

Erythropoietin Responsive Cardiomyogenic Cells Contribute to Heart Repair Post Myocardial Infarction

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Abstract

The role of erythropoietin (Epo) in myocardial repair after infarction remains inconclusive. We observed high Epo receptor (EPOR) expression in cardiac progenitor cells (CPCs). Therefore, we aimed to characterize these cells and elucidate their contribution to myocardial regeneration on Epo stimulation. High EPOR expression was detected during murine embryonic heart development followed by a marked decrease until adulthood. EPOR-positive cells in the adult heart were identified in a CPC-enriched cell population and showed coexpression of stem, mesenchymal, endothelial, and cardiomyogenic cell markers. We focused on the population coexpressing early (TBX5, NKX2.5) and definitive (myosin heavy chain [MHC], cardiac Troponin T [cTNT]) cardiomyocyte markers. Epo increased their proliferation and thus were designated as Eporesponsive MHC expressing cells (EMCs). In vitro, EMCs proliferated and partially differentiated toward cardiomyocyte-like cells. Repetitive Epo administration in mice with myocardial infarction (cumulative dose 4 IU/g) resulted in an increase in cardiac EMCs and cTNT-positive cells in the infarcted area. This was further accompanied by a significant preservation of cardiac function when compared with control mice. Our study characterized an EPO-responsive MHCexpressing cell population in the adult heart. Repetitive, moderate-dose Epo treatment enhanced the proliferation of EMCs resulting in preservation of post-ischemic cardiac function. STEM CELLS 2014;32:2480-2491

INTRODUCTION

Due to the limited regenerative capacity of the heart, cardiovascular diseases remain the main cause of morbidity and mortality in the world (WHO, Fact sheet N°317, September 2011). Recent evidence suggests that significant myocardial regeneration post-injury in the early neonatal mammalian heart [1], which is lost later mainly due to low cardiomyocyte turnover as shown in the human heart [2]. Two different mechanisms of endogenous cardiomyocyte regeneration have been suggested including proliferating dedifferentiated cardiomyocytes [3] and endogenous progenitor cell differentiation [4]. Neither of these mechanisms is sufficient to replace damaged myocardium in the adult heart. To exploit the dormant regenerative potential of the heart, it is necessary to delineate signaling pathways, which govern the activity of regenerative cell niches within the heart.

Erythropoietin (Epo) is a growth hormone that binds to a classical transmembraneous

Epo receptor (EPOR) and promotes the survival and proliferation of late erythroid progenitor cells [5]. However, the broad expression of EPOR in nonhematopoietic tissues such as the heart [6] suggested a role for Epo beyond erythropoiesis. In the developing murine heart, EPOR is expressed from embryonic day (ED) 10.5 and its complete knockdown leads to in utero death around ED13.5 due to anemia [6]. Erythroid-rescued EPOR-null mice reach adulthood with no apparent cardiac morphological defects [7]. However, on coronary artery ligation and reperfusion (I/R), these mice exhibit exacerbated cardiac dysfunction [8], indicating that the endogenous Epo system may beneficially affect cardiac remodeling. Exogenous Epo administration after myocardial ischemia (MI) in animal models improved global cardiac function by reducing apoptosis in the infarct border zone [9] and enhancing angiogenesis [10, 11]. However, Epo administration showed ambiguous results in large cohort clinical trials concerning its protective effects against cardiac

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remodeling [12, 13], highlighting the need for a better understanding of the molecular mechanisms underlying the cardioprotective action of Epo.

We found EPOR to be predominantly expressed in a cardiac progenitor-enriched cell pool of the adult heart. A proliferating subpopulation with biochemical distinct cardiomyogenic characteristics, which was responsive to Epo stimulation in vitro and in vivo was identified. Epo treatment increased the abundance of these cells in the chronic ischemic heart along with a preservation of cardiac function. Collectively, our data define a target cell type mediating the protective potential of Epo in the adult heart.

MATERIALS AND METHODS

RNA Isolation, Reverse Transcription, and Quantitative PCR Analysis

Cardiac crescent (ED7.5), heart tube structures (ED8.5, ED9.5), and hearts from ED10.5 were microdissected and pooled from three independent experimental series for RNA isolation. Hearts from later stage embryos (ED13.5 and ED15.5), neonatal (P1, P3, P6, P10, and P13), and adult NMRI mice were excised and individually subjected to RNA isolation. Total RNA was isolated with the NucleoSpin RNA II kit (Macherey-Nagel, Dueren/Germany, http://www.mn-net.com), according to manufacturer's instructions. Oligo(dT)₂₀ primer and M-MLV reverse transcriptase (Promega, Mannheim/Germany, http://www. promega.de) were used for reverse transcription. Primers were designed using primer3 Software. Quantitative polymerase chain reaction (qPCR) analysis was performed with SYBR Green (Promega, Mannheim/Germany, http://www.promega. de) with a 7900 HT Fast real-time PCR system (Applied Biosystems, Darmstadt/Germany, http://www.lifetechnologies.com/ de/de/home.html). Transcript abundance was calculated using the standard curve [14]. All reactions were performed in triplicates and normalized to reference control genes (Gapdh and Tpt1). Primers used in this study are listed in Supporting Information Table S1.

Immunoblot Analysis

Immunoblot analysis was performed as described previously [15]. The following primary and secondary antibodies were used: EPOR (1:1000; Sigma SAB2100693, Hamburg/Germany, http://www.sigmaaldrich.com/germany.html), GAPDH (1:50,000; Millipore, Darmstadt/Germany, http://www.merck-millipore.de/); p-AKT and AKT (1:1000; Cell Signaling, Leiden/The Netherlands, http://www.cellsignal.com); p-ERK (1:1000; Cell Signaling, Leiden/The Netherlands, http://www.cellsignal.com); and ERK (1:1000; SCBT, Heidelberg/Germany, http:// www.scbt.com/), p-STAT3 and STAT3 (1:1000; Cell Signaling, Leiden/The Netherlands, http://www.cellsignal.com); rb-HRP (1:5000; DAKO, Hamburg/Germany, http://www.dako.com/de/), ms-HRP (1:10,000; DAKO, Hamburg/Germany, http://www.dako.com/de/).

