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C-kit$^+$ resident cardiac stem cells improve left ventricular fibrosis in pressure overload

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ABSTRACT

To investigate the effect of resident cardiac stem cells (RCSC) on myocardial remodeling, c-kit$^+$ RCSC were isolated from hearts of C57Bl/6-Tg (ACTb-EGFP)1Osb/J mice expressing green fluorescent protein and expanded in vitro. C57/Bl6N wildtype mice were subjected to transverse aortic constriction (TAC, 360 μm) or sham-operation. 5×10^5 c-kit$^+$ RCSC or c-kit$^-$ cardiac cells or cell buffer were infused intravenously 24 h post-surgery (n = 11–24 per group). Hypoxia-inducible factor-1α-mRNA in left ventricles of TAC mice was enhanced 24 h after transplantation. 35 days post-TAC, the density of c-kit$^+$ RCSC in the myocardium was increased by two-fold. Infusion of c-kit$^+$ resident cardiac stem cells post-TAC markedly reduced myocardial fibrosis and the expression of collagen Iα2 and connective tissue growth factor. Infusion of c-kit$^-$ cardiac cells did not ameliorate cardiac fibrosis. In parallel, expression of pro-angiogenic mediators (FGFb, IL-4, IL-6, TGFß, leptin) and the density of CD31$^+$ and CD31$^+$ GFP$^+$ endothelial cells were increased. Transplantation reduced brain- and atrial natriuretic peptides and the cardiomyocyte cross-sectional area. Infusion of c-kit$^+$ resident cardiac stem reduced the rate of apoptosis and oxidative stress in cardiomyocytes and in non-cardiomyocyte cells.

1. Introduction

Hypertrophy, cardiac fibrosis and deterioration of myocardial capillarization are major components of cardiac remodeling e.g. caused by sustained pressure overload that can ultimately lead to heart failure (Burchfield et al. 2013; Segura et al. 2014). There is evidence that cardiomyocytes can repopulate in the human heart, but this process appears to be very slow and only a low number of cardiomyocytes appears to be exchanged over the course of a human life span (Bergmann and Jovinge 2014; Buja and Vela 2008; Senyo et al. 2013). Moreover, adverse tissue remodeling, particularly cardiac fibrosis, in pathological conditions disturbs renewal of cardiomyocytes and other cardiac cells (Bergmann and Jovinge 2014; Buja and Vela 2008; Kazakov et al. 2013; Kazakov et al. 2012; Müller et al. 2009; Müller et al. 2008; Senyo et al. 2013). Recent investigations suggest participation of bone-marrow derived stem cells and resident cardiac stem cells (RCSC) during normal and pathological remodeling of the adult heart (Beltrami et al. 2003; Bergmann and Jovinge 2014; Ellison et al. 2013; Hierlihy et al. 2002; Kazakov et al. 2013; Kazakov et al. 2012; Müller et al. 2009; Müller et al. 2008; Senyo et al. 2013; van Berlo et al. 2014). The adult endogenous c-kit$^+$ CD45$^-$ tryptase$^-$ CD31$^-$ CD34$^-$ cardiac stem cells (c-kit$^+$ RCSC) participate in myocardial adaptation to physiological and pathological stimuli (Beltrami et al. 2003; Ellison et al. 2013; Hierlihy et al. 2002; van Berlo et al. 2014). Furthermore, intramyocardial and intracoronary transplantation of c-kit$^+$ RCSC has been observed to improve cardiac regeneration (Beltrami et al. 2003; Ellison et al. 2013; Li et al. 2011). However, their role in maladaptive cardiac remodeling caused by sustained pressure overload is not completely understood (Rupp et al. 2012). Pressure overload, e.g. induced by aortic stenosis or arterial hypertension is a common cause of myocardial remodeling. Therefore, we investigated the influence of intravenous transplantation of c-kit$^+$ resident cardiac stem cells on cardiac remodeling induced by transverse aortic constriction.

2. Methods

2.1. Animals, transverse aortic constriction

The study was approved by the animal Ethics Committee of the Universität des Saarlandes and conforms with the Guide for the Care

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and Use of Laboratory Animals published by the Association for Assessment and Accreditation of Laboratory Animal Care (NRC 2011). Ten-week-old male C57/Bl6 (Charles River Laboratories, Sulzfeld, Germany) mice were housed under standard conditions. For surgery and left ventricle (LV)-pressure measurements animals were intraperitoneally anesthetized with 100 mg/kg body weight ketaminehydrochloride (Ketanest®, Pfizer, Berlin, Germany) and 10 mg/kg body weight xylazinehydrochloride (Rompun® 2%, Bayer, Leverkusen, Germany). Anesthetic monitoring was performed by testing of rear foot reflexes before and during procedures, observation of respiratory pattern, mucous membrane color, and responsiveness to manipulations throughout the procedures. After orotracheal intubation using a 20 G catheter, the tube was connected to a volume cycled rodent ventilator (Harvard Apparatus, USA) on supplemental oxygen with a tidal volume of 0.2 mL and respiratory rate of 110 min^{-1}. The chest cavity was entered in the second intercostal space at the left upper sternal border through a small incision and aortic constriction was performed by tying a 7–0 nylon suture ligature against a 27 G needle to yield a narrowing 360 μm in diameter and a transverse aortic constriction (TAC) of 65–70%. Control mice underwent a sham operation. Intravenous transplantation of c-kit$^+$ RCSC was performed 24 h post-surgery as described below. After 5 weeks, LV-pressure measurements were performed with 1.4 Fr pressure-transducing catheter (Mikro Tip Catheter, Millar instruments, USA). Mice were sacrificed 3 h, 24 h and 5 weeks after stem cell transplantation by i.p. injection of ketamine (1 g/kg body weight) and xylazine (100 mg/kg) and hearts were rapidly excised. Hearts and other internal organs were partly snap-frozen in liquid nitrogen and stored at −80 °C and partly embedded in paraffin after fixation in PBS-buffered formalin (4%).

