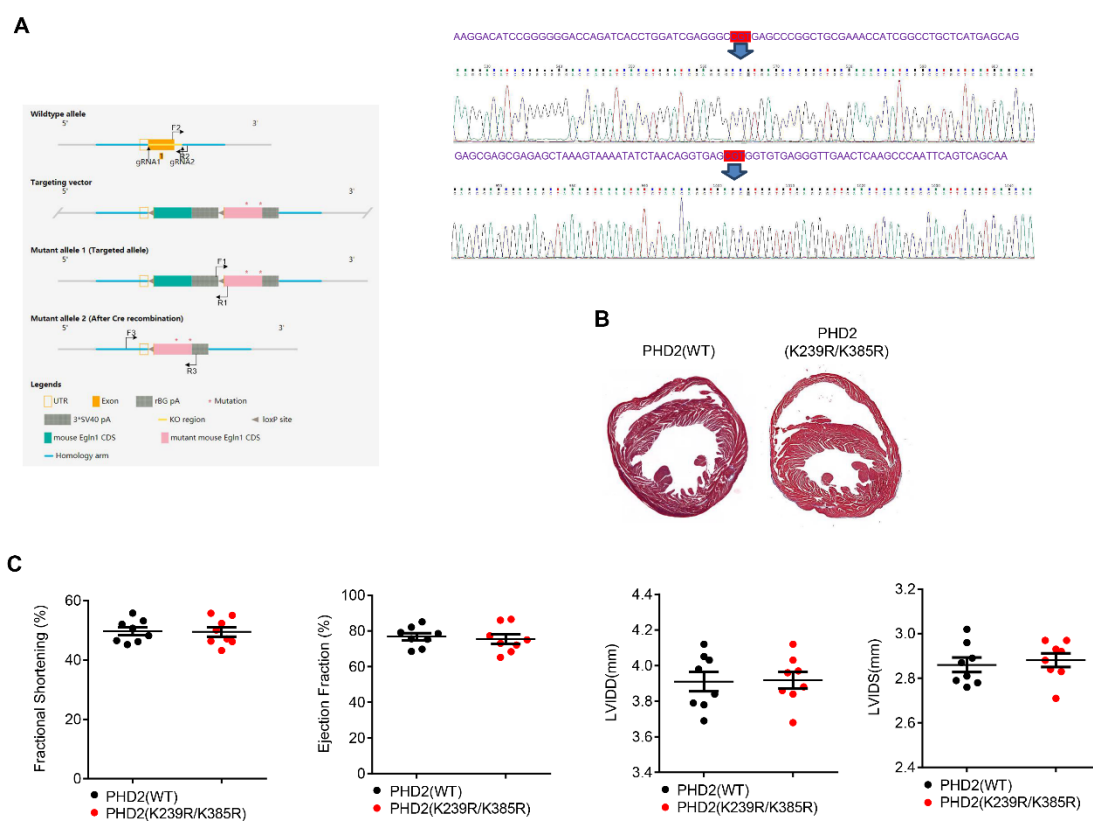


Figure S9. Structures of negative control beads (Beads), β-OHB and AcAc-conjugated Sepharose beads (β-OHB-Beads and AcAc-Beads).



FigureS10. Generation of PHD2 K239R and K385R knock-in mice. (A) Genotyping strategy and confirmation by sequencing. (B) Representative histomorphology of cardiac tissue and (C) echocardiographic analysis of FS, EF, LVIDD and LVIDS were obtained from PHD2 K239R and K385R knock-in mice. (n=8) Data were analyzed by Student *t* test.

