



# Endothelial $\beta$ 1 Integrin-Mediated Adaptation to Myocardial Ischemia

Carina Henning<sup>1,\*</sup> Anna Branopolski<sup>1,2,\*</sup> Paula Follert<sup>1</sup> Oksana Lewandowska<sup>1</sup> Aysel Ayhan<sup>2</sup>  
Marcel Benkhoff<sup>2</sup> Ulrich Flögel<sup>3</sup> Malte Kelm<sup>2</sup> Christian Heiss<sup>2,4,5,\*</sup> Eckhard Lammert<sup>1,6,7,\*</sup>

<sup>1</sup>Institute of Metabolic Physiology, Department of Biology, Heinrich-Heine-University, Düsseldorf, Germany

<sup>2</sup>Division of Cardiology, Pulmonology, and Vascular Medicine, Heinrich-Heine-University, Düsseldorf, Germany

<sup>3</sup>Institute for Molecular Cardiology, University Hospital Düsseldorf, Heinrich-Heine-University, Düsseldorf, Germany

<sup>4</sup>Department of Clinical and Experimental Medicine, Faculty of Health and Medical Sciences, University of Surrey, Guildford, United Kingdom

<sup>5</sup>Surrey and Sussex Healthcare NHS Trust, Redhill, Surrey, United Kingdom

<sup>6</sup>Institute for Vascular and Islet Cell Biology, German Diabetes Center (DDZ)—Leibniz Center for Diabetes Research, Düsseldorf, Germany

<sup>7</sup>German Center for Diabetes Research (DZD e.V.), Neuherberg, Germany

**Address for correspondence** Dr. rer. nat. Eckhard Lammert, Institute of Metabolic Physiology, Heinrich-Heine-University Düsseldorf, Universitätsstr. 1, 40225 Düsseldorf, Germany (e-mail: lammert@hhu.de).

Dr. med. Christian Heiss, Department of Clinical and Experimental Medicine, Faculty of Health and Medical Sciences, University of Surrey, Stag Hill, Guildford U2 7XH, United Kingdom (e-mail: c.heiss@surrey.ac.uk).

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## Abstract

**Background** Short episodes of myocardial ischemia can protect from myocardial infarction. However, the role of endothelial  $\beta$ 1 integrin in these cardioprotective ischemic events is largely unknown.

**Objective** In this study we investigated whether endothelial  $\beta$ 1 integrin is required for cardiac adaptation to ischemia and protection from myocardial infarction.

**Methods** Here we introduced transient and permanent left anterior descending artery (LAD) occlusions in mice. We inhibited  $\beta$ 1 integrin by intravenous injection of function-blocking antibodies and tamoxifen-induced endothelial cell (EC)-specific deletion of *Itgb1*. Furthermore, human *ITGB1* was silenced in primary human coronary artery ECs using small interfering RNA. We analyzed the numbers of proliferating ECs and arterioles by immunohistochemistry, determined infarct size by magnetic resonance imaging (MRI) and triphenyl tetrazolium chloride staining, and analyzed cardiac function by MRI and echocardiography.

**Results** Transient LAD occlusions were found to increase EC proliferation and arteriole formation in the entire myocardium. These effects required  $\beta$ 1 integrin on ECs, except for arteriole formation in the ischemic part of the myocardium. Furthermore, this integrin subunit was also relevant for basal and mechanically induced proliferation of human coronary artery ECs. Notably,  $\beta$ 1 integrin was needed for cardioprotection induced by transient LAD occlusions, and the absence of endothelial  $\beta$ 1 integrin resulted in impaired growth of blood vessels into the infarcted myocardium and reduced cardiac function after permanent LAD occlusion.

**Conclusion** We showed that endothelial  $\beta$ 1 integrin is required for adaptation of the heart to cardiac ischemia and protection from myocardial infarction.

## Keywords

- ▶ endothelial  $\beta$ 1 integrin
- ▶ cardiac vascular growth
- ▶ myocardial infarction
- ▶ cardioprotection

\* These authors contributed equally to this work.

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## Introduction

Coronary artery disease (CAD) is the leading cause of death around the globe.<sup>1</sup> Due to diminished blood supply of the heart, it can result in myocardial infarction (MI), heart failure, and death. Current prevention strategies aim to reduce risk factors that negatively affect the cardiovascular system, such as hyperglycemia or diabetes mellitus, hyperlipidemia, smoking, hypertension, physical inactivity, and unhealthy dietary patterns.<sup>2,3</sup> Notably, cardiac vascular growth was also observed as an important factor to protect from MI or to improve its outcome. Hereby, functional growth of coronary vessels creates additional and alternative routes to provide the ischemic myocardium with fresh blood, thus reducing MI size or preserving cardiac function after MI.<sup>4–7</sup>

Hypoxic and mechanical stimuli are thought to be involved in vascular growth.<sup>8</sup> Hypoxia activates hypoxia-inducible factor-1 $\alpha$  to induce signals, such as vascular endothelial growth factor-A (VEGF-A) that preferentially triggers vascular growth in a paracrine manner.<sup>9,10</sup> In contrast, mechanical stimuli directly activate mechanosensitive proteins, including integrins, in cells of the blood vessels.<sup>11–14</sup> There, they stimulate vascular growth by cross-activating VEGF receptors,<sup>10,15,16</sup> endothelial cell (EC) reorientation,<sup>17</sup> and trigger the release of angiocrine signals needed for tissue growth and survival.<sup>18</sup>

The mechanosensitive  $\beta 1$  integrin is part of several integrin receptors, acts as an essential “bridge” between the basement membrane (BM) or extracellular matrix and cytoskeleton of ECs, and—along with some of its mechanosensitive  $\alpha$  subunits—is required for embryonic and postnatal vascular development.<sup>19–23</sup> The  $\beta 1$  integrins are of particular interest, since they bind collagen type I, which is involved in vascular growth and left ventricular (LV) remodeling and dysfunction.<sup>24</sup> They also influence fibrosis and wound repair,<sup>25</sup> and to date the  $\beta 1$  integrin has been targeted among others in cardiomyocytes, myofibroblasts, and pericytes,<sup>26–29</sup> but not in the adult cardiac endothelium, to uncover its function in ischemia-induced protection of the heart from MI and preservation of cardiac function.

Here, we studied the hypothesis that endothelial  $\beta 1$  integrin is relevant for the ischemia-induced coronary vascular growth and cardiac adaptation to transient and chronic ischemia. Therefore, transient left anterior descending artery (LAD) occlusions were performed to mimic temporary ischemia observed in patients with CAD and discussed as a trigger for coronary growth.<sup>4</sup> Besides, short periods of ischemia induce ischemic preconditioning, which involves multiple different signaling pathways, including those activated by endothelial nitric oxide synthase (eNOS).<sup>30–33</sup> Additionally, a permanent LAD occlusion was performed to mimic the situation in patients with MI, but without receiving proper reperfusion.<sup>34</sup> To this end, both models were combined with pharmacologic and genetic manipulation of  $\beta 1$  integrin and analyses using different imaging techniques were performed. We first asked whether transient occlusions of the LAD induced EC proliferation and vascular growth only in the ischemic myocardium or also in the non-ischemic, right ventricular (RV) and septal myocardium. We then studied

by infusion of  $\beta 1$  integrin function-blocking antibodies and induction of EC-specific genetic deletion of *Itgb1* whether  $\beta 1$  integrin on the endothelium is required for cardiac vascular growth induced by transient LAD occlusion and preservation of heart function. Further, we wished to find out whether  $\beta 1$  integrin and eNOS interact with each other and if  $\beta 1$  integrin is needed for basal and mechanically induced proliferation of human coronary artery ECs (HCAECs) as a human correlate of the experiments on the adult mouse heart. Finally, we studied whether endothelial  $\beta 1$  integrin had a general function to protect the heart and preserve cardiac function upon MI, independent of short episodes of ischemia.

Our study uncovers an important role of endothelial  $\beta 1$  integrin in cardiac adaptation to LV ischemia as well as preservation of cardiac function after an MI.

## Methods

### Mice

For wild-type studies with antibody treatment, 10- to 15-week-old male C57BL/6J mice from Janvier were used.  $\beta 1$  integrin blocking antibody (BD Bioscience, 555002) and control antibody (BD Bioscience, 553957) were intravenously injected (1 mg/mL, 100  $\mu$ L) 1 day before each 15-minute ischemia and before and after permanent occlusion. For genetic deletion experiments, global *NOS3* (*eNOS* KO) mice<sup>35</sup> were used. For endothelial *Itgb1* deletion (*Itgb1*<sup>IECKO</sup>), *Cdh5-Cre*<sup>ERT2</sup>; homozygous *Itgb1-loxP* mice<sup>36,37</sup> were crossed with homozygous *Itgb1-loxP* mice. As controls for *eNOS* KO mice, the corresponding C57BL/6J mice from Jackson Laboratory were used. As controls for *Itgb1*<sup>IECKO</sup> mice, *Cdh5-Cre*<sup>ERT2</sup> mice were used, which received the same tamoxifen injections. Both mouse lines were treated with 100  $\mu$ L tamoxifen (75 mg/kg body-weight) for 5 consecutive days. All experiments were performed according to the German animal protection laws (Animal Ethics Committee of the Landesamt für Natur, Umwelt und Verbraucherschutz, North Rhine-Westphalia).