Immunohistochemistry

Tissues were rinsed in phosphate-buffered saline (PBS), fixed O/N in 4% paraformaldehyde (PFA) at 4° C, embedded in paraffin, and sectioned at 4 μ m. Sections were deparaffinized, microwaved for antigen retrieval in citrate buffer, blocked in bovine serum albumin

5% and incubated O/N with different primary antibodies: EPOR (1:100; Sigma, Hamburg/Germany, http://www.sigmaaldrich.com/ germany.html), cTNT (1:200; Abcam, Cambridge/UK, http://www. abcam.com). Slides were washed in PBS and incubated with Alexa-Fluor488- or AlexaFluor594-conjugated secondary antibodies (1:200; Invitrogen, Darmstadt/Germany, http://www.lifetechnologies.com/de/de/home.html). The slides were counterstained with DAPI and mounted with Mowiol 488 (Carl Roth, Karlsruhe/Germany, www.carlroth.de). Images were captured using epifluore-

sence (IX70, Olympus, Hamburg/Germany, http://www.olympus. de/) or confocal (Zeiss 710 NLO, Jena/Germany, http://www.zeiss. de/corporate/de_de/home.html) microscopes. Fetal liver (E16.5– E17.5), where erythropoiesis takes place and therefore contains high EPOR levels [16], served as positive control for the EPOR antibody (Supporting Information Fig. S2B, S2C). Particularly strong EPOR expression was detected around the cardiac vessels (Supporting Information Fig. S2C).

CPC Isolation, SCA1, and EPOR Magnetic Cell Sorting

CPCs were isolated according to a protocol originally described by Oh et al. [17] with slight modifications [18]. Briefly, mice were anesthetized by ketamine/xylazine (30 mg/19 mg/kg body weight) intraperitoneal (i.p.) and hearts were dissected. Following enzymatic digestion with 0.1% collagenase IIa (Worthington, Lakewood, NJ, http://www.worthington-biochem.com/default. html) and 2.4 IU/ml dispase (BD biosciences, Heidelberg/Germany, http://www.bdbiosciences.com/eu/index.jsp) in Hank's Balanced Salt Solution (HBSS) for 30 minutes at 37°C, the CPC pool was isolated by sequential straining through 40 μ m (BD biosciences, Heidelberg/Germany, http://www.bdbiosciences. com/eu/index.jsp) and 30 μ m meshes (Miltenyi Biotec, Bergisch Gladbach/Germany, https://www.miltenyibiotec.com/en/). SCA1pos cells were purified by magnetic cell sorting. CPCs were stained with anti-SCA1-Fluorescein isothiocyanate (FITC) (1:10; Miltenyi Biotec, Bergisch Gladbach/Germany, https://www.miltenyibiotec. com/en/) and labeled with anti-FITC-MicroBeads (1:5; Miltenyi Biotec, Bergisch Gladbach/Germany, https://www.miltenyibiotec. com/en/). SCA1pos cells were enriched by passing the labeled CPCs 2-3 rounds through an MS column (Miltenyi Biotec, Bergisch Gladbach/Germany, https://www.miltenyibiotec.com/en/). Flow cytometry (FC) was used to verify 95% purity followed by mRNA extraction. EPORpos CPCs were isolated by magnetic cell sorting using triple labeling, i.e.,: anti-EPOR (1:100; Sigma SAB2100693, Hamburg/ Germany, http://www.sigmaaldrich.com/ germany.htm), anti-rabbit APC (1:250, Jackson Immunoresearch, Suffolk/England, http://www.jireurope.com/home.asp), and finally, anti-APC-MicroBeads (1:5; Miltenyi Biotec, Bergisch Gladbach/Germany, https://www.miltenyibiotec.com/en/). $\ensuremath{\mathsf{EPOR}^{\mathsf{pos}}}$ cells were enriched by passing the labeled CPCs 2-3 rounds through an LS Column (Miltenyi Biotec, Bergisch Gladbach/Germany, https:// www.miltenyibiotec.com/en/). FC was used to verify 80% purity followed by mRNA extraction. Appropriate isotype controls were used. Propidium iodide was used for dead cell exclusion.

Flow Cytometry

CPCs were isolated as described above. For cocultured CPCs, cells were enzymatically digested and preplated to remove feeders. Isolated cells were labeled with CD31-FITC (1:200; eBioscience, Frankfurt/Germany, www.eBioscience.com), FLK1-PE (1:200; eBioscience, Frankfurt/Germany, www.eBioscience.com), CD34-FITC (1:100; eBioscience, Frankfurt/Germany,



Figure 1. Prominent erythropoietin (Epo) receptor (EPOR) expression in the embryonic developing heart. **(A):** EPOR transcript abundance from cardiac crescent stage (embryonic day [ED] 7.5), heart tube formation, and looping stages (ED8.5 and ED9.5), embryonic heart (ED10.5, ED13.5, and ED15.5), neonatal heart (P1, P3, P6, P10, and P13), and adult mouse heart (NMRI-strain) were analyzed by quantitative polymerase chain reaction ($n \ 5$ 3/developmental stage). Note that for the early stages, the aforementioned cardiac structures were micro-dissected from 10 to 12 embryos for ED7.5 and ED 8.5 and 5–8 embryos for ED 9.5 and ED10.5 and subsequently pooled for each of the three independent experiments. **(B, C):** EPOR transcript and protein expression levels in cardiac tissue of representative embryonic, post-natal stages of C57BL/6 mice ($n \ 5$ 3). Fetal liver served as a positive control tissue for EPOR and GAPDH was used as a loading control. **(D):** Confocal immunofluorescence analysis showing EPOR expression in the adult heart being confined to small interstitial cells (white arrows). ED10.5 is a representative picture from the common ventricle, whereas the rest of the photos show the left ventricular myocardium. Scale bar 5 20 µm. Abbreviations: ED, embryonic day post coitum; EPOR, erythropoietin receptor; P: postnatal day; NMRI, naval medical research institute.