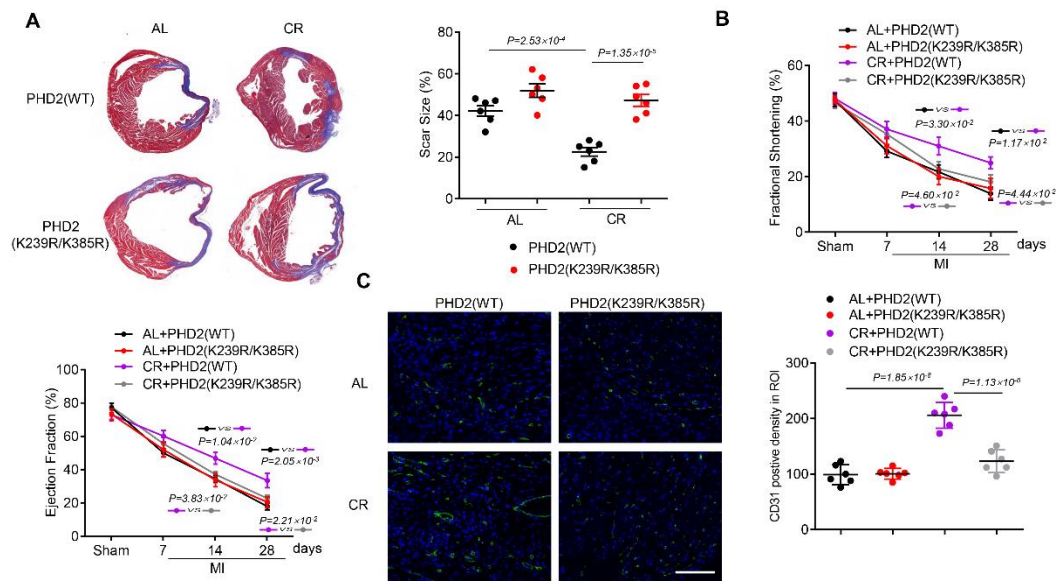


Figure S11. PHD2 Lys239 and Lys385 β -hydroxybutyrylation mediates the protective effect of CR on heart function after MI. (A) Representative Masson's trichrome staining of cardiac tissues obtained from WT or PHD2 K239R/K385R knock-in mice after MI (left). Quantitative analysis of scar size (right) and wall thickness (bottom) at Day 28 post-MI in the indicated mice. (B) Echocardiographic analysis of FS and EF at Days 0, 7, 14 and 28 after MI. (C) Immunohistochemical analyses of CD31 in the heart tissue at Day 28 post-MI. Nuclei were stained with DAPI (blue). (n=6) scale bar= 20 μ m. Data depicted in **B** were analyzed via repeated measures 2-way ANOVA. Data in **A** and **C** were analyzed by 2-way ANOVA followed by the Bonferroni post hoc test.

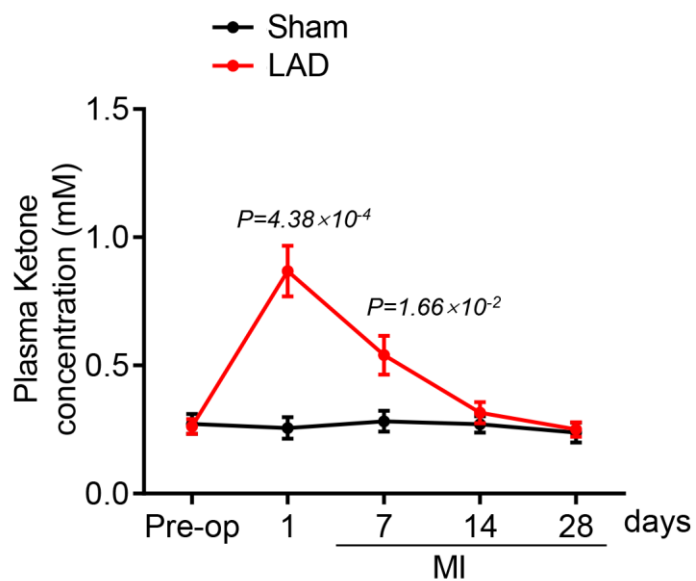


Figure S12. Blood ketone levels in Sham and LAD-treated mice at Days 0, 7, 14 and 28. (n=6) Data were analyzed by repeated measures 2-way ANOVA.