### Myocardial Ischemia

For the induction of transient LAD occlusions, a closed-chest model was used, as previously described.<sup>4</sup> Briefly, in an initial operation an occluder was implanted for temporary occlusion of the LAD, therefore a 7–0 prolene suture (Ethicon, Johnson and Johnson) was placed around the proximal LAD, ends were threaded through a polyethylene tube, the thorax was closed, and the suture ends were placed into a subcutaneous pocket. For permanent LAD occlusion, mice were treated the same way but the LAD was closed with a 7–0 silk suture (Seraflex, Serag-Wiessner) and myocardial ischemia was induced directly. In general, 7 days after the initial operation, the first ischemia for transient LAD occlusions was induced. However, during the proliferation analysis in blocking AB-treated mice, and when Repl/R and MI were combined for magnetic resonance imaging (MRI), the first ischemia was induced 2 days after the initial operation, so as to shorten the experimental procedure. Mice were anesthetized in an inhalation anesthetic set with oxygen-enriched air (O<sub>2</sub> 40%) and isoflurane (3% vol., Piramal

Healthcare). After anesthesia, the mice were placed in a supine position on a 37°C preheated surgery table, followed by respiration with oxygen-enriched air (O<sub>2</sub> 40%) and isoflurane (2% vol., Piramal Healthcare). After skin disinfection, electrocardiogram (ECG) electrodes were fixed to the fore- and hindfeet and the skin was reopened to pull out the suture ends. Afterwards, 15-minute or 60-minute ischemia was induced by sticking the suture ends to two magnets and setting them under tension to ligate the LAD. Induction of ischemia was controlled in ECG by ST-segment elevation followed by reperfusion of the LAD. Suture ends were placed back into the subcutaneous pocket or cut off after the last ischemia. The 15-minute ischemia was repeated three times every other day. Additional 60-minute ischemia was followed only if the infarct size (IS) and cardiac function were quantified. All mice received an occluder implantation and afterward were randomized to sham or ischemia/reperfusion (I/R) procedure. Mice were intraperitoneally (IP) injected with ketamine (100 mg/kg bodyweight, Ketanest S., Pfizer Pharma GmbH) and xylazine (10 mg/kg bodyweight, Rompun TM, Bayer Healthcare) for anesthesia and treated with Temgesic (0.05–0.1 mg/kg bodyweight) for analgesia.

### Cardiac Function

For analysis of IS and cardiac function, <sup>1</sup>H MRI measurements were performed at 400.13 MHz using a Bruker AVANCE III 9.4 T Wide Bore NMR spectrometer (Bruker) driven by ParaVision 5.1, as described previously.<sup>38</sup> In the case of late gadolinium enhancement (LGE) measurements, mice received an IP injection of 0.2 mmol Gd-DTPA per kg bodyweight immediately prior to the measurement. To calculate the LV ejection fraction (EF), defined as the difference between end-diastolic volume (EDV) and end-systolic volume (ESV) divided by EDV, the ventricular borders were selected manually by means of the ParaVision region-of-interest tool (Bruker). For quantification of IS, LGE-positive areas were selected manually and related to the LV volume. Transthoracic echocardiography was also used to analyze LV cardiac function, as previously described.<sup>39</sup> Therefore, measurements were performed with a MS-400 scan head (Vevo 2100, VisualSonics, FUJIFILM). EDV, ESV, and EF were calculated from long-axis B mode in the Vevo laboratory software 1.7.1 (VisualSonics). Only mice that survived until the end of the experiment were used for analysis.

### Osmotic Pump Implantation

Osmotic pumps (Alzet, model 1002) were filled with 20 mg/mL 5-bromo-2'-deoxyuridine (BrdU, Sigma) in phosphate buffer saline (PBS) and were implanted IP during the first 15-minute ischemia. The pumps remained in the mice until heart isolation.

### Pimonidazole Treatment

To determine hypoxic regions in the myocardium during ischemia, mice were treated with pimonidazole (Hypoxyprobe Green Kit, Hypoxyprobe, Inc., Burlington, Massachu-

setts, United States). More specifically, after anesthesia, pimonidazole (60 mg/kg bodyweight) dissolved in PBS was injected IP and the LAD was occluded as described before, without reperfusion. After ischemia induction, hearts were isolated, perfused with ice-cold PBS, deep-frozen in nitrogen, and embedded in Tissue-Tek O.C.T. (Sakura Finetek GmbH) for cryosectioning.

### Immunohistochemistry

Heart isolation was performed 7 days after the last 15-minute ischemia, except for the proliferation analysis in AB-treated mice, which was 2 days after the last 15-minute ischemia. For proliferation analysis, sections were pretreated with 2 M HCl for 30 minutes at 37°C and neutralized with 0.1 M sodium tetraborate, followed by primary and secondary antibody incubation. The following primary antibodies were used in this study: rat anti-BrdU (Abcam, ab6326), goat anti-PECAM-1 (R&D Systems, AF3628), mouse anti- $\alpha$ SMA-Cy3 (Sigma, C6198), mouse anti- $\alpha$ SMA (Sigma, A5228), and rat anti- $\beta$ 1 integrin (Millipore, MAB1997), and incubated overnight at 4°C. Secondary antibodies conjugated with fluorophores AF488 (Invitrogen Molecular probes), Cy3 (Jackson ImmunoResearch), and Cy5 (Jackson ImmunoResearch) were incubated in the dark for 1 or 3 hours at room temperature. Cell nuclei were stained with DAPI (Sigma). For hypoxia analysis, sections were stained with fluorophore-coupled antibodies for HP-FITC-MAb (detects pimonidazole adducts) and mouse anti- $\alpha$ SMA-Cy3 (Sigma, C6198) overnight at 4°C. Cell nuclei were stained with DAPI (Sigma).

Hearts with considerable tissue damage and areas of cryosections containing part of the occlusion system were excluded from quantification.

### Triphenyl Tetrazolium Chloride Staining

The triphenyl tetrazolium chloride (TTC) staining was performed to determine the IS and area at risk (AAR), after 60-minute I/R. After anesthesia, hearts were isolated, cannulated over the aorta and washed with NaCl solution to remove remaining blood clots. Afterwards, the LAD was permanently closed with a silk suture and the hearts were perfused with 1% Evans blue solution (in NaCl). Hearts were frozen at –20°C for approximately 1 hour, transversally cut, transferred into TTC solution containing 8 parts 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, 2 parts 0.1 M NaH<sub>2</sub>PO<sub>4</sub> (pH 7.4), and 10 mg/mL TTC and incubated at 37°C for 5 to 10 minutes under shaking. Images were taken with a stereomicroscope (Leica MZ6 with Leica KL 1500 LCD), and infarcted area and AAR in the LV were analyzed with the software DISKUS (Vers. 4.30.7, Carl H. Hilgers, DISKUS mikroskopische Diskussion).

### Microfil Perfusion

To image the entire coronary vasculature, hearts were washed and retrograde-perfused via the thoracic aorta with Microfil compound (Flow Tech Inc.), previously described but modified.<sup>40</sup> For optical clearing, hearts were incubated in methyl salicylate (Sigma Aldrich) and afterward imaged under a stereomicroscope (Leica MZ6 with Leica KL 1500 LCD).

### Primary Human Cell Culture, Transfection, and Mechanical Stretching

Primary HCAECs (male, 21 years old), purchased from PELO-Biotech, were cultured in a humidified atmosphere at 5% CO<sub>2</sub> and 37 °C. HCAECs were grown in a microvascular EC growth medium kit enhanced (PELOBiotech), plated on stretch chambers (STEX) or on 12-well plates, and used up to passage 6. To selectively knock down the expression of  $\beta 1$  integrin, HCAECs were transfected with one of the following *ITGB1*-siRNAs ( $\beta 1$ -siRNA) (Invitrogen) via electroporation (4D-Nucleofector System, LONZA) and incubated for 48 hours:

$\beta 1$ -siRNA1: 5'-CCUAAGUCAGCAGUAGGAACAUUUAU-3'  
 $\beta 1$ -siRNA2: 5'-UGCGAGUGUGGUGUCUGUAAGUGUA-3'  
 $\beta 1$ -siRNA3: 5'-GGGAGCCACAGACAUUUAUUAUAAA-3'

As control, a non-targeting small interfering RNA (siRNA; Invitrogen) with a similar GC content was used. Additionally, HCAECs were mechanically stretched for 30 minutes in the case of proliferation studies and for 15 minutes to analyze  $\beta 1$  integrin activation by means of a manual cell-stretching system (STREX, STB-10). Knockdown efficiencies were analyzed by quantitative real-time polymerase chain reaction (PCR) and Western blotting.

### In Vitro Proliferation Analysis and $\beta 1$ Integrin Activation

For analysis of in vitro proliferation, 10  $\mu$ M BrdU (Sigma) was added to the transfected HCAECs and incubated for 2 hours. In the case of stretching experiments, BrdU was added at the beginning of the stretching. After pretreatment of fixed cells with 2 M HCl and subsequent neutralization with 0.1 M sodium tetraborate, immunofluorescence staining was performed using mouse anti-BrdU antibody (BD Bioscience, 555627) as primary and anti-mouse antibody conjugated with AF488 (Invitrogen Molecular probes) as secondary antibodies. For  $\beta 1$  integrin activation analysis, HCAECs were fixed in 4% PFA after mechanical stretching, and the immunostaining was performed using mouse anti-human activated  $\beta 1$  integrin antibody (Merck Millipore, MAB2079Z) as primary and anti-mouse antibody conjugated with Cy5 (Jackson ImmunoResearch) as secondary antibodies.

### Microscopy and Analysis

All immunofluorescence stainings were imaged using a confocal laser scanning microscope (LSM 710 or LSM 880, Zeiss). Proliferating ECs and arterioles were counted manually and related to the myocardial area in mm<sup>2</sup>. Proliferating HCAECs were quantified either by manual or a semiautomated counting method in FIJI (ImageJ, NIH). The myocardial area was determined applying the freehand selection tool in FIJI, area or intensity of PECAM-1, DAPI or  $\beta 1$  integrin in heart sections and in HCAECs after mechanical stretching was analyzed with FIJI as well.