2.2. Cardiac c-kit$^+$ cells: isolation, culture and intravenous transplantation

Ten-week old C57Bl/6-Tg(ACTbEGFP)1Osb mice (Jackson laboratory; expressing green fluorescent protein (GFP) ubiquitously) were sacrificed by i.p. injection of ketamine (1 g/kg body weight) and xylazine (10 mg/kg). Cardiac cells isolated by heart perfusion with 0.1% collagenase (Worthington, USA) for 23 min and mincing in Ham's F-12 medium (Sigma-Aldrich, Germany) were centrifuged, supplemented with the fresh medium, sorted with mouse anti-c-kit antibody conjugated with magnetic beads (Miltenyi Biotec, Germany) for 15 min and separated by magnet-activated cell sorting into c-kit$^+$ and c-kit$^-$ fractions (Fig. 1). c-kit$^+$ RCSC and c-kit$^-$ cardiac cells (c-kit$^-$CC) were expanded in cell culture until the second passage and partly immunostained for stem cell markers c-kit (Santa Cruz Biotechnology, USA) Oct3/4 and Kruppel-like factor 4 (Klf4) (abcam, UK) (Fig. 1). Cells at 85% confluence in cell culture dishes were harvested by trypsinization (Invitrogen, Germany) and passed through a 40 μm cell strainer (BD Bioscience, USA). 5×10^5 c-kit$^+$ RCSC resuspended in stem cell buffer (provided in detail in the Supplementary material online) were infused in the tail vein of SHAM + cells (c-kit$^+$) and TAC + cells (c-kit$^+$) mice 24 h post-surgery (n = 15–24 per group). Control mice received an injection of the stem cell buffer (SHAM + buffer, TAC + buffer n = 11–16 per group). To control the specificity of the effects observed after c-kit$^+$ RCSC transplantation, 5×10^5 c-kit$^-$ cardiac cells were infused in the tail vein of SHAM + c-kit$^-$ (n = 3) and TAC + c-kit$^-$ (n = 4) mice. For this purpose animals were anesthetized by inhalation of 2% isoflurane and the adequacy of anesthesia was evaluated as described above. To prevent embolization, all mice prior to stem cells or vehicle injection were intravenously injected with 200 μl (20 USP units) heparin (B.Braun Melsungen AG, Germany) and 50 μl of 0.5 mg/ml sodium nitroprusside (1 mg/kg body weight) (Schwarz Pharma, Germany) (Boomsma et al. 2007). For transplantation of Tie-2-GFP c-kit$^+$ RCSC, Tie-2-GFP mice (FVB/NTgN[TIE2GFP]287Sato; Jackson Laboratory) were used as donors of c-kit$^+$ RCSC, and unoperated wild-type FVB/NJ mice (Charles River, Germany), n = 5, were used as recipients (Müller et al. 2009). To investigate differentiation capability, c-kit$^+$ RCSC isolated from 8 mice were cultivated until the third passage in stem cell medium or for 3 days and 5 weeks in differentiation medium (Minimum essential medium eagle, alpha modification Sigma-Aldrich, Germany) supplemented with 10% calf serum (PAA, Austria) and dexamethasone (Sigma-Aldrich, Germany) and immunostained for marker of stem cells stem cell antigen-1(Sca-1) (Cedarlane, Canada), CD34 (DAKO, Germany), mast cell tryptase (DAKO, Germany), leucocytes CD45 (abcam, UK), endothelial cell podocalyxin (R&D Systems, USA), von Willebrand factor (abcam, UK), CD31 (abcam, UK), fibroblast marker discoidin domain-containing receptor 2 (DDR2) (Santa Cruz Biotechnology, USA), smooth muscle cell marker α-smooth muscle actin (Sigma-Aldrich, Germany), cardiomyocyte marker cardiac troponin T–C (Santa Cruz Biotechnology, USA) and receptor of SDF-1 CXCR4 (abcam, UK). To estimate the extent of c-kit$^+$ RCSC differentiation each immunostaining was performed on three cell culture dishes per mouse and the number of cells immunostained for analyzing marker per 300 cells were counted in each cell culture dish.