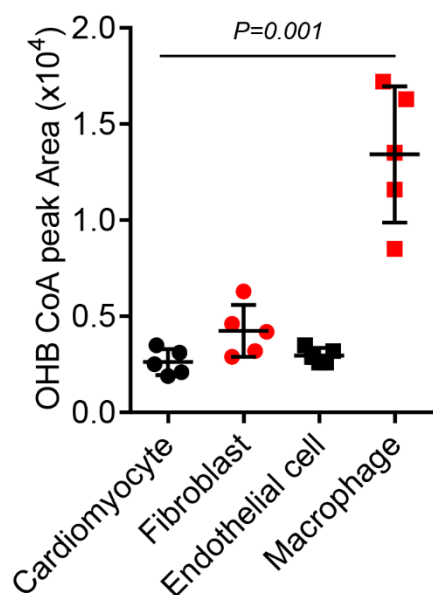


Figure S13. The levels of β -hydroxybutyryl-CoA in macrophages, cardiomyocytes, endothelial cells or fibroblasts from mice were analyzed using LC-MS. (n=5) Data were analyzed by Kruskal-Wallis test followed by a Dunn's multiple comparison tests.

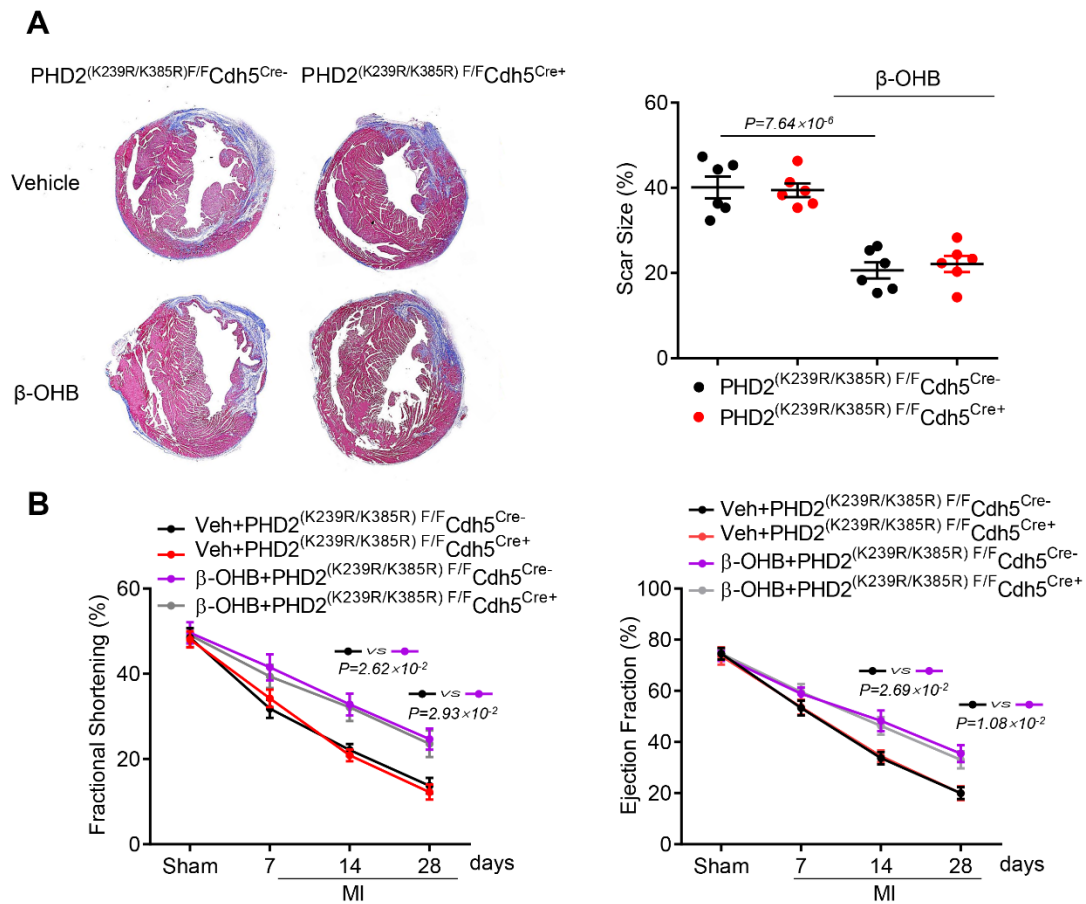


Figure S14. (A) Representative Masson's trichrome staining of cardiac tissue obtained from endothelial PHD2 K239R/K385R knock-in mice at Day 28 after MI. (n=6) Quantitative analysis of scar size at Day 28 post-MI in the indicated mice. Data were analyzed by 2-way ANOVA followed by the Bonferroni post hoc test. (B) Echocardiographic analysis of FS and EF at Days 0, 7, 14 and 28 after MI or sham operation. Data presented in **A** were analyzed by 2-way ANOVA followed by the Bonferroni post hoc test. Data depicted in **B** were analyzed via repeated measures 2-way ANOVA.