www.eBioscience.com), cKIT-FITC (1:100; eBioscience, Frankfurt/ Germany, www.eBioscience.com), and EPOR (1:100; Sigma, Hamburg/Germany, http://www.sigmaaldrich.com/germany. html) in FC-buffer. For EPOR staining, the cells were further incubated with secondary antibody anti-rabbit IgG-APC (1:500; Jackson ImmunoResearch). Following PBS washes, the cells were fixed in 1% PFA/PBS and permeabilized in FC-buffer containing 0.5% Saponin (Sigma, Hamburg/Germany, http://www. sigmaaldrich.com/germany.html). For intracellular labeling. MHC (1:500; Abcam Cambridge/UK, http://www.abcam.com), GATA4 (1:200; SCBT, Heidelberg/Germany, http://www.scbt.com/), TBX5 (1:500, Abcam, Cambridge/UK, http://www.abcam.com), KI67 (1:100; Abcam, Cambridge/UK, http://www.abcam.com), αACT (1:200; Sigma, Hamburg/Germany, http://www.sigmaaldrich.com/germany.html), and cTNT (1:500; Abcam, Cambridge/ UK, http://www.abcam.com) antibodies as well as appropriate isotype controls were used. As secondary antibodies anti-rabbit (or mouse) IgG-APC (1:500; Jackson ImmunoResearch, Suffolk/ England, http://www.jireurope.com/home.asp) and anti-mouse F(ab)₂-FITC (1:500; Jackson ImmunoResearch, Suffolk/England, http://www.jireurope.com/home.asp) were applied. For cell cycle analysis, the cells were incubated with 1 μ g/ml DAPI in

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0.1% Triton X-100 PBS solution for 30 minutes at RT. Fluorescence was detected with a FACS-Calibur or FACS LSRII systems (Becton Dickinson, Heidelberg/Germany, http://www.bdbiosciences.com/eu/index.jsp). Cell doublets were excluded from the analysis using DAPI width to area scatter.

Cell Culture

CPCs were seeded either on adult fibroblasts or neonatal rat cardiomyocytes (NRCMs). CPCs on fibroblast were cultured for 14 days at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 2% fetal calf serum (FCS), 20 µmol/l L-glutamine, 1% penicillin streptomycin (Invitrogen, Darmstadt/Germany, http://www.lifetechnologies.com/de/de/home.html), and 100 µmol/l ascorbic acid (Sigma, Hamburg/Germany, http://www.sigmaaldrich.com/germany.html). Microscopic observation revealed that CPCs form little clusters on top of the fibroblasts up until day 5 and subsequently elongate, migrate, and differentiate (Supporting Information Fig. S1). For the cell signaling experiments, the cocultures were serum starved with 0.5% FCS. For the analysis of the EPOR^{pos}, CPCs were plated on collagen-coated coverslips. The cells were maintained in culture for 2 days with

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Figure 2. Erythropoietin (Epo) receptor (EPOR) expression in immature cardiac cells of the adult heart. **(A):** Confocal immunofluorescence analysis of isolated cardiac progenitor cells (CPCs) showing coexpression of EPOR with the endothelial CD34, FLK1; mesenchymal Vimentin (VIM); cardiomyogenic myosin heavy chain (MHC); and stem cell SCA1 and cKIT markers. **(B):** FC analysis showed abundant MHC and CD34 expression in the EPOR^{pos} subpopulation, followed by SCA1, FLK1, and cKIT, respectively (*n* 5 3). **(C):** EPOR expression in small undifferentiated CD31^{pos}, VIM^{pos}, or MHC^{pos} cells is lost on differentiation. Isotype IgG was used as control (ISO). Scale bar 5 20 μm. Data represent mean 6 SEM. Abbreviations: APC, allophycocyanin; CPC, cardiac progenitor cells; DAPI, cEPOR, erythropoiet in receptor; FITC, fluorescein isothiocyanate; MHC, myosin heavy chain; VIM, Vimentin.

fibroblast conditioned medium containing 2% FCS. NRCMs derived from Wistar or AsRed-transgenic Lewis rats. They were isolated using a cardiomyocyte isolation kit and gentleMACS dissociator (Miltenyi Biotech, Bergisch Gladbach/Germany, https://www.miltenyibiotec.com/en/) according to the manufacturer's instructions. NRCMs were cultured on collagencoated eight-well Millicell EZ glass slides (Millipore, Darmstadt/Germany, http://www.merckmillipore.de/) with DMEM/ F12 medium supplemented with 5% horse serum. 20 umol/l Lglutamine, 1% penicillin streptomycin (Invitrogen, Darmstadt/ Germany, http://www.lifetechnologies.com/de/de/home.html), 100 µmol/l ascorbic acid (Sigma, Hamburg/Germany, http:// www.sigmaaldrich.com/germany.html), 100 mM sodium pyruvate (Invitrogen, Darmstadt/Germany, http://www.lifetechnologies.com/de/de/home.html), and 1% insulin-transferrinselenium (Invitrogen, Darmstadt/Germany, http://www.lifetechnologies.com/de/de/home.html). Subsequently, CPCs derived from α MHC-EGFP C57BL/6 mice were plated on the NRCMs and cultured with the same medium. One day after culture, the cells were washed well with PBS to remove debris and replenished with fresh medium. Daily medium changes were performed.