2.3. Immunofluorescence analysis

To detect c-kit$^+$ RCSC, cardiomyocytes, endothelial cells, smooth muscle cells, fibroblasts, hepatocytes, nestin$^+$ cells, GFP$^+$ cells, Ki67$^+$ cycling cells and level of oxidative stress immunostainings on 3 μm paraffin sections of the LV were performed using heat-mediated antigen retrieval with citraconic anhydride solution followed by overnight incubation at 4 °C with the primary antibody and incubation with the appropriate secondary antibody at 37 °C for 1 h. The detailed methods are described in the Supplementary material online.

2.4. Apoptosis

Apoptosis detection was performed on 3 μm thin sections of formalin-fixed heart sections with the ApopTag Peroxidase In Situ Oligo Ligation Kit (Millipore, Germany). To evaluate apoptosis rate in cardiomyocytes and non-cardiomyocytes, immunostaining for cardiomyocyte marker α-sarcomeric actin (Sigma-Aldrich, Germany) was performed. The analysis protocol is described in more detail in the Supplementary material online.

2.5. Tissue morphometry

The procedures used for morphometric analyses are provided in detail in the Supplementary material online.

2.6. Expression analysis

Gene expression was measured by the real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) using the TaqMan system (AB Step One Plus, Applied Biosystems, Germany). Briefly, RNA from the LV myocardium was extracted using the peqGOLD RNAPure (Peqlab Biotechnologie, Germany). Oligo (dT) primed cDNA synthesis was performed with High capacity cDNA reverse transcription kit (Life Technologies, Germany). Expression of collagen Iα2 (Mm01165187_m1), brain natriuretic peptide (BNP) (Mm01255770_g1), atrial natriuretic peptide (ANP) (Mm01255747_g1), connective tissue growth factor (CTGF), (Mm01192931_g1), hypoxia-inducible factor-1α (Hif1α) (Mm00468869_m1), Oct3/4 (Mm03053917_g1), hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (Mm 01545399_m1) and 18S rRNA was analyzed with TaqMan gene expression assays-on-demand purchased from Life Technologies. For quantification, mRNA amount of the respective gene was normalized to the amount of 18S rRNA and HGPRT using the $2^{-\Delta\Delta CT}$ method. To assess activators and inhibitors of angiogenesis in the LV myocardium, tissue protein extracts were analyzed using the Mouse Angiogenesis Antibody Array Kit (Affymetrix, UK). The BNP level in the peripheral blood was analyzed by enzyme immunoassay (RayBiotech, USA).