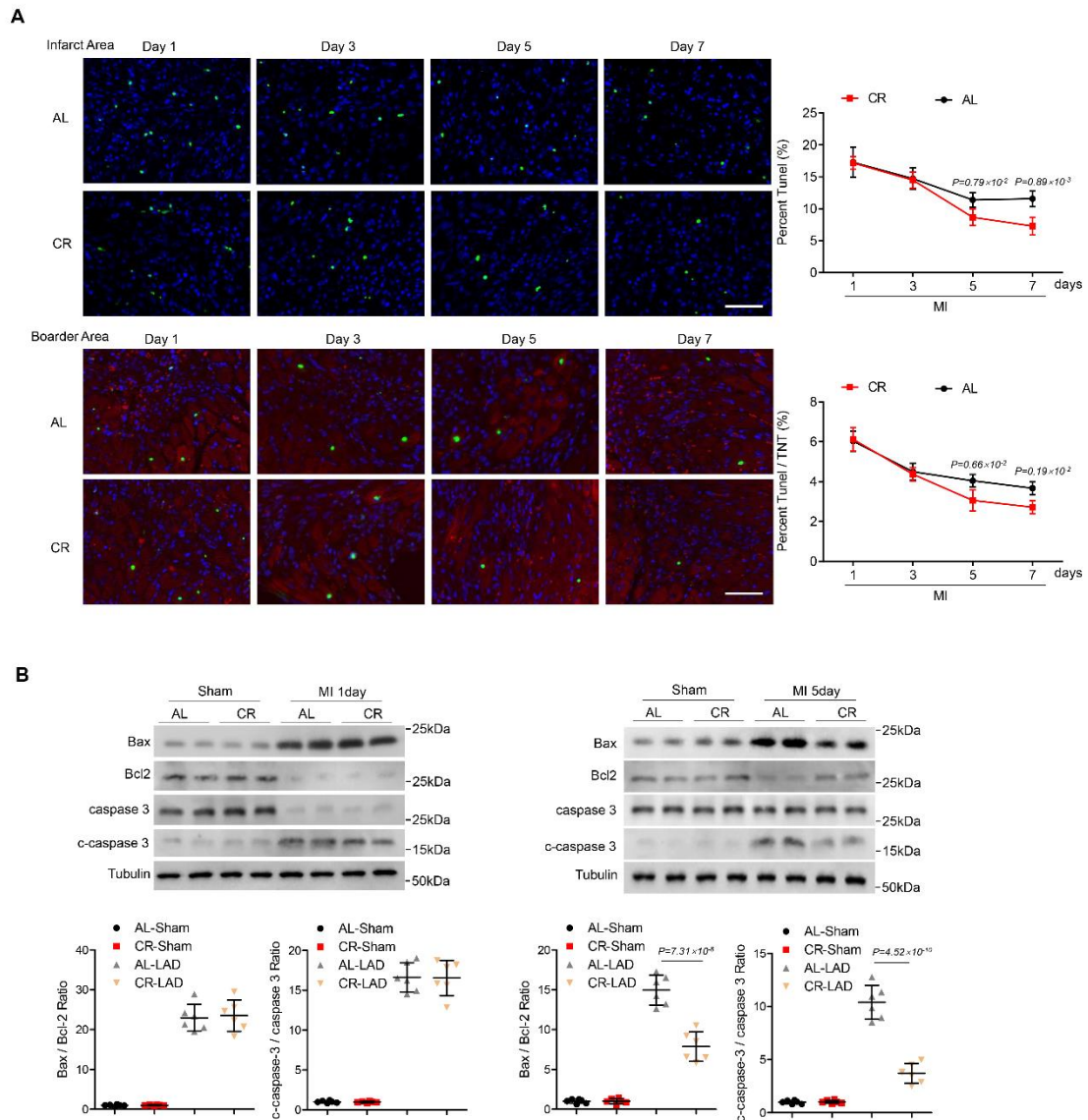


Figure S15. (A) Representative TUNEL staining of the heart tissue sections obtained from AL and CR mice in infarct and boarder area at Day 1, 3, 5 and 7 post-MI. (n=6) scale bar= 20 μ m. (B) Bax, Bcl-2, c-caspase-3 and caspase-3 protein expression at Day 1 and 5 post-MI in peri-infarct tissues isolated from AL and CR mice. (n=6) Data presented in **A** were analyzed by repeated measures 2-way ANOVA. Data in **B** were analyzed by 2-way ANOVA followed by the Bonferroni post hoc test.