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Adult Cardiac Fibroblast Isolation and Preparation of Conditioned Medium

Adult cardiac fibroblasts were isolated by enzymatic digestion of minced heart tissue in HBSS, containing 0.1% trypsin (Invitrogen, Darmstadt/Germany, http://www.lifetechnologies.com/ de/de/home.html) and 100 IU/ml collagenase IIa (Worthington, Lakewood, NJ, http://www.worthington-biochem.com/ default.html), and cultured at 37°C and 5% CO₂ in DMEM/F12 medium supplemented with 10% FCS, 20 µmol/l L-glutamine, 1% penicillin streptomycin (Invitrogen, Darmstadt/Germany, http://www.lifetechnologies.com/de/de/home.html) antibiotics (Invitrogen, Darmstadt/Germany, http://www.lifetechnologies. com/de/de/home.html), and 100 $\mu mol/l$ ascorbic acid (Sigma, Hamburg/Germany, http://www.sigmaaldrich.com/germany. html). The feeders were expanded for three passages, growth arrested by mitomycin C treatment (Sigma, Hamburg/Germany, http://www.sigmaaldrich.com/germany.html) or two rounds 30 Gy irradiation and frozen for use. Cell cycle arrest was confirmed by BrdU staining. Coculture experiments were performed with passage #4 fibroblasts. The conditioned medium was prepared by mixing supernatant from 48 hours fibroblast cultures with fresh fibroblast medium at a 2:1 ratio.

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Figure 3. Erythropoietin (Epo) receptor (EPOR)^{pos}/myosin heavy chain (MHC)^{pos} cells can proliferate and partially differentiate into cardiomyocyte-like cells. EPOR^{pos}/ α ACT^{pos} cells (white arrows) in transversal sections of the adult mouse heart. (**B**): Cell cycle analysis of adult cardiac progenitor cells (CPCs). Central plot represents the different cell populations with their respective cell cycle histograms. (**C**): Quantification of this analysis showing higher proliferation index of EPOR^{pos}/MHC^{pos} and EPOR^{pos}/ α ACT^{pos} cells. (**D**): Confocal immunofluorescence (IF) of proliferating EPOR^{pos}/KI67^{pos} cells (white arrows). EPOR^{pos} (PCS coexpressed cardiac Troponin T (cTNT), TBX5, HAND1, and NKX2.5. (**E**): IF of CPCs isolated from α MHC-enhanced green fluorescent protein (EGFP) mice cocultured on NRCMs. Small GFP^{pos} cells on day (d)1 of coculture expressed the cardiac transcription factor NKX2.5 (white arrows, panels i–ii). GFP^{pos} cells elongated on d2 and were EdU^{pos} (white arrows, panels iv–v). GFP^{pos} cells expressed EPOR (panel vi). On d5, they showed cTNT-positive sarcomeric structures (white arrow panel vii). GFP^{neg} NRCMs (control, panel iii). EPOR-sorted cells cultured on NRCMs for 6 days (panel x–xii). DAPI was used for nuclear staining. Panels i, vii, x–xii represent Z-stack overlays generated from individual photos, presented in Figure S4D. Scale bar 5 20 µm. Data represent me a 6 SEM, two-tailed Student's t-test, **, *p* < .01, ***, *p* < .001. Abbreviations: α ACT, alpha Actinin; DAPI, 4',6-diamidino-2-phenylindole; EPOR, erythropoietin receptor; MHC, myosin heavy chain; NRCM, neonatal rat cardiomycyte.

Immunocytochemistry

CPCs were grown on collagen coated coverslips, adult mouse fibroblasts or NRCMs. The cells were washed with 2% FCScontaining PBS (FC-buffer) and stained against CD31-FITC (1:100; eBioscience, Frankfurt/Germany, www.eBioscience.com), FLK1-PE (1:100; eBioscience, Frankfurt/Germany, www.eBioscience.com), SCA1-FITC (1:100; eBioscience, Frankfurt/Germany, www.eBioscience.com), CD34-FITC (1:100; eBioscience, Frankfurt/Germany, www.eBioscience.com), cKIT-FITC (1:100; eBioscience, Frankfurt/ Germany, www.eBioscience.com), and EPOR (1:100; SCBT, Heidelberg/Germany, http://www.scbt.com/). For EPOR staining, an additional secondary antibody incubation was required (Alexa-Fluor488 or 594 1:500; Invitrogen, Darmstadt/Germany, http:// www.lifetechnologies.com/de/de/home.html). Next, the cells were washed with PBS and fixed in 4% PFA, permeabilized in 0.1% TritonX-100 and blocked with 2% FCS. For intracellular staining cells were incubated with respective antibodies against MHC (1:500; Abcam, Cambridge/UK, http://www.abcam.com), TBX5 (1:500; Abcam, Cambridge/UK, http://www.abcam.com), KI67 (1:100; Abcam, Cambridge/UK, http://www.abcam.com),

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cTNT (1:500; Abcam, Cambridge/UK, http://www.abcam.com), Vimentin ([VIM] 1:500; Abcam, Cambridge/UK, http://www. abcam.com), HAND1 (1:200; SCBT, Heidelberg/Germany, http:// www.scbt.com/), NKX2.5 (1:200; SCBT, Heidelberg/Germany, http://www.scbt.com/) or respective isotype controls. Cells were incubated with AlexaFluor488 and AlexaFluor594 conjugated secondary antibodies (1:500; Invitrogen, Darmstadt/Germany, http://www.lifetechnologies.com/de/de/home.html), counterstained with DAPI (Sigma, Hamburg/Germany, http://www.sigmaaldrich.com/germany.htm), and mounted with ProlongGold (Invitrogen, Darmstadt/Germany, http://www.lifetechnologies. com/de/de/home.html). Microscopic images were captured with a digital (IX70, Olympus, Hamburg/Germany, http://www.olympus.de/) or confocal (Fluo View TMFV 1000, Olympus, Hamburg/Germany, http://www.olympus.de/) microscope.