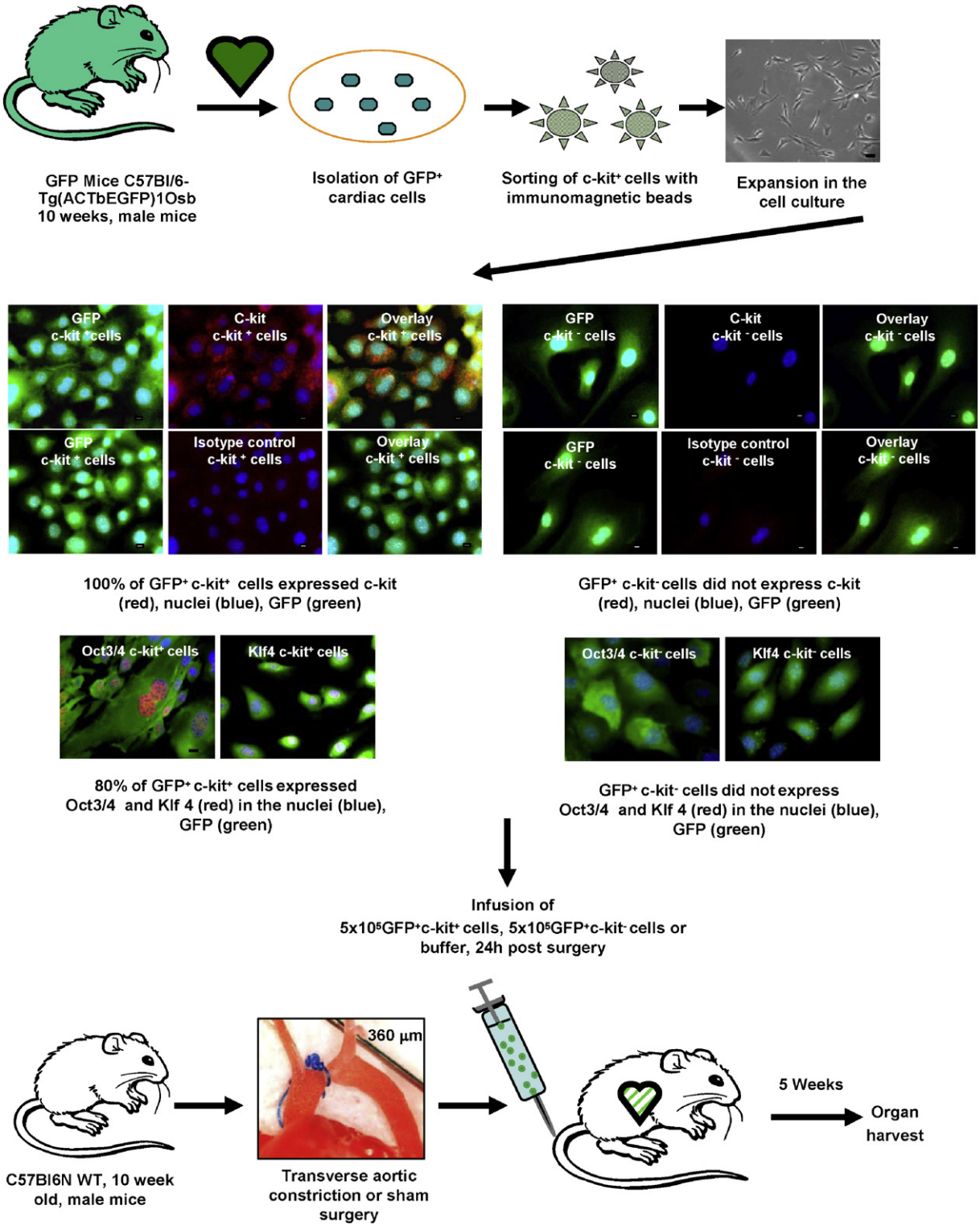

Fig. 1. Isolation and transplantation of GFP$^+$ c-kit$^+$ cells. Cardiac cells from C57Bl/6-Tg(ACTbEGFP)1Osb mice were isolated by heart perfusion with collagenase, sorted with mouse anti-c-kit antibody conjugated with magnetic beads and separated by magnet-activated cell sorting into c-kit$^+$ and c-kit$^-$ fractions. c-kit$^+$ RCSC were expanded in the cell culture and immunostained for stem cell markers c-kit (red) and Oct3/4 (red), Klf4 (red), and GFP (green). Nuclei are stained blue by DAPI. Each immunostaining was performed on 3 cell culture dishes per mouse (n = 8) and number of cells immunostained for stem cell marker per 300 cells were counted in each cell culture dish. 5×10^5 c-kit$^+$ RCSC resuspended in stem cell buffer were infused in the tail vein of ten-week-old male C57/Bl6 SHAM + cells (n = 15) and TAC + cells (n = 24) mice 24 h post-surgery. Control mice SHAM + buffer (n = 11) and TAC + buffer (n = 16) received an injection of the stem cell buffer. To exclude unspecific effects of c-kit$^+$ RCSC transplantation, 5×10^5 c-kit$^-$ cardiac cells were infused in the tail vein of SHAM + c-kit$^-$ (n = 3) and TAC + c-kit$^-$ (n = 4) mice. Mice were sacrificed after 5 weeks and organs were harvested. Bars = 10 µm.

2.7. Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Mann–Whitney tests were used for the comparison of two groups. For experiments with more than two groups, one-way ANOVA with Fisher-LSD post-hoc tests were used. Correlations were assessed with Spearman analyses. Values of $p < 0.05$ were considered significant. SPSS version 18.0 (SPSS Inc., Chicago, Illinois) was used for statistical calculations.

3. Results

3.1. C-kit$^+$ RCSC differentiate into main cardiac lineages and migrate to the myocardium

Immunostaining revealed that all isolated and expanded in cell culture resident cardiac stem cells were positive for c-kit and 80% expressed Oct3/4 and Klf4 in the nucleus (Fig. 1, Suppl. Fig. 3). C-kit$^-$ cardiac cells were negative for Oct3/4 and Klf4. Transplanted cells were c-kit$^+$ Sca-1$^-$ CD45$^-$ CD31$^-$ CD34$^-$ tryptase$^-$ (Fig. 2). Stem cells cultivated in Ham's F-12 stem cell medium until the third passage spontaneously differentiated and expressed markers of main cardiac linages, the endothelial markers podocalyxin, CD31 and von Willebrand factor, the cardiomyocyte marker troponin T–C, the smooth muscle cell marker α-smooth muscle actin, the fibroblast marker DDR2 and SDF-1 receptor CXCR4 (Fig. 2A–E). The most pronounced increase was observed for the endothelial cell markers podocalyxin and von Willebrand factor (20 to 30-fold, Fig. 2A–B).

Longer cultivation for 5 weeks in differentiation medium induced differentiation of c-kit$^+$ GFP$^+$ RCSC mainly towards a smooth muscle phenotype. 28.2 ± 5.2% of the cells expressed α-smooth muscle actin, 9.7 ± 2% expressed cardiac troponin T–C, 16.7 ± 5% and 12.1 ± 3% expressed podocalyxin and von Willebrand factor respectively and 15.5 ± 2% expressed DDR2 (Fig. 2A–E).