### Magnetic-Activated Cell Sorting of ECs

For collection of cardiac ECs from *Itgb1*<sup>IECKO</sup> and control mice, hearts were isolated and dissociated with a gentleMACS dissociator (Miltenyi Biotec). All further steps were performed

according to the customer protocol (Miltenyi Biotec; "Preparation of single-cell suspensions from mouse heart," "CD45 MicroBeads," and "CD31 MicroBeads"). EC pellets were used either for quantitative real-time PCR and therefore homogenized with peqGold TriFast (Peqlab) or lysed in RIPA buffer for Western blot analysis.

### Quantitative Real-Time PCR

Total RNA from cultivated HCAECs was extracted with an RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. Total RNA isolation from magnetic-activated cell sorting (MACS)-sorted murine ECs was performed by using the phenol/chloroform extraction. Quantitative real-time PCR was performed with Brilliant III Ultra-Fast SYBR Green QPCR Master Mix and the thermal cycler Stratagene Mx3000P (Agilent Technologies). All samples were analyzed in triplicates. The following primers were used:

human *ITGB1* forward: 5'-CATCTGCGAGTGTGGTGTCT-3'  
 human *ITGB1* reverse: 5'-GGGGTAATTTGTCGGACTT-3'  
 human *RPLP0* forward: 5'-GCAGCATCTACAACCCTGAAG-3'  
 human *RPLP0* reverse: 5'-CACTGGCAACATTGCGGAC-3'  
 human *HPRT* forward: 5'-TGACACTGGCAAAACAATGCA-3'  
 human *HPRT* reverse: 5'-GGTCCTTTTACCAGCAAGCT-3'  
 human *B2M* forward: 5'-TTTCATCCATCCGACATTGA-3'  
 human *B2M* reverse: 5'-CCTCCATGATGCTGCTTACA-3'  
 mouse *Itgb1* forward: 5'-AATGCCAAGTGGGACACGGG-3'  
 mouse *Itgb1* reverse: 5'-TGACTAAGATGCTGCTGCTGTGAGC-3'  
 mouse *Rplp0* forward: 5'-GATGCCCAGGGAAGACAG-3'  
 mouse *Rplp0* reverse: 5'-ACAATGAAGCATTTGGATAATCA-3'  
 mouse *Hprt*: forward: 5'-CACAGGACTAGAACACCTGC-3'  
 mouse *Hprt* reverse: 5'-GCTGGTGAAAAGGACCTCT-3'  
 mouse  $\beta 2m$  forward: 5'-GAGCCCAAGACCGTCTACTG-3'  
 mouse  $\beta 2m$  reverse: 5'-GCTATTTCTTTCTCGTGCAT-3'

The efficiencies of  $\beta 1$  integrin knockout (in vivo) or knockdown (in vitro) were determined by means of the  $\Delta\Delta CT$  method, where *RPLP0/Rplp0*, *HPRT/Hprt*, and *B2M/ $\beta 2m$*  were used as housekeeping genes for normalization.

### Western Blotting

Cultivated human or MACS-sorted murine ECs were lysed in RIPA buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% IGEPAL, 0.25% Na-deoxycholate, 1 mM EDTA) containing protease inhibitors (cOmplete Protease Inhibitor Cocktail Tablets, Roche) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail, Roche). Total protein concentrations were determined by means of the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein were used for the SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). For protein separation, 4 to 15% SDS-gels (Bio-Rad) and Mini-PROTEAN Tetra Cell (Bio-Rad) were used. Western blotting was performed by using the Trans-Blot Turbo Transfer System (Bio-Rad) and rabbit anti-GAPDH (Abcam, ab9485), rat anti- $\beta 1$  integrin (Millipore, MAB1997) for MACS-sorted murine ECs or goat anti- $\beta 1$  integrin (Santa Cruz, sc-6622) for human ECs as primary antibodies and HRP-conjugated donkey anti-rabbit (Cell Signaling Technologies, 7074), donkey anti-rat (Jackson ImmunoResearch, 712-035-150) and donkey anti-goat (Jackson

ImmunoResearch, 705–035–147) as secondary antibodies. To determine the efficiencies of  $\beta 1$  integrin-knockout (in vivo) or knockdown (in vitro), a semiquantitative band density analysis was performed (FIJI, ImageJ, NIH), where GAPDH was used as a housekeeping protein for normalization.

### Statistical Analysis

Statistical significance was determined by using Excel (Microsoft) or Prism (GraphPad Inc.). To compare two groups, an unpaired two-tailed Student's *t*-test was used, whereas for multiple comparisons a one-way analysis of variance with subsequent Dunnett's posthoc test was performed. To compare survival rates, in a Kaplan–Meier curve a log-rank (Mantel–Cox) test was used. *p*-Values less or equal to 0.05 are shown either in the figures or mentioned in the figure legend. All values are shown as means  $\pm$  standard error of the mean.

## Results

### Transient Episodes of LV Cardiac Ischemia Lead to $\beta 1$ Integrin-Dependent Cardiac EC Proliferation and Arteriole Formation in the Entire Myocardium

We used a closed-chest mouse model to introduce transient LAD occlusions, leading to repetitive episodes of ischemia followed by reperfusion (Repl/R) in the LV myocardium ( $\blacktriangleright$ Fig. 1), similar to a previously published mouse model.<sup>4</sup> The procedure employed an occluder to block the blood flow through the LAD three times every other day for 15 minutes each ( $\blacktriangleright$ Fig. 1A–G). The LAD occlusions resulted in a transient transmural ischemia. This was indicated by ST-segment elevation on ECG that resolved when the LAD was opened again ( $\blacktriangleright$ Fig. 1D). Hypoxia staining revealed that only the LV (“ischemic myocardium”), but not the RV and septum (“non-ischemic myocardium”), turned hypoxic during the LAD occlusions ( $\blacktriangleright$ Supplementary Fig. S1A–D, available in the online version and  $\blacktriangleright$ Fig. 1E, F). As a control (RepSham), an occluder was implanted around the LAD, and the surgery was performed three times as described above ( $\blacktriangleright$ Fig. 1A–D), but without occluding the LAD.

First, we studied the proliferation of cardiac ECs in response to the repeated LAD occlusions and the role of  $\beta 1$  integrin in this proliferative response by intravenously injected  $\beta 1$  integrin blocking antibodies ( $\beta 1$ -B-AB) versus isotype-matched control antibodies (ctrl-AB) into mice ( $\blacktriangleright$ Fig. 1H–O). When mice were treated with ctrl-AB, Repl/R increased cardiac EC proliferation in both the ischemic ( $\blacktriangleright$ Fig. 1R) and non-ischemic myocardia ( $\blacktriangleright$ Fig. 1H–K, P). In contrast,  $\beta 1$ -B-AB abolished the Repl/R-induced cardiac EC proliferation in both, the ischemic and non-ischemic myocardia ( $\blacktriangleright$ Fig. 1L–O, Q, S).

Next, we counted the number of arterioles in the myocardium of the hearts undergoing either Repl/R or RepSham treatments ( $\blacktriangleright$ Fig. 2). Notably, the arterioles increased in number, both in the ischemic and non-ischemic myocardia at the level of papillary muscles upon Repl/R ( $\blacktriangleright$ Fig. 2A–D, I, K). In contrast, no increase in the number of arterioles was observed upon Repl/R when mice were treated with  $\beta 1$ -B-AB ( $\blacktriangleright$ Fig. 2E–H, J, L).

### Transient Cardiac Ischemia Protects from Myocardial Infarction and Subsequent Cardiac Dysfunction via $\beta 1$ Integrin

We then investigated whether the Repl/R treatment reduced the IS of an MI induced by an additional 60-minute ischemia ( $\blacktriangleright$ Fig. 3A). Consistent with previous results,<sup>4</sup> transient cardiac ischemia reduced the IS as compared with the RepSham treatment, analyzed in MRI and TTC staining ( $\blacktriangleright$ Fig. 3B–E, J;  $\blacktriangleright$ Supplementary Fig. S2A–E; and  $\blacktriangleright$ Video 1, available in the online version). Repl/R also improved cardiac function after MI, as determined by preserved LV EF ( $\blacktriangleright$ Fig. 3L).