Proliferation Assays

Mice received daily i.p. injections of BrdU (50 mg/kg BW) for 5 days before Epo treatment. One month after injections, the hearts were dissected, digested, and subjected to FC.



Figure 4. Erythropoietin (Epo) augments mouse Epo-responsive MHC expressing cell number in vitro. **(A):** AKT, ERK, and STAT3 activation confirmed in erythropoietin (Epo)-treated (0.5 IU/ml) cell cocultures. Semiquantitative densitometry showed enhanced AKT and ERK phosphorylation (p) in Epo-treated cocultures (n 5 4). **(B):** Epo treatment increased numbers of GATA4^{pos} and cardiac Troponin T (cTNT)^{pos} on coculture day (d)6 and d14 by enhancing cell proliferation (KI67) (n 5 3). **(C):** Mitomycin C-arrested cells subjected to a similar analysis showed no effect of EPO on cell cycle arrest as confirmed by loss of KI67 expression (n 5 3). Data represent mean 6 SEM, two-tailed Student's t-test, *, p < .05, ***, p < .001. Abbreviations: AKT, RAC-alpha serine/threonine-protein kinase; CPCs, cardiac progenitor cells; cTNT, cardiac Troponin T; Epo, erythropoietin; ERK, extracellular-signal-regulated kinases.

Proliferation was detected with the BrdU Assay Kit (Roche BrdU labeling, Mannheim/Germany, http://www.roche.de/ diagnostics/index.htm) according to the manufacturer's instructions. For the in vitro assays the cells were incubated with 10 μ mol/l EdU for 24 hours. For the in vivo experiments, 200 μ g EdU was applied by a single i.p. injection a week before left anterior descending artery (LAD) ligation and Epo treatment. At the endpoint, hearts were analyzed by immuno-histochemistry (IHC). EdU-positive cells were detected with Click-it EdU Assay kit (Invitrogen, Darmstadt/Germany, http:// www.lifetechnologies.com/de/de/home.html) according to the manufacturer's instructions. Both BrdU and EdU were used in such concentration, which would intercalate into DNA to mark proliferating cells in the S-phase, but would not negatively affect their proliferation.

Myocardial Infarction

Chronic occlusion of the LAD was performed in 20 weeks old C57BL/6 female mice. LAD ligation was induced on anesthesia (2% isoflurane) with a 7-0 prolene suture (Ethicon, Norderstedt/ Deutschland, http://de.ethicon.com/healthcare-professionals). Following surgery, pain relief was administrated (buprenorphine 2 mg/kg). Myocardial infarction was confirmed by echocardiography. After 2, 4, and 10 weeks of surgery, mice were killed and the hearts were removed for further examination.

Echocardiography

Mice were anesthetized by 2% isoflurane inhalation and ventricular measurements were done with a Visual Sonics Vevo 2100 Imaging System equipped with a 45 MHz MS-550D MicroScan transducer. The observer was unaware of the treatments.

Statistical Analysis

Differences between experimental groups were analyzed using two-tailed Student's *t*-test. Data are presented as mean-6 SEM. p < .05 values were considered significant.

RESULTS

EPOR Expression Is Pronounced in the Developing Heart

Since EPOR is predominantly expressed in late stage erythroid progenitors we analyzed its expression during embryonic cardiac development (embryonic day postcoitum (ED) ED7.5, ED8.5, ED9.5, ED10.5, ED13.5, and ED15.5), and postnatal (P) maturation (P1, P3, P6, P10, P13, and 20 weeks old) in NMRI mice. The highest Epor transcript expression was detected at ED10.5 and the lowest at 20 weeks (Fig. 1A; Supporting Information Fig. S2A). Similarly, in C57BL/6 mice, the highest Epor mRNA and protein levels were detected at ED10.5, which



Figure 5. Erythropoietin (Epo) enhances Epo-responsive MHC expressing cell abundance in vivo. (A): C57BL/6 mice were administered with BrdU for 1 week followed by two intraperitoneal injections of 2 IU/g Epo. (B, C): Hemoglobin (Hb) levels, heart/body weight ratio, fractional shortening (FS), and ejection fraction (EF) were unaffected by Epo administration. (D): AKT activation was confirmed in Epo-treated hearts 12 hours post-injection (n 5 3/group). (E): Significant increase in cardiac Troponin T (cTNT)^{pos}, myosin heavy chain (MHC)^{pos} (n 5 7), and BrdU^{pos} as well as enhanced BrdU/NKX2.5^{pos} cardiac progenitor cells (CPCs) (n 5 3) in Epo-treated versus saline mice. (F): Elevated levels of MHC as well as significant augmentation of cTnt and Nkx2.5 transcripts in Epo-treated mice (n 5 6). All data apart from that in 5D refer to mice killed at 4 weeks post Epo injection. Data represent mean 6 SEM, two-tailed Student's *t*-test, *, p < .05, **, p < .01. Abbreviations: AKT, RAC-alpha serine/threonine-protein kinase; BrdU, Bromodeoxyuridine; cTNT, cardiac Troponin T; Epo, erythropoietin; WHC, myosin heavy chain; rhEPO, recombinant human erythropoietin; WB, western blot.

decreased in later stages and reached a minimum expression in the adult heart (Fig. 1B, 1C). In the latter, immunofluorescence (IF) showed EPOR expression to be confined in interstitial nonmyocyte cells with a high nucleus to cytosol ratio (Fig. 1D). Antibody specificity was corroborated by high EPOR expression in fetal liver, where erythropoiesis occurs (Supporting Information Fig. S2B, S2C) and transcript enrichment in EPOR^{pos} sorted cells (80%) (Supporting Information Fig. S2D).