c-kit$^+$ GFP$^+$ RCSC injected intravenously migrated to the heart and other internal organs where they were detectable as early as 3 h after injection (Fig. 3A, D, E, J). The mRNA-expression level of Oct3/4 was significantly increased in the pressure-overloaded myocardium 24 h after c-kit$^+$ RCSC-transplantation (Fig. 3F). Pressure overload for 3 h, 24 h and 5 weeks led to a two-fold increase of GFP$^+$ injected cells per mm^2 in the LV myocardium (Fig. 3A–C). GFP$^+$ cardiomyocytes were identified in 3 of 24 (12%) TAC + cell mice (Fig. 3K). GFP$^+$ cells were also found in the lungs, spleen, kidneys and liver of RCSC-transplanted mice 5 weeks after transplantation (Fig. 3L). In the liver of four (12.8%) RCSC-transplanted animals, GFP$^+$ cells expressing the hepatocyte marker cytokeratin 8 were detected (Fig. 3M). About 25% of injected GFP$^+$ cells expressed c-kit or α-smooth muscle actin 5 weeks after transplantation (Fig. 3G, H, N, O). Injection of c-kit$^+$ GFP$^+$ RCSC increased the number of nestin-positive GFP-negative cardiomyocytes during pressure overload for 5 weeks (Fig. 3I, P).

3.2. Intravenous transplantation of c-kit$^+$ RCSC improves parameters of LV hemodynamics and reduces cardiomyocyte cross-sectional area

Transverse aortic constriction increased LVSP, LVEDP, $dP/dt_{max,}$ the ratio of wet lung weight to tibia length, and decreased dP/dt_{min} (Table 1). As expected, TAC induced both myocardial (increased ratio of heart weight to tibia length) and cardiomyocyte hypertrophy (increased cardiomyocyte cross-sectional area). Transplantation of c-kit$^+$ RCSC improved LV systolic and diastolic function (elevated $LVdP/dt_{max}$ and diminished $LVdP/dt_{min}$), reduced the cardiomyocyte cross-sectional area and increased cardiomyocyte density (Table 1). Transplantation of c-kit$^-$ cardiac cells did not improve parameters of LV hemodynamics and cardiomyocyte hypertrophy during TAC (Table 1).

3.3. Increase of endothelial cell number and Hif1α-expression in the pressure-overloaded myocardium after c-kit$^+$ RCSC transplantation

Transplantation of c-kit$^+$ RCSC increased the number of CD31$^+$ endothelial cells per mm^2 but not the ratio of CD31$^+$ endothelial cells to α-sarcomeric actin-positive cardiomyocytes in TAC mice (Fig. 4A, B, E). Transplanted cells differentiated into CD31$^+$ cells and pressure overload significantly increased their percentage (Fig. 4C, D, F). To assess whether transplanted c-kit$^+$ RCSC were transdifferentiating into endothelial cells in our model and to exclude the possibility that the findings were due to non-endothelial cells lying in the vicinity of true endothelial cells, transplantation studies of c-kit$^+$ RCSC from Tie-2-GFP FVB/N mice into their wild-type FVB/N mice were performed. After c-kit$^+$ RCSC transplantation from Tie-2-GFP mice, all animals showed GFP-positive cells in the heart (Fig. 4G). GFP expression in these mice is under the control of the endothelial promoter Tie-2. In the c-kit$^+$ RCSC recipients, GFP-positive cells therefore represent endothelial cells from injected c-kit$^+$ RCSC.

Real-time PCR of myocardial tissue revealed increased Hif1α mRNA expression 24 h after c-kit$^+$ RCSC transplantation in pressure-overloaded myocardium (SHAM + buffer 100 ± 18%, SHAM + cells 104 ± 20%, TAC + cells 209 ± 41% vs. TAC + buffer 108 ± 10%, $p = 0.01$) (Fig. 4H). Five weeks after the transplantation, Hif1α mRNA was down-regulated in all groups with the exception of SHAM + buffer (SHAM + buffer 100 ± 30%, SHAM + cells 58 ± 30%, TAC + cells 50 ± 15% vs. TAC + buffer 50 ± 18%, n.s.) (Fig. 4H).

3.4. Transplantation of c-kit$^+$ RCSC shifts the balance between activators and inhibitors of angiogenesis towards activators

Total protein concentration of all pro-angiogenic mediators in the pressure-overloaded LV myocardium was not significantly changed 24 h after c-kit$^+$ RCSC transplantation (Fig. 4I). Specifically, the summarized expression of epidermal growth factor (EGF), acidic fibroblast growth factor (FGFa), basic fibroblast growth factor (FGFb), granulocyte-colony stimulating factor (G-CSF), interleukin-1alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin-4 (IL-4), interleukin-6 (IL-6), leptin, transforming growth factor alpha (TGFα), and transforming growth factor beta (TGFβ), tumor necrosis factor alpha (TNFα), vascular endothelial growth factor (VEGF) remained unchanged (Fig. 4I). Five weeks post-transplantation, protein expression of all pro-angiogenic mediators in the pressure-overloaded LV was significantly increased, $p = 0.0004$ (Fig. 4I). Specifically, the expression of FGFb, IL-4, IL-6, leptin, TGFβ was significantly increased by two- to four-fold (Fig. 4K).