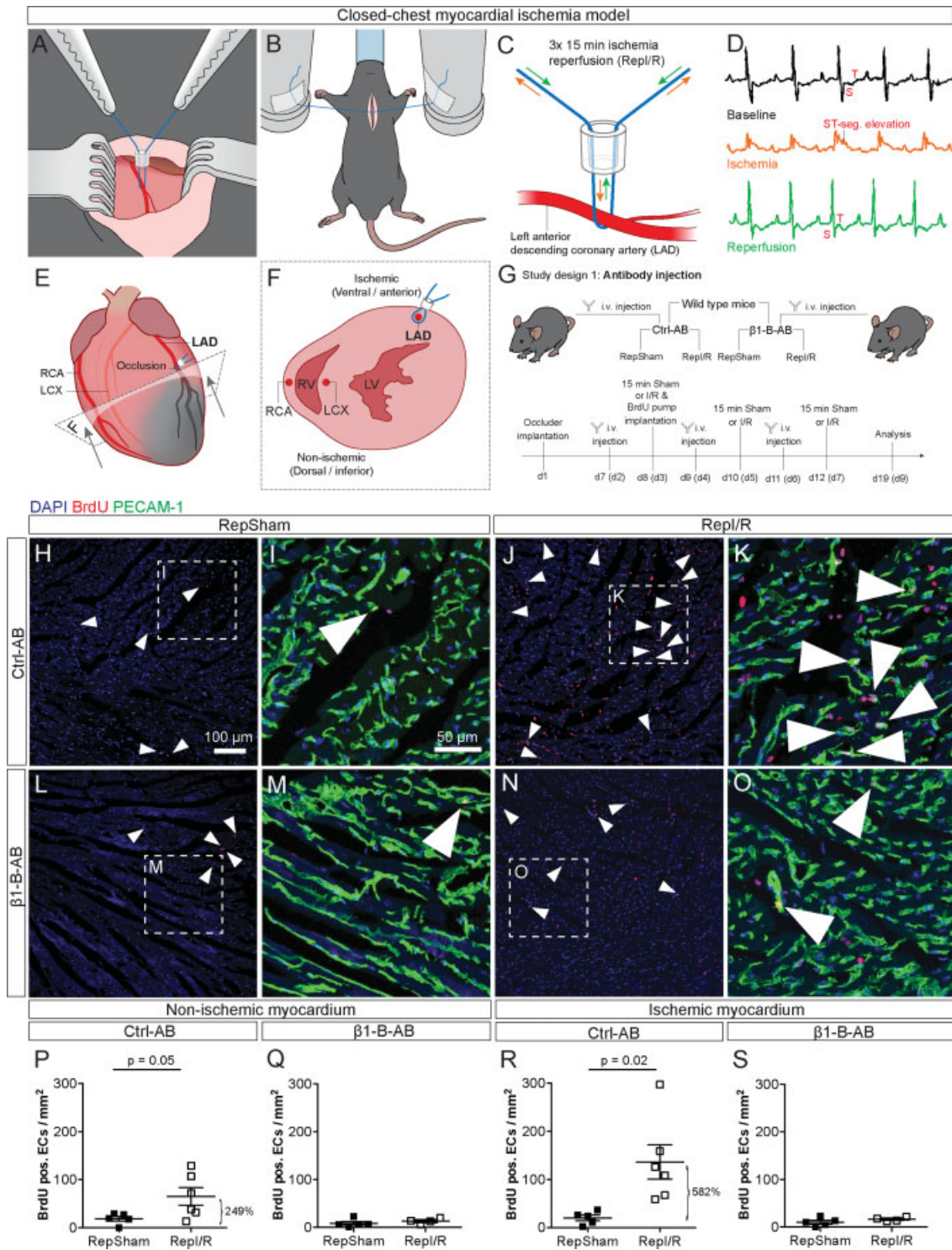
#### Video 1

Comparison of myocardial function upon Repl/R treatment and myocardial infarction (MI) in adult mice. MRI-based visualization of myocardial function in adult mice. Mice received either a ctrl-AB (*top-left* and *top-right panels*) or a  $\beta 1$ -B-AB (*bottom-left* and *bottom-right panels*) before RepSham or Repl/R treatments followed by MI (60-minute ischemia) with reperfusion. MRI, magnetic resonance imaging. Online content including video sequences viewable at: <https://www.thieme-connect.com/products/ejournals/html/10.1055/s-0040-1721505>.

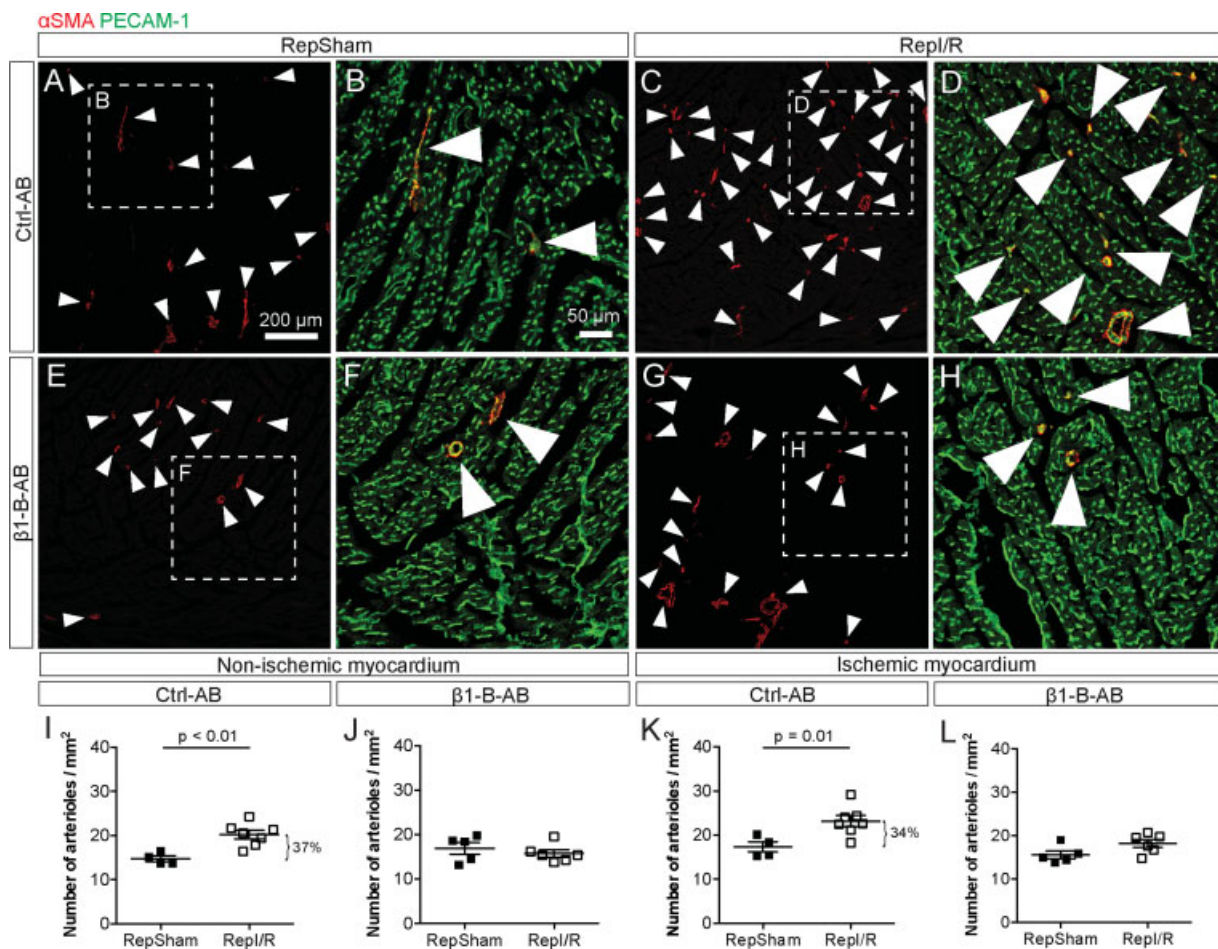
Next, we analyzed the role of  $\beta 1$  integrin function in myocardial protection from MI. When mice were treated with  $\beta 1$ -B-AB before induction of an MI ( $\blacktriangleright$ Fig. 3A), transient cardiac ischemia no longer reduced the IS to a significant extent and no longer protected the heart from infarct-related LV dysfunction ( $\blacktriangleright$ Fig. 3F–I, K, M;  $\blacktriangleright$ Supplementary Fig. S2F–J; and  $\blacktriangleright$ Video 1, available in the online version).

### Requirement of Endothelial $\beta 1$ Integrin for Cardiac EC Proliferation and Arteriole Formation in the Myocardium

To investigate whether  $\beta 1$  integrin in the endothelium was required for Repl/R-induced cardiac EC proliferation, we selectively deleted the *Itgb1* gene in the ECs of adult mice (*Itgb1*<sup>IECKO</sup>) using tamoxifen-inducible *Cdh5-Cre*<sup>ERT2</sup> expression ( $\blacktriangleright$ Fig. 4). These adult mice expressed *Cre* recombinase only in the endothelium, but not in myeloid cells,<sup>36</sup> and were compared with tamoxifen-injected *Cre* control mice ( $\blacktriangleright$ Fig. 4A). An up to 70% knockdown efficiency of  $\beta 1$  integrin in ECs sorted from tamoxifen-treated mouse hearts was observed on both mRNA and protein levels ( $\blacktriangleright$ Supplementary Fig. S3; for uncropped Western blots, see  $\blacktriangleright$ Supplementary Fig. S6, available in the online version). In the controls, transient ischemia induced a twofold increase in cardiac EC proliferation in the non-ischemic myocardium as well as a three- to fourfold increase in EC proliferation in the ischemic myocardium ( $\blacktriangleright$ Fig. 4B–E, J, L). In contrast, tamoxifen-induced EC-selective gene depletion of  $\beta 1$  integrin strongly reduced the proliferative response of cardiac EC to the short periods of cardiac ischemia ( $\blacktriangleright$ Fig. 4F–I, K, M).



**Fig. 1** Transient episodes of left ventricular (LV) cardiac ischemia require  $\beta 1$  integrin to induce cardiac EC proliferation in the entire myocardium. (A–C) Illustrations of (A) an implanted occluder at the LAD to introduce (B, C)  $3 \times 15$ -minute myocardial ischemia treatment with reperfusion (Repl/R) in a closed-chest mouse model. (D) ECG recording before (baseline; black line), during (ischemia; orange line), and after the LAD occlusion (reperfusion; green line). (E) Illustration of an adult mouse heart, including the LAD with an occluder installed. The location of cross-section through the mouse heart (as illustrated in panel F and used for immunohistochemical analyses) is also indicated. (F) Schematic illustration of a cross-section through the ischemic and non-ischemic myocardium. (G) Study design to inhibit  $\beta 1$  integrin by antibody injection. A shortened experimental protocol is shown in brackets. (H–O) Representative immunofluorescence images of sections through the non-ischemic myocardium; mice received either (H–K) ctrl-AB or (L–O)  $\beta 1$ -B-AB before each 15-minute ischemia or sham treatment. Sections were stained for BrdU (red), platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31; green), and DAPI (blue). (P–S) Quantification of BrdU-positive ECs per mm<sup>2</sup> in (P, Q) the non-ischemic and (R, S) the ischemic myocardium upon ctrl-AB or  $\beta 1$ -B-AB treatment. Reported values are means  $\pm$  SEM. Ctrl-AB with RepSham:  $n = 5$ ; ctrl-AB with Repl/R:  $n = 6$ ;  $\beta 1$ -B-AB with RepSham:  $n = 5$ ;  $\beta 1$ -B-AB with Repl/R:  $n = 4$ . Statistical significance was determined using unpaired two-tailed Student’s *t*-test. EC, endothelial cell; LAD, left anterior descending artery; LCX, left circumflex artery; RCA, right coronary artery; SEM, standard error of mean.