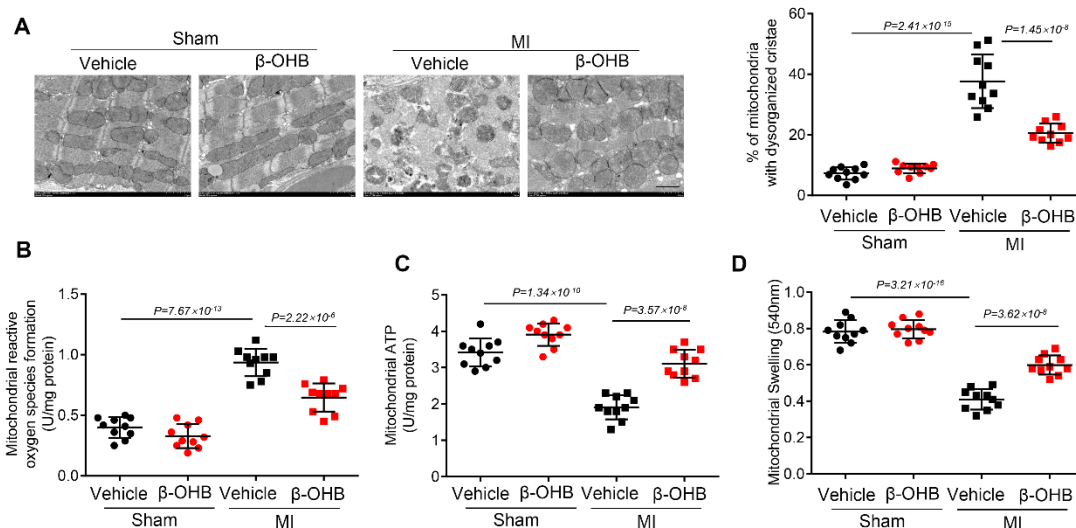


Figure S16. Mitochondrial morphology observed using transmission electron microscopy in the Veh- and β -OHB- treated mice at Day 7 post-MI (A) Scale bar = $1\mu\text{m}$. Graphs showed the mitochondrial formation of reactive oxygen species (B) and ATP (C), mitochondrial swelling (D). (n=10) Data were analyzed by 2-way ANOVA followed by the Bonferroni post hoc test.