In contrast, expression of all anti-angiogenic factors was markedly reduced 24 h post-transplantation, $p = 0.04$ (Fig. 4I). Specifically, the expression of interferon gamma (IFNγ) and IFNγ-inducible protein 10 (IP-10) was reduced by 30–60% (Fig. 4J). Although five weeks after stem cell transplantation, an over-all increased expression of all anti-angiogenic factors IFNγ; interleukin-12 (IL-12), IP-10, tissue inhibitor of metalloproteinases 1 and 2 (TIMP-1,TIMP-2) was observed, $p = 0.04$, protein concentration of pro-angiogenic mediators in the pressure-overloaded LV was higher, $p = 0.04$ (Fig. 4I). In summary, the intravenous transplantation of c-kit$^+$ RCSC shifted the balance between activators and inhibitors of angiogenesis towards activators in the pressure-overloaded myocardium.

3.5. Intravenous transplantation of c-kit$^+$ RCSC reduces myocardial level of oxidative stress and cardiac fibrosis in TAC

Intravenous infusion of c-kit$^+$ RCSC diminished oxidative stress induced by pressure-overload both in cardiomyocytes and in non-cardiomyocytes evaluated by immunostaining for 8-hydroxyguanosine (Fig. 5A, B, K, Suppl. Fig. 4) and reduced myocardial collagen Iα2 and CTGF mRNA expression (Fig. 5C, D). Spearman correlation