**Fig. 2** Transient episodes of cardiac ischemia require  $\beta 1$  integrin to induce arteriole formation in the entire myocardium. (A–H) Representative immunofluorescence images of sections through the non-ischemic myocardium of adult mouse hearts; mice received either (A–D) ctrl-AB or (E–H)  $\beta 1$ -B-AB before RepSham or Repl/R treatments. Sections were stained for  $\alpha$ -smooth muscle actin ( $\alpha$ SMA; red) and PECAM-1/CD31 (green). (I–L) Quantification of  $\alpha$ SMA/PECAM-1-positive arterioles per  $\text{mm}^2$  in (I, J) the non-ischemic and (K, L) the ischemic myocardium upon ctrl-AB or  $\beta 1$ -B-AB treatment. Reported values are means  $\pm$  SEM. Ctrl-AB with RepSham:  $n = 4$ ; ctrl-AB with Repl/R:  $n = 7$ ;  $\beta 1$ -B-AB with RepSham:  $n = 5$ ;  $\beta 1$ -B-AB with Repl/R:  $n = 6$ . Statistical significance was determined using unpaired two-tailed Student's  $t$ -test. SEM, standard error of mean.

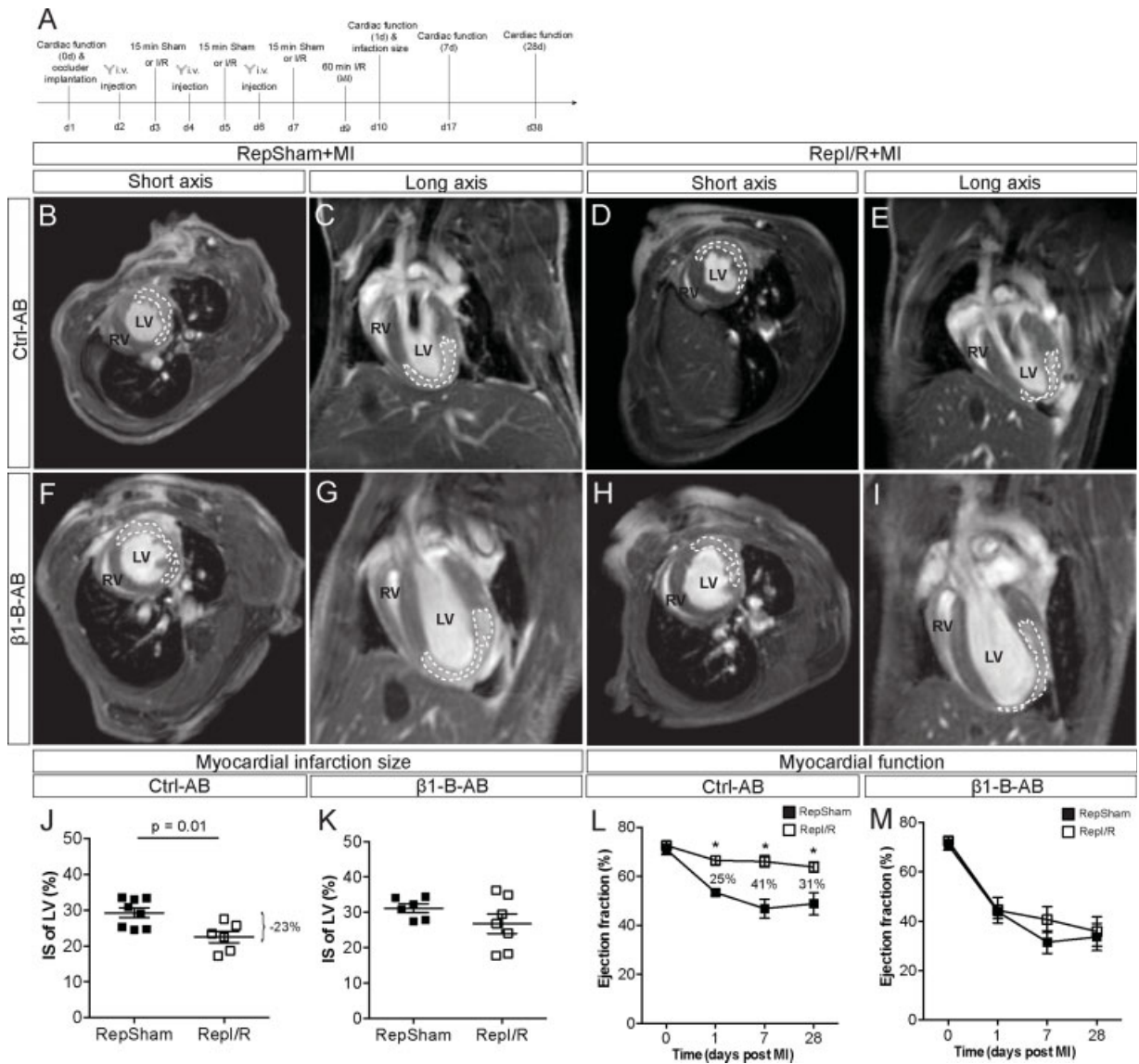
Next, we analyzed the number of arterioles within the ischemic and non-ischemic myocardia in the *Itgb1*<sup>IECKO</sup> mice ( $\rightarrow$ Fig. 5). Consistent with the previous experiments, more arterioles were found in both parts of the myocardium upon Repl/R ( $\rightarrow$ Fig. 5A–D, I, K). In contrast to the situation in the ischemic myocardium ( $\rightarrow$ Fig. 5K, L), endothelial  $\beta 1$  integrin was required for a statistically significant Repl/R-induced arteriole formation in the non-ischemic myocardium ( $\rightarrow$ Fig. 5E–H, I, J).

### Contribution of Endothelial $\beta 1$ Integrin to Ischemia-Induced Cardioprotection

We next analyzed the role of endothelial  $\beta 1$  integrin in cardioprotection conveyed by transient cardiac ischemia. To this end, an MI was induced after Repl/R treatments ( $\rightarrow$ Fig. 5O), and the hearts were analyzed over the following days for IS and LV EF. Notably, the IS in adult *Itgb1*<sup>IECKO</sup> mice was found to be substantially larger compared with that of tamoxifen-induced controls ( $\rightarrow$ Fig. 5M–R), and the LV EF was reduced on the first day after the MI ( $\rightarrow$ Fig. 5S).

### Growth of Blood Vessels into the Infarcted Myocardium and Maintenance of Cardiac Function upon MI Require Endothelial $\beta 1$ Integrin

Next, we occluded the LAD permanently to induce a severe MI (without reperfusion), with the goal to study the role of endothelial  $\beta 1$  integrin in vascularization and function of the infarcted myocardium in the absence of Repl/R ( $\rightarrow$ Fig. 6). Growth of blood vessels into the infarcted myocardium was analyzed by Microfil compound injection ( $\rightarrow$ Supplementary Fig. S1E–H, available in the online version). Here,  $\beta 1$ -B-AB was found to reduce vessel density in the infarcted area and to numerically reduce heart function ( $\rightarrow$ Fig. 6A–G). Tamoxifen-induced EC-specific depletion of  $\beta 1$  integrin prevented the growth of blood vessels into the infarcted area ( $\rightarrow$ Fig. 6H–K), and worsened heart function ( $\rightarrow$ Fig. 6L–N). Notably, 3 of 8 mice (37.5%) without endothelial  $\beta 1$  integrin died in the first 4 days after the MI induction, while 2 of 10 control animals (20%) died only after 7 days ( $\rightarrow$ Fig. 6S). Histology of the hearts revealed myocardial rupture due to thinning of the LV myocardium as the likely reason for cardiac death ( $\rightarrow$ Fig. 6O–R, exemplified for *Itgb1*<sup>IECKO</sup> mice).



**Fig. 3** Transient episodes of cardiac ischemia require  $\beta 1$  integrin to reduce infarct size (IS) and preserve LV heart function after myocardial infarction (MI). (A) Timeline of experimental procedure. (B–I) Representative cardiac MRI of mouse hearts after RepSham or Repl/R treatments and MI (60-minute ischemia) in short and long axis. IS or infarcted areas (as revealed by LGE) in the left myocardium are indicated by dashed lines. Mice received (B–E) ctrl-AB or (F–I)  $\beta 1$ -B-AB before RepSham or Repl/R treatments followed by MI. (J, K) Quantification of the IS 1 day after MI with reperfusion in (J) ctrl-AB and (K)  $\beta 1$ -B-AB treated mice. (L, M) Quantification of the LV ejection fraction at 0, 1, 7, and 28 days after MI with reperfusion in (L) ctrl-AB and (M)  $\beta 1$ -B-AB treated mice. At day 0, wild-type mice without AB treatment were used as controls. Reported values are means  $\pm$  SEM. Ctrl-AB with RepSham:  $n = 8$ ; ctrl-AB with Repl/R:  $n = 6$ ;  $\beta 1$ -B-AB with RepSham:  $n = 6$ ;  $\beta 1$ -B-AB with Repl/R:  $n = 7$ . Statistical significance was determined using unpaired two-tailed Student’s  $t$ -test; \* $p$ -values: 1 d  $< 0.01$ ; 7 d  $< 0.01$ ; 28 d = 0.01. LGE, late gadolinium enhancement; LV, left ventricular; MRI, magnetic resonance imaging; SEM, standard error of mean.