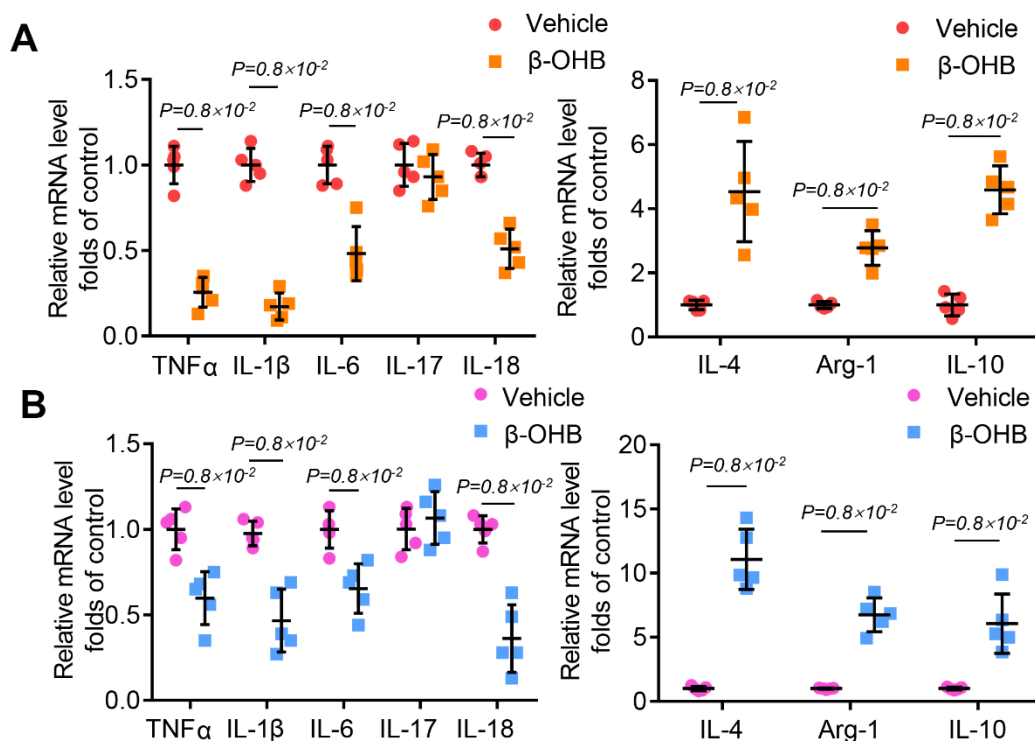


Figure S17. (A) The mRNA level of TNF α , IL-1 β , IL-6, IL-17, IL-18, IL-4, Arg-1 and IL-10 in peri-infarct tissues isolated from Veh- and β -OHB- treated mice post-MI. (B) The

mRNA level of TNF α , IL-1 β , IL-6, IL-17, IL-18, IL-4, Arg-1 and IL-10 in cardiac macrophages under Veh- and β -OHB supply. (n=5) Data were analyzed by Mann-Whitney U test.