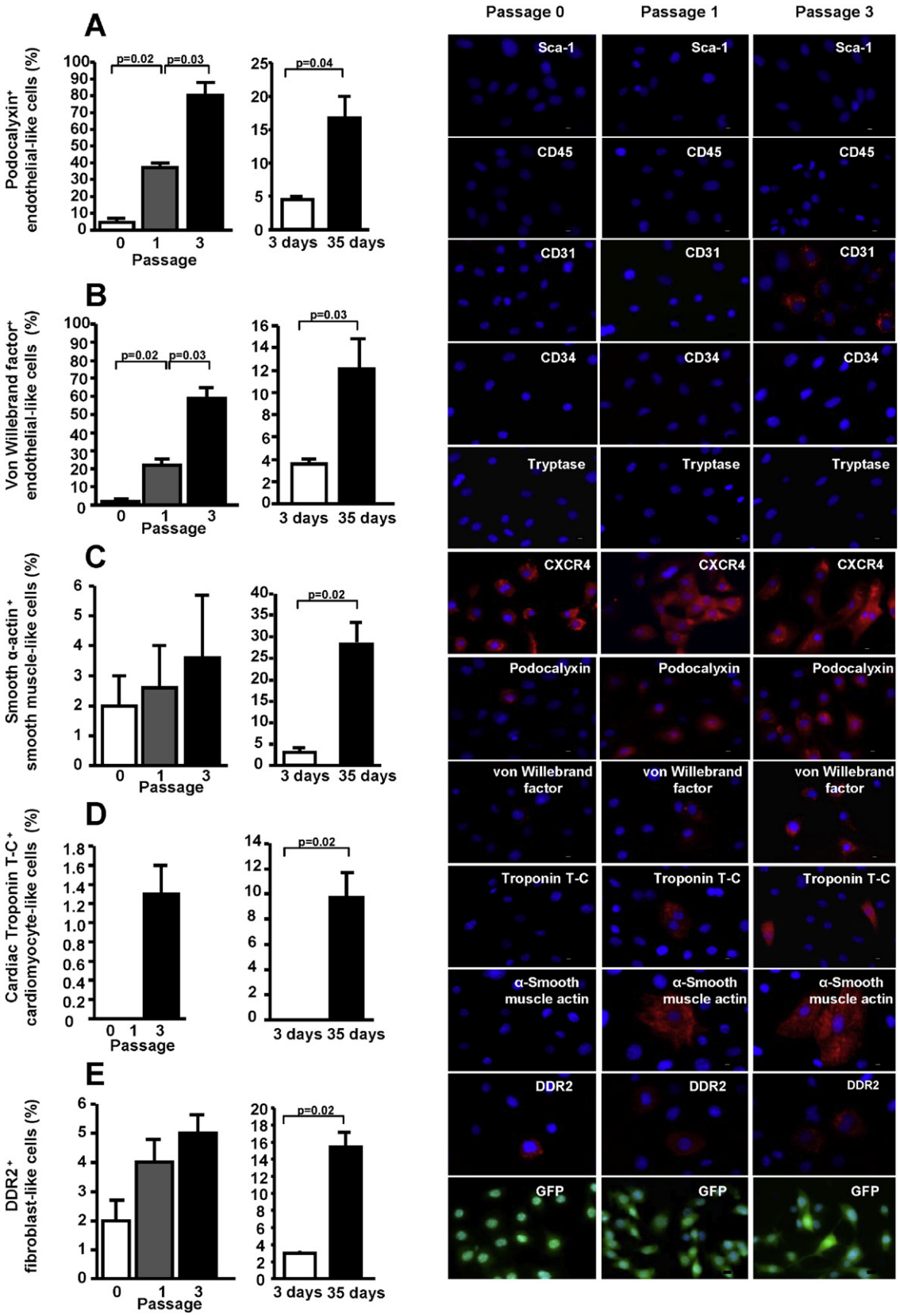

Fig. 2. Cultivated c-kit$^+$ resident cardiac stem cells acquire an endothelial phenotype in stem cell medium and a smooth myocyte phenotype in differentiation medium. Cultivation of c-kit$^+$ GFP$^+$ RCSC until the third passage in Ham's F-12 stem cell medium and for 5 weeks in differentiation medium increases the percentage of podocalyxin$^+$ (A) (red) and von Willebrand factor$^+$ endothelial-like cells (B), (red). Cultivation in differentiation medium also significantly increases the percentage of smooth α-actin$^+$ smooth muscle-like cells (C) (red), cardiac troponin T–C$^+$ cardiomyocyte-like cells (D) (red) and DDR2$^+$ fibroblast-like cells (E) (red). Transplanted cells do not express stem cell antigen 1, leucocyte marker CD45, endothelial cell marker CD31, CD34 and tryptase (red). All cells exhibit GFP fluorescence (green) and CXCR4 expression (red). Each immunostaining was performed on 3 cell culture dishes per mouse (n = 8) and number of cells immunostained for differentiation marker per 300 cells were counted in each cell culture dish. Nuclei are stained blue by DAPI. Bars = 10 μm.