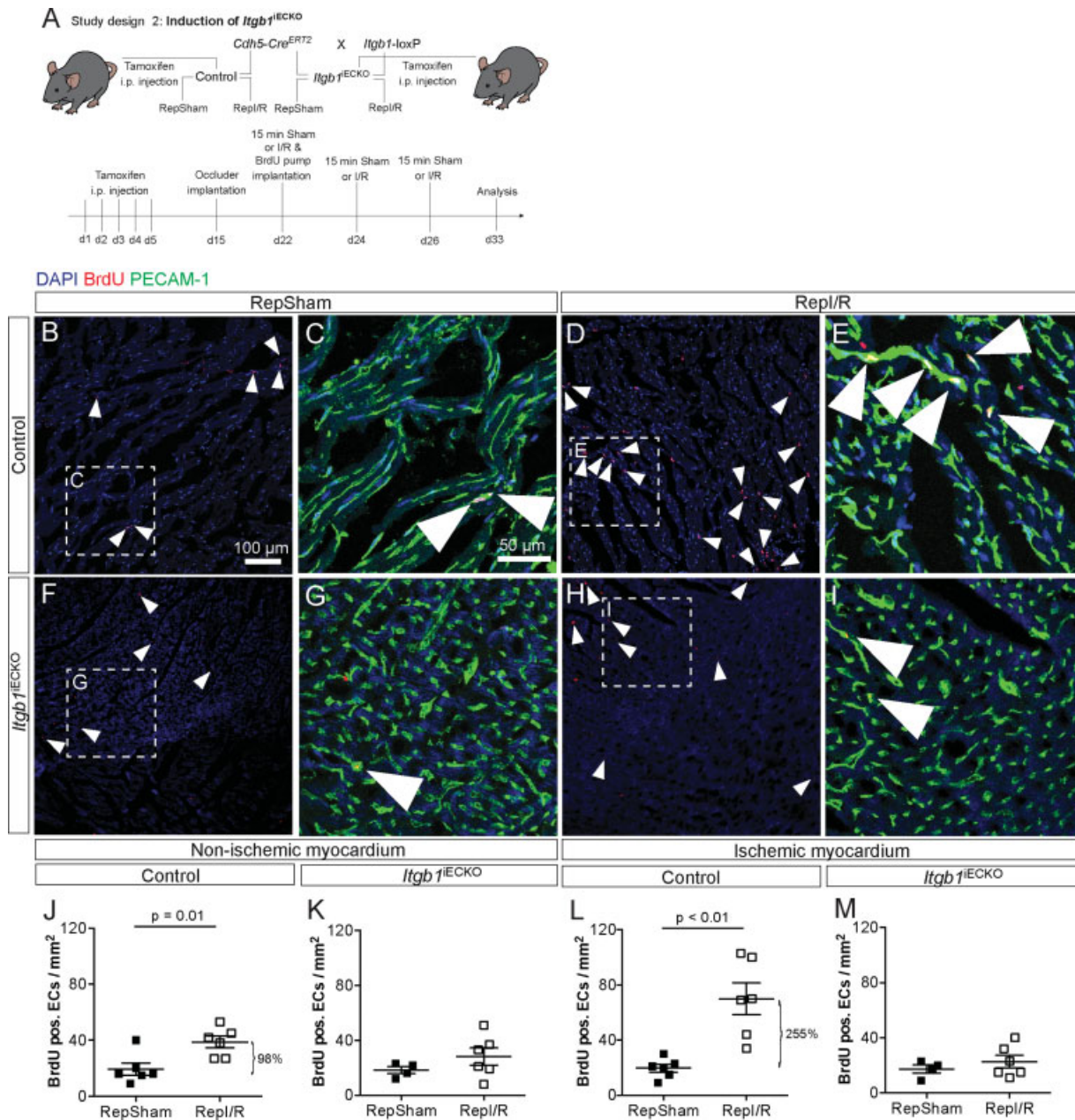
**Identification of eNOS-Regulated  $\beta 1$  Integrin Expression in Cardiac Endothelium**

Previously others and we provided evidence for eNOS as a possible downstream effector of  $\beta 1$  integrin in vitro.<sup>41–43</sup> To analyze the relevance in vivo, we checked  $\beta 1$  integrin expression in eNOS KO mice. Here, the intensity of endothelial  $\beta 1$  integrin expression was found to be increased in eNOS KO mice when their myocardium was compared with that of control mice ( $\blacktriangleright$  **Supplementary Fig. S4A–C**, available in the online version), whereas the EC area was not substantially different ( $\blacktriangleright$  **Supplementary Fig. S4D**, available in the online version).

**Requirement of  $\beta 1$  Integrin for Basal and Mechanically Induced Proliferation of Primary HCAECs**

To investigate whether our in vivo results on endothelial  $\beta 1$  integrin in adult mouse hearts were (in principle) applicable to the human situation, *ITGB1*, the gene for human  $\beta 1$  integrin, was silenced in primary HCAECs. Using three different siRNAs, we obtained a more than 90% knockdown efficiency on the mRNA level, even when the *ITGB1* gene expression was normalized to three different housekeeping genes ( $\blacktriangleright$  **Supplementary Fig. S5A–C**, available in the online version). The siRNAs also reduced the human  $\beta 1$  integrin protein to a substantial extent ( $\blacktriangleright$  **Supplementary Fig. S5D, E**;





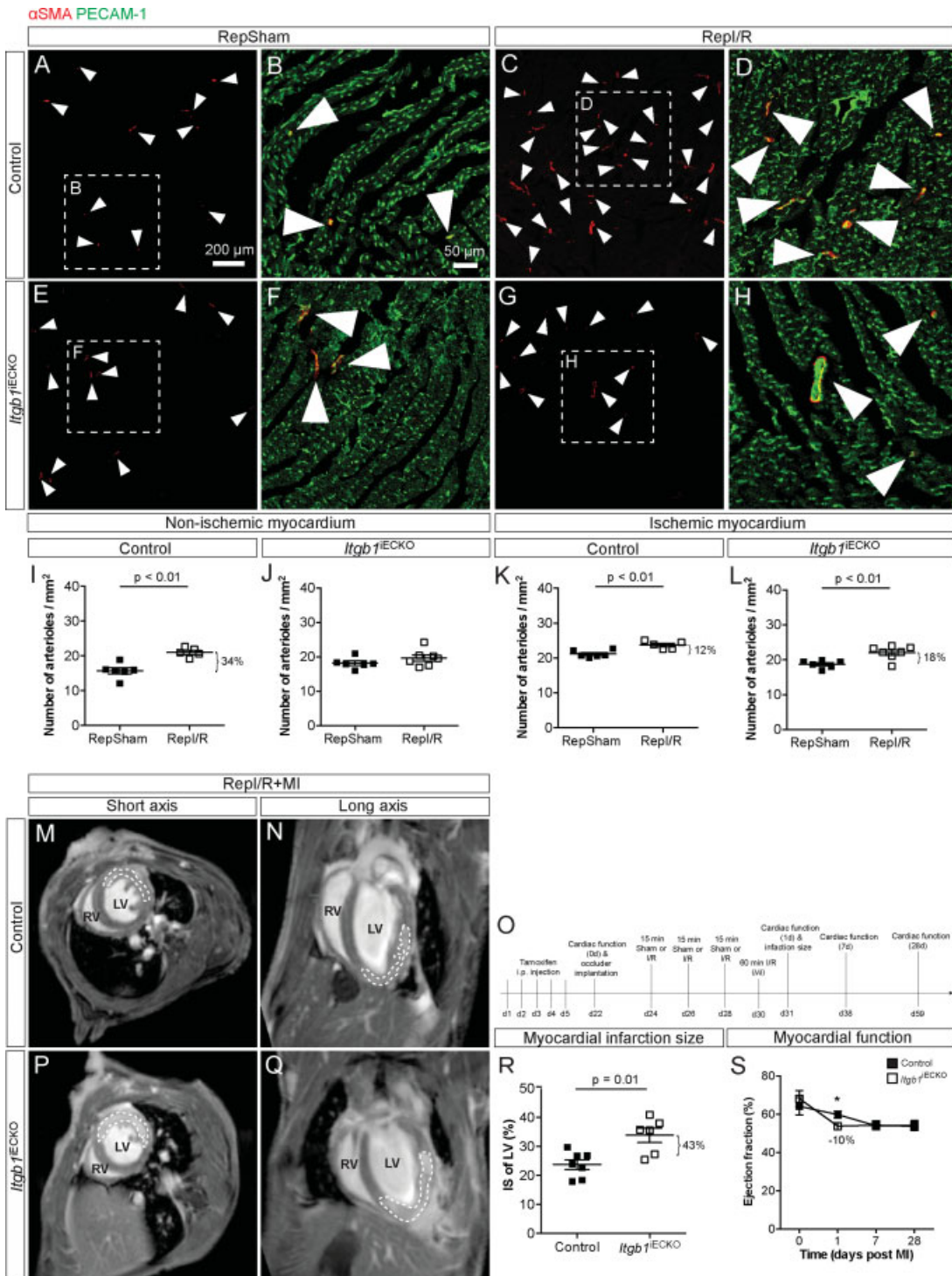
**Fig. 4** Induction of cardiac EC proliferation by transient episodes of cardiac ischemia strictly requires endothelial  $Itgb1$  expression. (A) Study design to knock out the gene for  $\beta 1$  integrin ( $Itgb1$ ) by tamoxifen treatment in ECs of mice. (B–I) Representative immunofluorescence images of sections through the non-ischemic myocardium of mouse hearts taken from tamoxifen-induced (B–E) Cre control mice (indicated as control) and (F–I) EC-specific  $\beta 1$  integrin KO (indicated as  $Itgb1^{IECKO}$ ) mice upon RepSham or Repl/R treatments. Sections were stained for BrdU (red), PECAM-1/CD31 (green), and DAPI (blue). (J–M) Quantification of the BrdU-positive ECs per  $\text{mm}^2$  (J, K) non-ischemic and (L, M) ischemic myocardium of control or  $Itgb1^{IECKO}$  mice. Reported values are means  $\pm$  SEM. Control with RepSham:  $n = 6$ ; control with Repl/R:  $n = 6$ ;  $Itgb1^{IECKO}$  with RepSham:  $n = 4$ ;  $Itgb1^{IECKO}$  with Repl/R:  $n = 6$ . Statistical significance was determined using unpaired two-tailed Student's *t*-test. EC, endothelial cell; SEM, standard error of mean.