EPOR Marks a Heterogeneous Immature Cell Population in the Adult Heart

Since EPOR was highly expressed during cardiogenesis and confined to interstitial nonmyocytes in the adult heart, we investigated whether these cells represent immature cells of the cardiac lineage. The 20-week-old C57BL/6 hearts were enzymatically depleted from adult myocytes and cells smaller than 30 µm were isolated. The markedly low cardiac Troponin T (cTnt) expression and the 15-fold enrichment in Sca1 expression indicated negligible cardiomyocyte contamination and CPC enrichment, respectively (Supporting Information Fig. S3A, S3B). Interestingly, a fourfold higher Epor expression was detected in both, the CPC and the SCA1-purified fractions as compared to total heart (Supporting Information Fig. S2B). CPCs expressing EPOR were characterized by coimmunostaining for EPOR and cell specific markers (Fig. 2A). EPOR was detected in MHC^{pos} immature cells; endothelial-like CD34^{pos} and FLK1^{pos} cells; in adult cardiac stem cell populations positive for SCA1 or cKIT; and in mesenchymal like-cells expressing VIM (Fig. 2A). Flow cytometry (FC) quantification showed 24% of the CPCs expressing EPOR (Fig. 2B). Among the EPOR^{pos} CPCs, 11% and 10% coexpressed MHC and CD34, respectively. In line with the aforementioned pronounced Epor transcript expression in the SCA1-purified fraction (Supporting Information Fig. S3B) 7% of the EPOR^{pos} cells coexpressed SCA1. Minor cell fractions were represented by FLK1 (1.6 %) and cKIT (0.6 %) EPOR^{pos} cells (Fig. 2B). Consistent with our in vivo observations, in vitro differentiation of EPOR^{pos} immature cells (coexpressing CD31^{pos}, VIM^{pos} or MHC^{pos}) led to a strong EPOR downregulation (Fig. 2C). Altogether these findings indicate that EPOR marks a heterogeneous immature cell population in the adult heart.

EPOR/MHC Double-Positive Cells Represent Proliferating Cardiomyogenic Cells

IHC analysis of adult cardiac tissue revealed several clusters of small EPOR^{pos} cells with an atypical α ACT expression (Fig. 3A). To investigate the proliferation potential of these cells, isolated CPCs costained for EPOR and MHC or αACT were subjected to cell cycle analysis (Fig. 3B; Supporting Information Fig. S4A). The threefold increase in EPOR^{pos}/MHC^{pos} cells in the S- and G2-phase indicated a higher proliferation potential (Fig. 3B, 3C). Similarly, MHC^{pos} cells derived from transgenic mice expressing EGFP under the α MHC promoter [19] showed an increased proliferation index (Supporting Information Fig. S4B). Additionally, 126 3% MHCpos cells costained with KI67 (Supporting Information Fig. S4C) and in culture several EPOR/MHC/KI67 triple-positive cells were detected (Fig. 3D). The cardiomyogenic nature of these cells was further verified by coexpression of typical structural proteins as cTNT and transcription factors as TBX5, HAND1, and NKX2.5 (Fig. 3D). CPCs isolated from the aforementioned α MHC-EGFP mice were labeled with EdU and cocultured on NRCMs for 5-6

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Figure 6. Erythropoietin (Epo) reduces cardiac function deterioration on cardiac ischemia. (A): Infarcted and sham C57BL/6 mice were administered twice. 2 IU/g Epo and were subjected to analysis 1, 2, 4, and 10 weeks on left anterior descending artery ligation ($n \ 5 \ 8$). (B): Hemoglobin (Hb) levels were insignificantly elevated in Epo-treated mice. (C): Kaplan-Meier curve showing improved survival of Epo-treated mice ($n \ 5 \ 14 \$ saline control; $n \ 5 \ 14 \$ Epo-treated mice). (D): Partially preserved EF and fractional area shortening (FAS) of Epo-treated mice at 1, 4, and 10 weeks post myocardial ischemia (MI) (saline $n \ 5 \ 6, \ Epo \ n \ 5 \ 5)$. (E): DEF and DFAS, representing the EF and FS differences between pre-MI and 10 weeks post-MI cardiac function. Data represent mean 6 SEM, two-tailed Student's t-test, *, p < .05. Abbreviations: EF, ejection fraction; Epo, erythropoietin; FAS, fractional area shortening; FC, flow cytometry; Hb, haemoglobin; IHC, immunohistochemistry; MI, myocardial ischemia; OP, operation; rhEPO, recombinant human erythropoietin.

days. The first day of culture (d1) clusters of small GFP^{pos}/ NKX2.5^{pos} cells with high nucleus to cytoplasm ratio were observed (Fig. 3E). In the following days, GFP^{pos} cells expanded, elongated, exhibited EdU incorporation, and only small immature cells coexpressed EPOR (Fig. 3E). Next, EPORpos cells were purified from the CPC-enriched fraction to 80% purity (Supporting Information Fig. S2D) and cocultured on NRCMs. Microscopic semiquantification of EPOR^{pos} cells showed 89% purity and 28% GFP content (Supporting Information Fig. S4C). Some EPOR^{pos}/GFP^{pos} cells differentiated into contracting cardiomyocyte-like cells (Fig. 3E; Supporting Information Fig. S4D; Supporting Information Video 1). To examine whether the cardiomyocyte-like cells are a result of cell fusion, we cocultured EPOR^{pos}/GFP^{pos} cells with NRCMs isolated from transgenic rats that ubiquitously express AsRed [20]. No GFP^{pos} cardiomyocyte-like cells coexpressed AsRed corroborating that bona fide differentiation rather than cell fusion had occurred (Supporting Information Fig. S4E). These data collectively support the hypothesis that EPOR^{pos}/MHC^{pos} cells of the adult heart are cardiomyocyte-committed cells with a proliferation and differentiation potential.