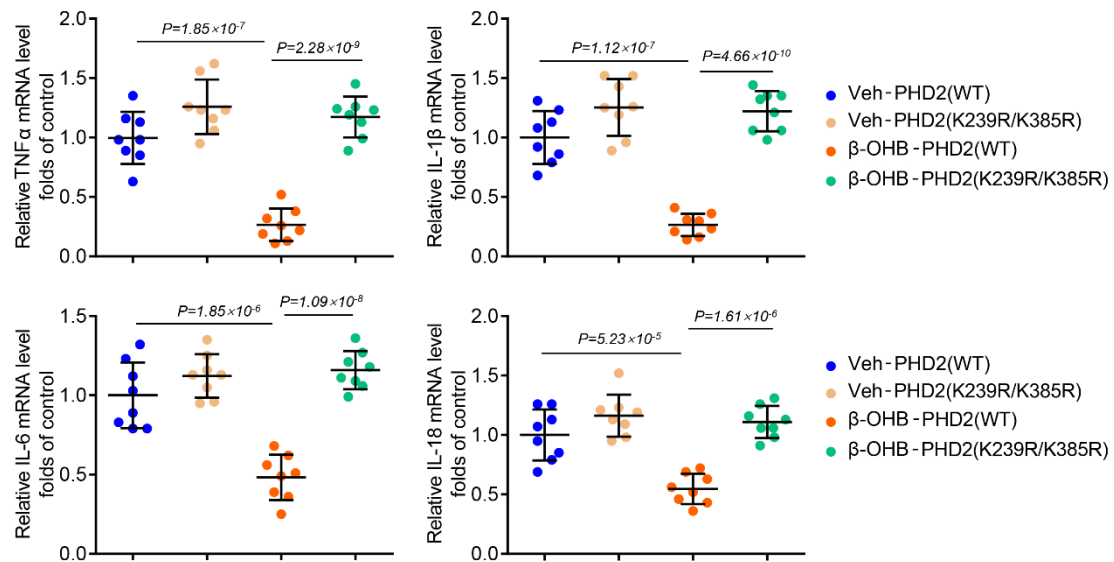


Figure S18. The mRNA level of TNF α , IL-1 β , IL-6, and IL-18 in scar tissue isolated from Veh- and β -OHB- treated WT or PHD2 K239R/K385R knock-in mice post-MI. (n=8) Data were analyzed by 2-way ANOVA followed by the Bonferroni post hoc test.

Table S1. β -OHB-bound proteins by LC-MS/MS

Accession	Protein Name	Gene	Length
Q00PI9	HNRL2_MOUSE	HNRNPUL2	745
Q7TMK9	HNRPQ_MOUSE	SYNCRIP	623
Q91YE3	EGLN1_MOUSE	EGLN1	400
Q6P1F6	2ABA_MOUSE	PPP2R2A	447
Q6R891	NEB2_MOUSE	PPP1R9B	817
Q8K4G5	ABLM1_MOUSE	ABLIM1	861
O54962	BAF_MOUSE	BANF1	89
Q99104	MYO5A_MOUSE	MYO5A	1853
Q9CZU3	MTREX_MOUSE	MTREX	1040
D3YXK2	SAFB1_MOUSE	SAFB	937
Q69ZX8	ABLM3_MOUSE	ABLIM3	682
Q62191	RO52_MOUSE	TRIM21	470
P62806	H4_MOUSE	H4C1	103
P97315	CSRP1_MOUSE	CSRP1	193
Q60668	HNRPD_MOUSE	HNRNPD	355
Q9D8Y0	EFHD2_MOUSE	EFHD2	240
Q9D1R9	RL34_MOUSE	RPL34	117
P11103	PARP1_MOUSE	PARP1	1013
Q9Z204	HNRPC_MOUSE	HNRNPC	313
Q5F2E7	NUFP2_MOUSE	NUFIP2	692
Q62167	DDX3X_MOUSE	DDX3X	662
P62717	RL18A_MOUSE	RPL18A	176
P16858	G3P_MOUSE	GAPDH	333
P21107	TPM3_MOUSE	TPM3	285
P52480	KPYM_MOUSE	PKM	531
Q99LC3	NDUAA_MOUSE	NDUFA10	355

Table S2. Sequence of primers used for RT-PCR studies

Gene	Forward primer	Reverse primer	Amplicon sizes (bp)
TNF α	GGTGCCTATGTCTCAGCCTCT T	GCCATAGAAGCTGATGAGAGG GAG	139
IL-1 β	TGGACCTTCCAGGATGAGGAC A	GTTTCATCTCGGAGCCTGTAGT G	146
IL-6	TACCACTTCACAAGTCGGAGG C	CTGCAAGTGCATCATCGTTGT TC	116
IL-17	CAGACTACCTCAACCGTTCCA C	TCCAGCTTTCCCTCCGCATTG A	130
IL-18	GACAGCCTGTGTTTCGAGGATA TG	TGTTCTTACAGGAGAGGGTAG AC	159
IL-4	ATCATCGGCATTTTGAACGAG GTC	ACCTTGGAAGCCCTACAGAC GA	125
Arg-1	CATTGGCTTGCGAGACGTAGA C	GCTGAAGGTCTCTTCCATCAC C	124
IL-10	CGGGAAGACAATAACTGCACC C	CGGTTAGCAGTATGTTGTCCA GC	130
GAPDH	CATCACTGCCACCCAGAAGAC TG	ATGCCAGTGAGCTTCCCGTTC AG	153