for uncropped Western blots, see **► Supplementary Fig. S6**, available in the online version). Notably, knockdown of  $ITGB1$  reduced the number of proliferating HCAECs by more than 20% (**► Supplementary Fig. S7A–E**, available in the online version), and even the total number of  $ITGB1$ -silenced HCAECs was numerically reduced compared with control-silenced cells within 2 days of cultivation (**► Supplementary Fig. S7F**, available in the online version). We also found that mechanical stretching (mimicking the effect of vasodilation on ECs<sup>18</sup>) activated  $\beta 1$  integrin in HCAECs (**► Supplementary Fig. S8A–E**, available in the online version) and that the

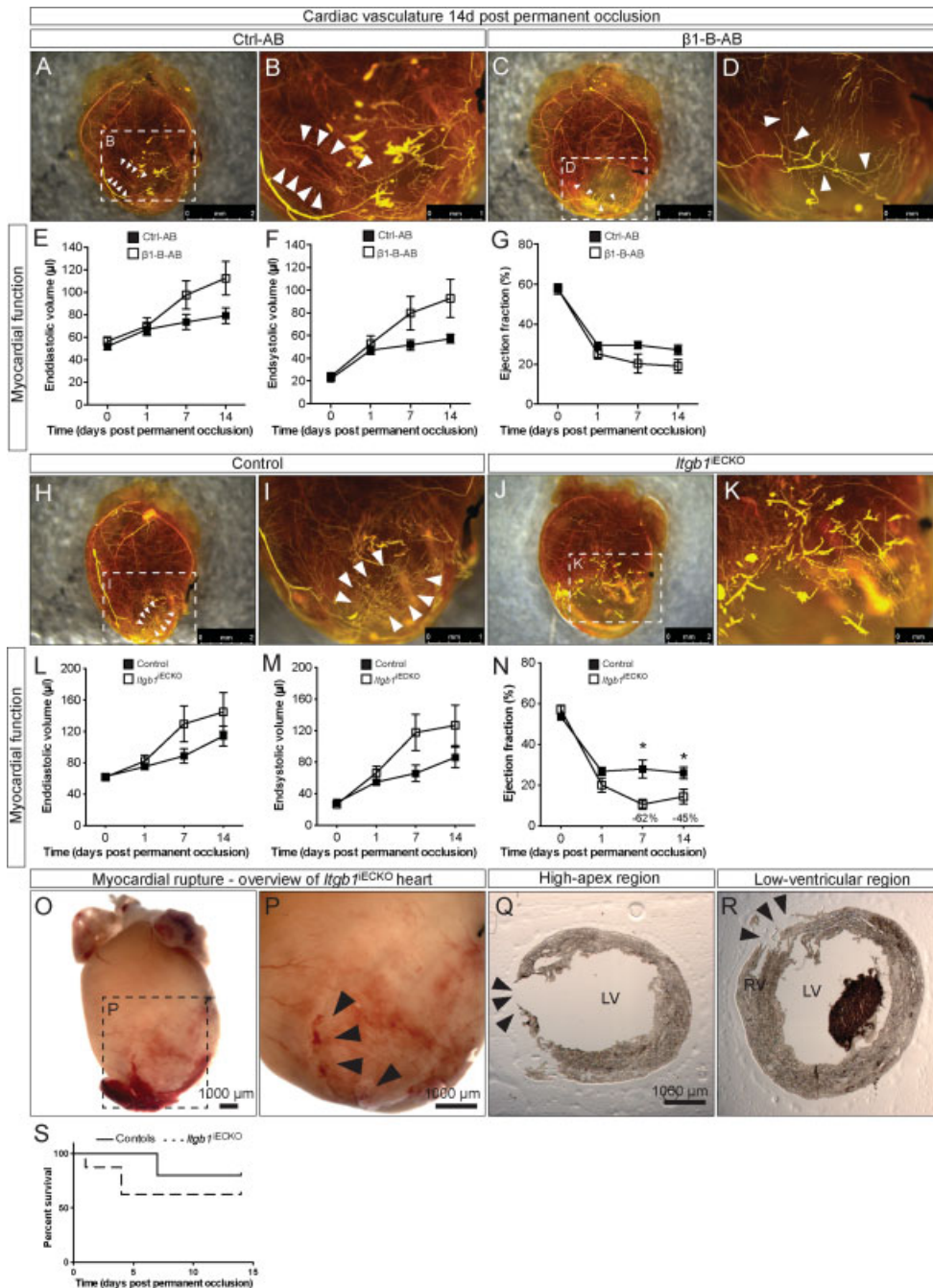
integrin was strictly required for mechanically induced proliferation of HCAECs (**► Supplementary Fig. S8F–O**, available in the online version).

## Discussion

The  $\beta 1$  integrin is part of the largest group of integrins and needed for assembly of BMs, cardiomyocyte function and differentiation, mechanosensing, EC reorientation, cross-activation of VEGF receptors, flow-mediated vasodilation, release of angiocrine signals, and other processes.<sup>10,14–23,41,44,45</sup> Studies could



**Fig. 5** Transient episodes of cardiac ischemia require endothelial *Itgb1* expression to induce arteriole formation in the non-ischemic myocardium and reduce infarct size (IS) after myocardial infarction (MI). (A–H) Representative immunofluorescence images through the non-ischemic myocardium of (A–D) control and (E–H) *Itgb1*<sup>IECKO</sup> mice after RepSham or Repl/R treatments. Sections were stained for  $\alpha$ SMA (red) and PECAM-1/CD31 (green). (I–L) Quantification of  $\alpha$ SMA/PECAM-1-positive arterioles per mm<sup>2</sup> (I, J) non-ischemic and (K, L) ischemic myocardium of control or *Itgb1*<sup>IECKO</sup> mice. Reported values are means  $\pm$  SEM. Control with RepSham: n = 6; control with Repl/R: n = 5; *Itgb1*<sup>IECKO</sup> with RepSham: n = 6; *Itgb1*<sup>IECKO</sup> with Repl/R: n = 7. (M, N, P, Q) Representative MRI of (M, N) control and (P, Q) *Itgb1*<sup>IECKO</sup> mouse hearts after Repl/R treatments and MI (60-minute ischemia) in short and long axis. IS or infarcted areas (as revealed by LGE) in the left myocardium are indicated by dashed lines. (O) Timeline of the experimental procedure. (R) Quantification of the IS 1 day after MI in control and *Itgb1*<sup>IECKO</sup> mice undergoing Repl/R treatment. (S) Quantification of the LV ejection fraction at 0, 1, 7, and 28 days after MI with reperfusion in control versus *Itgb1*<sup>IECKO</sup> mice. Reported values are means  $\pm$  SEM. Control with Repl/R: n = 7; *Itgb1*<sup>IECKO</sup> with Repl/R: n = 6. Statistical significance was determined using unpaired two-tailed Student's *t*-test. \**p*-Values: 1 d = 0.04. LGE, late gadolinium enhancement; LV, left ventricular; MRI, magnetic resonance imaging; SEM, standard error of mean.



**Fig. 6** Growth of blood vessels into the infarcted myocardium, LV function, and cardiac survival after permanent LAD occlusion require endothelial *Itgb1* expression. (A–D, H–K) Representative bright-field images of Microfil-perfused mouse hearts to visualize the coronary vasculature 14 days after permanent LAD occlusion. Mice were treated with (A, B) ctrl-AB and (C, D)  $\beta 1$ -B-AB. Alternatively, tamoxifen-induced (H, I) control and (J, K) *Itgb1*<sup>IECKO</sup> mice were used. (E–G, L–N) Quantification of LV myocardial function represented by (E, L) the end-diastolic volume, (F, M) the end-systolic volume, and (G, N) the LV ejection fraction at 0, 1, 7, and 14 days after LAD occlusion in wild-type mice treated with (E–G) ctrl-AB versus  $\beta 1$ -B-AB or in (L–N) control versus *Itgb1*<sup>IECKO</sup> mice. Reported values are means  $\pm$  SEM. (E–G) Ctrl-AB:  $n = 5$ ;  $\beta 1$ -B-AB:  $n = 5$ ; (L–N) control:  $n = 7$ ; *Itgb1*<sup>IECKO</sup>  $n = 5$ . Statistical significance was determined using unpaired two-tailed Student's *t*-test. \**p*-Values: (N) 7 d = 0.01; 14 d = 0.03. (O–R) Representative bright-field images of an *Itgb1*<sup>IECKO</sup> mouse heart with myocardial rupture as (O, P) overview or as (Q, R) transversal sections. Arrows indicate the rupture. In panels (Q) and (R), the LV and RV are labeled. (S) Quantification of survival in a Kaplan–Meier curve in control versus *Itgb1*<sup>IECKO</sup> mice. Control 0 d:  $n = 10$ , 7 d:  $n = 8$ ; *Itgb1*<sup>IECKO</sup> 0 d:  $n = 8$ , 1 d:  $n = 7$ , and upon 4 d:  $n = 5$ . Of note, one *Itgb1*<sup>IECKO</sup> mouse died after 0 d measurement of cardiac function, but before permanent LAD occlusion, and therefore was excluded from the Kaplan–Meier curve. Only mice which received a permanent LAD occlusion are shown. Statistical significance was determined using the log-rank (Mantel–Cox) test. LAD, left anterior descending artery; LV, left ventricular; SEM, standard error of mean.