Epo-Responsive MHC-Expressing Cells

To investigate the responsiveness of EPOR^{pos} cells to Epo, an enriched CPC fraction was cocultured on mitomycin C-arrested adult cardiac mouse fibroblasts and treated with 0.5 IU/ml Epo β for 30 minutes. Epo induced significant AKT phosphorylation and enhanced ERK activation exclusively in CPCs, while STAT-3 activation was observed in cocultures as well as in fibroblasts alone (Fig. 4A). Furthermore, cocultured CPCs were treated every 2 days with 0.5 IU/ml of Epo β and analyzed on days 4, 6, and 14. On a single Epo pulse, a significant increase in GATA4^{pos} and cTNT^{pos} cells was observed (Fig. 4B). On d14, i.e., after 5 consecutive Epo pulses, the Epotreated group showed approximately a doubling of $\mathsf{GATA4}^{\mathsf{pos}}$ and cTNT^{pos} cells compared with d4 (Fig. 4B); enhanced KI67^{pos} cell number suggested that Epo promoted cell proliferation. To verify this finding, mitomycin C-arrested CPCs, were cocultured under Epo treatment (Fig. 4C). On inhibition of proliferation, no effect of Epo on GATA4pos and cTNTpos cell number was observed confirming that Epo affects mainly immature cell survival and proliferation rather than commitment and differentiation. As Epo stimulated these immature



Figure 7. Erythropoietin (Epo) enhances Epo-responsive MHC expressing cell number and modifies scar cellularity on cardiac ischemia. **(A)**: Representative confocal immunofluorescence pictures, partially reconstructed from single pictures, of 2 weeks post-myocardial ischemia (MI) transversal heart sections showing Epo receptor (EPOR)^{pos}/cardiac Troponin T (cTNT)^{pos} cells. Border zone and scar areas showed several small EPOR^{pos}/cTNT^{pos} cells in both groups (white arrows) in contrast to remote areas as the right ventricular (RV) epicardium (insert). **(B)**: Flow cytometry analysis showed that MI hearts contained more EPOR^{pos} cardiac progenitor cells (CPCs) in comparison with sham animals. CD31^{pos}, TBX5^{pos}, myosin heavy chain (MHC)^{pos}, cTNT^{pos}, as well as double cTNT^{pos}/TBX5^{pos} cells were significantly increased in Epo-treated animals compared with saline (Sham: saline and Epo *n* 5 5, MI: saline *n* 5 7, Epo *n* 5 8). **(C)**: 10 weeks post-MI more EPOR^{pos} endocardial (white arrows) and interstitial EPOR^{pos}/cTNT^{pos} cells (white arrows with black outline) as well as a STNT^{pos} cardiac Student's t-test, *, *p* < .05, **, *p* < .01, ***, *p* < .001. Abbreviations: cTNT, cardiac Troponin T; DAPI, 4',6-diami-dino-2-phenylindole; Epo, erythropoietin; EPOR, erythropoietin receptor; LV, left ventricular; MHC, myosin heavy chain; MI, myocardial ischemia; RV, right ventricular.

myocyte-like cells in the adult heart, we designated this population as Epo-responsive MHC-expressing cells (EMCs).

Epo Amplifies EMCs In Vivo

We tested the effect of Epo on EMC expansion in vivo. C57BL/6 mice were injected twice with 2 IU/g Epo β (i.p.) in the first 2 weeks and BrdU was administered daily for 1 week before Epo administration (Fig. 5A). No difference was observed between the saline and Epo-treated group concerning hemoglobin (Hb) levels, heart to body weight ratio, and cardiac function (Fig. 5B, 5C). 12 hours after treatment enhanced AKT-phosphorylation was confirmed in heart tissue of the Epo-treated animals (Fig. 5D). Following 4 weeks of Epo-treatment, analysis of the CPCs showed a significant increase in cTNT^{pos} and MHC^{pos} cells as well as enhanced proliferation marked by BrdU^{pos} cells in Epo-treated mice. BrdU-^{pos}/NKX2.5^{pos} cell number was also augmented, supporting the in vitro findings of a mitogenic effect of Epo on EMCs

(Fig. 5E). This result was further verified by qPCR (Fig. 5F). Together these data suggest that Epo induces EMC proliferation in vivo.

Epo Effect on EMCs in the Injured Adult Heart

Finally, we investigated the effect of Epo on EMCs post ischemia. C57BL/6 mice were injected with EdU to label proliferation a week prior to LAD ligation (Fig. 6A). Immediately and 1 week after ligation mice were injected with 2 IU/g Epo β (i.p.) that did not affect Hb levels (Fig. 6B). Epo-receiving animals showed an improved survival (n 5 14 saline control; n 5 14 Epo-treated mice; Fig. 6C) and partially preserved myocardial function (DEF 5 2 116 1%, DFAS 5 2 106 2% n 5 5) compared with control (DEF 5 2 206 2%, DFAS 5 2 176 2% n 5 6; Fig. 6D–6E). Two weeks post-MI, IF showed several small EPOR^{pos/} cTNT^{pos} cells in the border zone of both saline and Epo-treated hearts (Fig. 7A). Interestingly, the latter seemed to contain more proliferating EPOR^{pos/} EdU^{pos} cells as compared

to the saline control (Supporting Information Fig. S6). Indeed, 4 weeks post-MI, FC analysis showed that Epo significantly augmented EMC number characterized by MHC, cTNT and TBX5 expression as well as CD31^{pos} cells in a lesser amount (Fig. 7B). In the same line, 10 weeks post-MI, visualization of the scar showed increased cardiomyocyte, EPOR^{pos} cell, and EMC content, preferentially located in the endocardium of Epo-treated mice (Fig. 7C). Finally, more EdU^{pos}/cTNT^{pos} and EdU^{pos}/TBX5^{pos} cells were detected in the scar and the border zone of Epo-treated animals (Supporting Information Fig. S7), suggesting that these myocytes derived from immature proliferating cells. Altogether these data suggest that Epo administration in ischemic hearts increases the number of immature cells, including EMCs. Activation of EMCs may have contributed to a better outcome in the Epo-treated group

DISCUSSION

The role for Epo in myocardial protection remains elusive despite comprehensive, but contradictory preclinical and clinical studies [21]. Thus, we find it necessary to go back to the bench-side and define Epo-responsive cells and the consequences of their activation in the heart. In this study, we identified a subpopulation of EPOR^{pos} immature cardiomyocyte-committed cells (EMCs). In response to Epo stimulation, EMCs activated the AKT-prosurvival pathway and demonstrated enhanced cell cycle activity; this was paralleled by better survival in a mouse model of myocardial infarction.