show that a global heterozygous knockout of  $\beta 1$  integrin worsened the outcome after MI.<sup>46</sup> Furthermore, it was shown that cardiomyocyte-specific deletion of  $\beta 1$  integrin causes fibrosis, cardiac failure, issues in mechanotransductive response,<sup>28,47</sup>

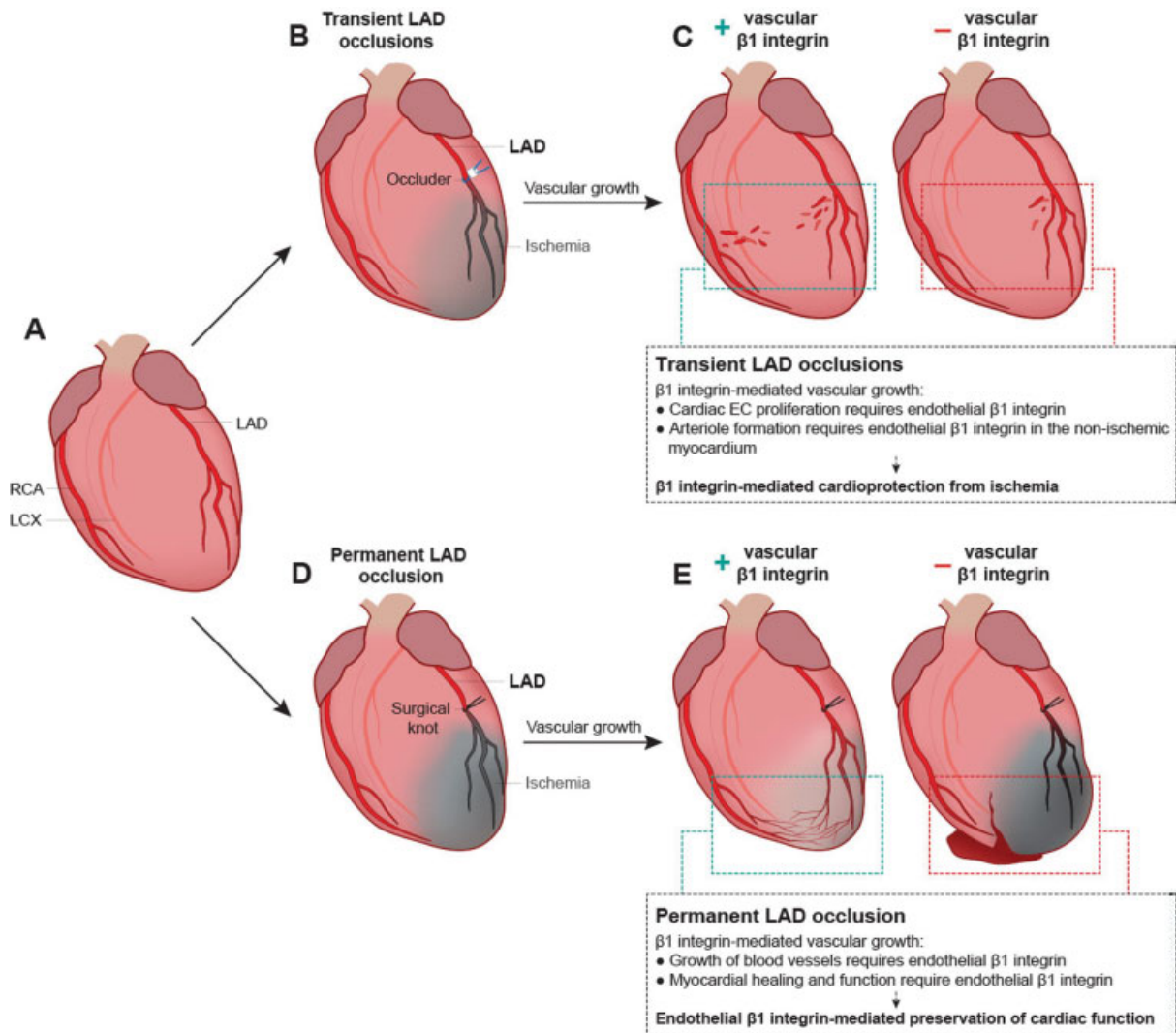
and bone mesenchymal stem cells overexpressing *Itgb1* revealed a protective role after MI for cardiomyocytes.<sup>48</sup> In addition, an EC-specific knockout of  $\beta 1$  integrin was used to study the role of this integrin in vascular leakage and endotoxemia in the mouse

heart.<sup>49</sup> However, the role of endothelial  $\beta 1$  integrin in cardio-protective ischemic events, ischemia-induced cardiac vascular growth, and preservation of LV cardiac function after MI has, to our knowledge, not yet been investigated.

Here, we provide evidence for an important role of endothelial  $\beta 1$  integrin in vascular growth and cardioprotection (see ►Fig. 7). We found that short episodes of myocardial ischemia are sufficient to induce proliferation of cardiac ECs as well as formation of arterioles in the ischemic and non-ischemic myocardia in a  $\beta 1$  integrin-dependent manner (see ►Fig. 7). In contrast to EC proliferation, arteriole formation depends on endothelial  $\beta 1$  integrin in the non-ischemic, but not in the ischemic part of the adult mouse heart. This is particularly striking given that Repl/R treatment more strongly induces cardiac EC proliferation in the ischemic myocardium compared with the non-ischemic one. These regional differences in the adult heart might be due to additional responses

taking place when the myocardium encounters hypoxia and subsequent inflammatory events.<sup>50,51</sup> For example, whereas arteriole formation in the non-ischemic myocardium is likely to predominantly depend on mechanical stimulation (e.g., via increased shear stress/mechanical stretch) and thus  $\beta 1$  integrin as a mechanosensory protein on ECs, hypoxia-induced responses also take place in the ischemic myocardium. The latter responses might trigger arteriole formation independent of local EC proliferation and  $\beta 1$  integrin on ECs.

One possible scenario might be the recruitment of smooth muscle cells (SMCs) by migration, causing an arterIALIZATION of capillaries,<sup>52</sup> which could be independent of EC proliferation. Further, it is noteworthy that Red-Horse and colleagues have recently shown that collaterals are also formed via “artery reassembly” in the neonatal heart,<sup>53</sup> and that Adams and colleagues have shown that ECs from sprouting capillaries (that develop in hypoxic areas) can relocate to participate in



**Fig. 7** Visual Summary: Endothelial  $\beta 1$  integrin is required for myocardial ischemia-induced vascular growth, growth of blood vessels into the infarcted myocardium and preservation of cardiac function. (A) Adult mouse heart, with a coronary vasculature similar to the human situation, received (B) transient LAD occlusions, mimicking temporary ischemia observed in patients with CAD. (C) These transient occlusions trigger  $\beta 1$  integrin-dependent vascular growth and cardioprotection. (D) Permanent LAD occlusion, mimicking the situation in patients with MI, not receiving proper reperfusion. (E) Endothelial  $\beta 1$  integrin is required for growth of blood vessels into the infarcted myocardium and preservation of cardiac function.

the formation of arteries,<sup>54</sup> thus representing possible scenarios by which arterioles might form in the ischemic myocardium without increased cardiac EC proliferation.

The here-presented data show that pharmacological inhibition of  $\beta 1$  integrin abolished Repl/R-induced cardioprotection, shown in MRI measurements and TTC staining. Since a simultaneous decrease in cardiac function is observed in the RepSham and the Repl/R group,  $\beta 1$  integrin seems to have a general role in cardioprotection that extends beyond its role in Repl/R-induced cardioprotection.

To validate this point, the role of  $\beta 1$  integrin was also analyzed in a chronic ischemia model without Repl/R. In this model, we could generate a stronger ischemia, which induced growth of blood vessels into the infarcted myocardium, comparable to a previous study.<sup>40</sup> Notably, our experimental results revealed that endothelial  $\beta 1$  integrin was required for proper vascular growth and maintenance of cardiac function independent of Repl/R. Further, we showed in the mouse heart that endothelial  $\beta 1$  integrin is regulated by eNOS (as indicated by increased  $\beta 1$  integrin expression in eNOS-deficient mice). Since eNOS contributes to ischemic preconditioning,<sup>30–33</sup> our findings also point to a possible role of  $\beta 1$  integrin in this relevant phenomenon.

Multiple different cell types, including SMCs, cardiomyocytes, myeloid cells (such as monocytes and macrophages), and endothelial progenitor cells, were previously reported to be involved in cardiac vascular growth.<sup>4,40,55,56</sup> Thus, our findings are remarkable in that  $\beta 1$  integrin on ECs, meaning a single-cell surface receptor on a single-cell type, is needed to induce vascular growth and cardioprotection. In summary, we uncover a key role of endothelial  $\beta 1$  integrin in cardiac vascular adaptation to ischemia, function, and protection from MI.

### What is known about this topic?

- Short episodes of myocardial ischemia can protect the heart from myocardial infarction (MI).
- To date,  $\beta 1$  integrin has been targeted among others in cardiomyocytes, myofibroblasts, and pericytes, but not in endothelial cells (ECs) to uncover its endothelial role in myocardial ischemia.
- A role of endothelial  $\beta 1$  integrin has been identified in vascular leakage and endotoxemia of the mouse heart.

### What does this paper add?

- Endothelial  $\beta 1$  integrin is shown to be required for cardiac EC proliferation during short episodes of left ventricular (LV) ischemia in mice in vivo and for proliferation of human coronary artery ECs in vitro.
- Endothelial  $\beta 1$  integrin is required for arteriole formation induced by short episodes of LV ischemia in the non-ischemic myocardium.
- Endothelial  $\beta 1$  integrin has a general role in vascular growth into the infarct region and preservation of cardiac function after chronic myocardial infarction.

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### Conflict of Interest

None declared.

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