We found EPOR to be strongly expressed in the early developing heart and in immature cardiac cells in the adult heart. Similarly, high EPOR expression was detected in the early developing nervous system [22], while low postnatal EPOR expression was confined to adult neural progenitor cells [23]. Immature cardiac cells showed strong downregulation of EPOR on differentiation, a phenomenon observed also in erythroid progenitor cells [24]. These data support the notion that Epo-EPOR system has mainly a role in undifferentiated cells.

 $\operatorname{\mathsf{EPOR}}^{\operatorname{\mathsf{pos}}}$ subpopulations in the adult heart consisted of MHC $^{\rm pos}$ (${\sim}11\%$), CD34 $^{\rm pos}$ (${\sim}10\%$), and SCA1 $^{\rm pos}$ (${\sim}7\%$) cells. Recent studies demonstrated that CD34pos cells from the bone marrow [10] and SCA1^{pos} cells in the heart [25] can be activated by Epo and contribute to neovascularization in the diseased heart. We were particularly interested in the MHC^{pos} subpopulation, which morphologically did not resemble bona fide cardiomyocytes. In vitro lineage tracing showed that these cells had the ability to expand and differentiate toward cardiomyocyte-like cells, suggesting a potential for cardiomyocvte generation. In line with previous data [26] only few cells differentiated into functional myocytes in the course of 6 day coculture, likely due to the limited time in culture and lack of proper environmental cues to trigger cardiomyocyte differentiation. Importantly, the observed cardiomyocyte differentiation events did not result from cell fusion. To further characterize and specifically expand the EPOR^{pos}/MHC^{pos} cells (EMCs) clonal analysis was attempted, but was not successful. Single EMCs did not survive without a feeder laver indicating that additional efforts are needed to define the proper microenvironment of EMC maintenance and amplification. Aside from the hypothesis that EMCs resemble a subpopulation of cardiac progenitor cells (CPCs) we acknowledge that EMCs

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may represent immature, but replication competent cardiomyocytes or dedifferentiated cardiomyocytes [3].

The fact that EPOR expression was found mainly in dividing rather than senescent cells indicated a role of the Epo-EPOR system in cell proliferation. Indeed, we observed in vitro and in vivo that Epo promoted EMC proliferation and activated AKT signaling, which typically mediates the protective and mitogenic properties of Epo [27] as shown in erythroid [28] and neural progenitors [29]. Accordingly, conditional brain-specific deletion of EPOR resulted in reduced cell proliferation in the subventricular zone where in vivo neurogenesis occurs in the adult mouse [23]. It should be noted that Epo does not exclusively affect EMCs since we found several noncardiomyogenic cell populations expressing EPOR including endothelial progenitors, which is in agreement with the role of Epo in angiogenesis in the cardiac system [11, 25].

To confirm the role for Epo in myocardial protection, we performed two Epo injections (total 4 IU/g; i.p.) directly and 1 week after myocardial infarction. This resulted in better survival and functional preservation of the infarcted hearts compared with the saline group; note, that the Epo dose used here was similar to Epo doses administered in previous studies (5-8 IU/g) [9, 10, 30]. Recent small cohort clinical trials demonstrated a similar beneficial role of Epo for cardiac function after MI [31, 32]. However, larger clinical studies using single or repetitive bolus Epo injections were neutral with respect to cardiac end-points and in part negative with respect to thromboembolic complications [12, 13]. These clinical trials differed in Epo formulation, dosing, and route as well as timing of administration. From the clinical trials we infer that additional preclinical data is needed to define the mechanism of action of Epo on the heart.

At the cellular level, we observed that Epo induced EPOR-^{pos} cell proliferation and EMCs accumulation in ischemic myocardium. In line with our data, intracardiac injections of Epo in infarcted rat myocardium enhanced proliferation of interstitial cells [33]. Ten weeks post-MI, we found a higher number of intracardiac EMCs located in between well-preserved myocytes in the scar of Epo-treated mice. Being aware of the difficulties to unambiguously demonstrate newly generated cardiomyocytes we attempted to identify proliferating EMCs by administering EdU prior to any intervention. Ten weeks post-MI, EdU-labeled cTNT^{pos} and TBX5^{pos} cells with a clear mature cardiomyocyte-like structure were observed in the Epo-treated hearts. Collectively these data suggest that application of Epo after myocardial infarction results in a favorable cardiac remodeling by affecting a variety of immature cell populations, including EMCs. These cells may not only replenish the heart with cardiac cells but may also secrete protective paracrine factors inducing cardiac growth [34, 35] .

CONCLUSION

In conclusion, we show that EPOR is highly expressed in undifferentiated embryonic and adult cardiac cells. Specifically, EMCs proliferate and partially differentiate toward cardiomyocyte-like cells in response to Epo stimulation in vitro and in vivo. Following MI, Epo administration resulted in an increase in EMCs and preservation of cardiac function. Collectively, our data point to a novel cellular mechanism for the

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cardioprotective action of Epo-namely, the activation of EMCs in the adult heart.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

M.P.Z.: conception and design, performed research, collection and/or assembly of data, data analysis and interpretation,

The authors indicate no potential conflicts of interest.

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