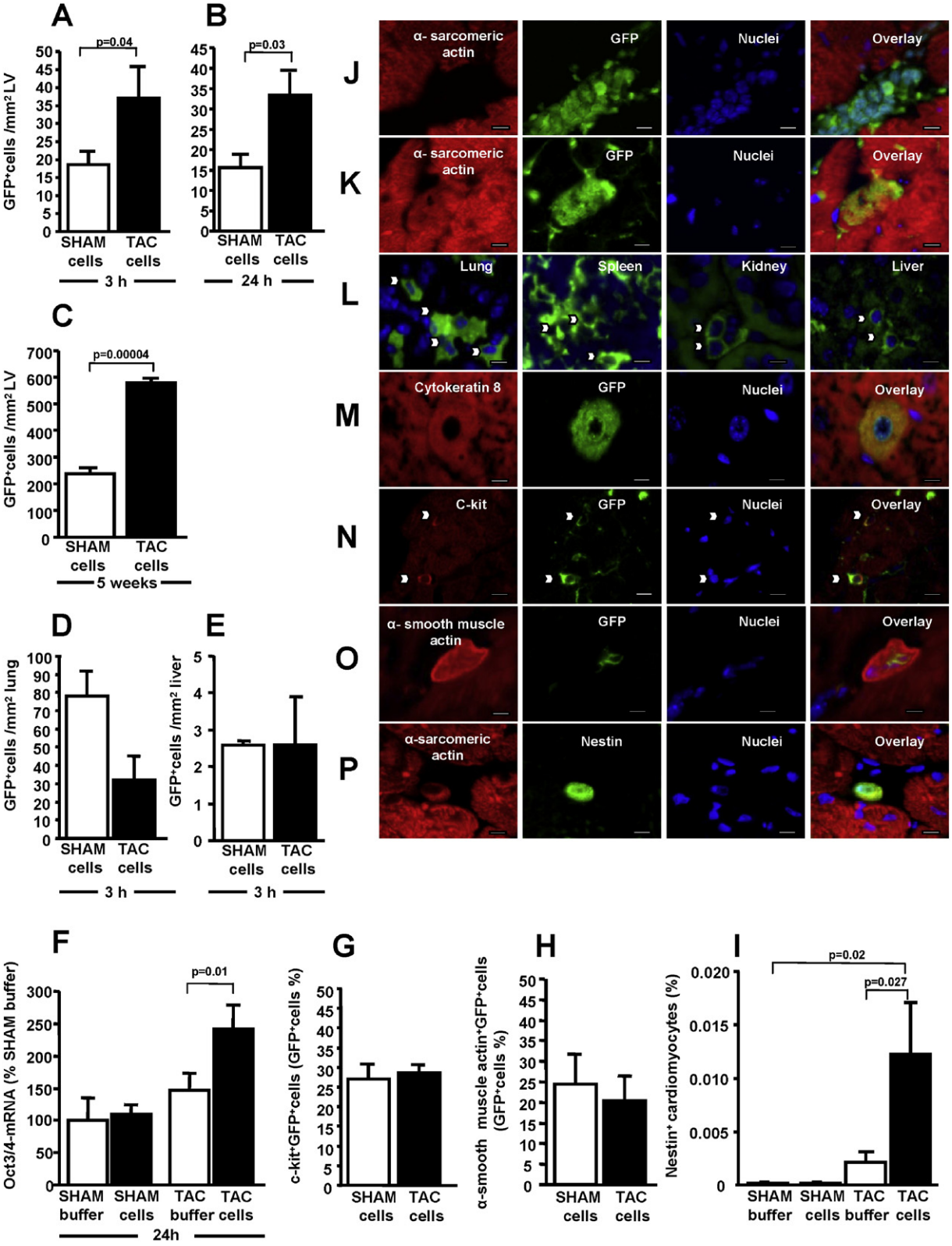

Fig. 3. Injected c-kit$^+$ resident cardiac stem cells migrate to the heart and other internal organs. Injected c-kit$^+$ GFP$^+$ RCSC migrate to the heart and other internal organs. Pressure overload significantly increases the number of GFP$^+$ cells per mm^2 3 h (A), 24 h (B) and 5 weeks (C) after stem cell transplantation, n = 4–5 per group. Transplanted c-kit$^+$ GFP$^+$ RCSC were detected in the lung (D) and liver (E) of SHAM and TAC mice. C-kit$^+$ GFP$^+$ RCSC transplantation increases mRNA-expression level of Oct3/4 in the LV myocardium of TAC + cell mice 24 h later, n = 4–5 per group (F). About 25% of injected GFP$^+$ cells express c-kit (G) or α-smooth muscle actin (H) 5 weeks after transplantation, n = 5 per group. Stem cell transplantation increases the percentage of GFP-negative nestin-positive cardiomyocytes in TAC + cell mice (I), n = 11–24 per group. GFP$^+$ cells in LV sections from a TAC + cell mouse 3 h after stem cell transplantation: GFP$^+$ cells between cardiomyocytes-overlay (J) and a GFP$^+$ cardiomyocyte-overlay in a TAC + cell mouse 5 weeks post-TAC (K): α-sarcomeric actin (red) and GFP (green). GFP$^+$ (green) cells in the lung, spleen, kidney and liver sections 5 weeks post-injection are marked by arrowheads (L). A GFP$^+$ hepatocyte-overlay 5 weeks after stem cell injection (M): cytokeratin 8 (red) and GFP (green). C-kit$^+$ GFP$^+$ cell-overlay in a TAC + cell mouse 5 weeks post-TAC (N): c-kit (red) and GFP (green). A GFP + smooth muscle cell overlay in a TAC + cell mouse 5 weeks post-TAC (O): α-smooth muscle actin (red) and GFP (green). A small nestin-positive GFP-negative cardiomyocyte-overlay in a TAC + cell mouse 5 weeks post-TAC (P): α-sarcomeric actin (red) and nestin (green). Nuclei are stained blue by DAPI. Bars = 10 μm.

Table 1
Effects of transverse aortic constriction (TAC) and transplantation on parameters of myocardial hypertrophy.

	SHAM + buffer	SHAM + c-kit$^-$	SHAM + cells	TAC + c-kit$^-$	TAC + buffer	TAC + cells
Animal number	11	3	15	4	16	24
Body weight (g)	25 ± 0.4	24 ± 1.4	26 ± 0.4	24.7 ± 0.6	24.9 ± 0.8	25.2 ± 0.4
Heart rate (b.p.m.)	164 ± 7	186 ± 15	173 ± 6	199 ± 4	167 ± 8	175 ± 15
LVSP (mm Hg)	75 ± 3	79 ± 6	79 ± 3	164 ± 4*,†	117 ± 6*	127 ± 5*,†,‡
LVdP/dt $_{max}$ (mm Hg/s)	2565 ± 179	3086 ± 319	2582 ± 145	4486 ± 90*	3106 ± 137*	3534 ± 140*,†,‡
LVdP/dt$_{min}$ (mm Hg/s)	−2233 ± 197	−2724 ± 329	−2286 ± 141	−5311 ± 161*	−3180 ± 168*	−3708 ± 206*,†,‡
LVEDP (mm Hg)	17 ± 2	12 ± 0.5	19 ± 2.5	29 ± 2*	27.5 ± 1.3*	30.7 ± 2.6*
Lung fluid weight/TL (mg/mm)	10.3 ± 0.6	9.3 ± 0.4	9.8 ± 0.5	18.7 ± 0.3	16.4 ± 1.8*	18.4 ± 1.7*
Heart weight/TL (mg/mm)	7.8 ± 0.4	7 ± 0.4	8.2 ± 0.3	11.8 ± 0.3*	12.7 ± 0.9*	13.7 ± 0.8*
CCSA (μm^2)	248 ± 22	220 ± 20	248 ± 15	435 ± 26*,†	358 ± 15*	312 ± 14*,†,‡
Cardiomyocytes (/mm^2)	5525 ± 469	6696 ± 514	5430 ± 259	3709 ± 219*	3874 ± 185*	4455 ± 227*,†,‡

CCSA, cardiomyocyte cross-sectional area; LV, left ventricle; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; and TL, tibia length.
Data are presented as means ± SEM. One-way ANOVA with a Fisher-LSD post-hoc test.
* $p < 0.05$ vs. corresponding control group.
† $p < 0.05$ vs. TAC + buffer.
‡ $p < 0.05$ vs. TAC + c-kit$^-$.

analysis revealed a strong positive correlation between mRNA-expression level of CTGF and collagen Iα2 ($r = 0.7$, $p = 0.6 \times 10^{-6}$).

Cardiac fibrosis was quantified morphometrically as fractional area of collagen content in % of myocardial content using picrosirius red staining. TAC led to a profound replacement fibrosis, which was significantly diminished by c-kit$^+$ RCSC transplantation (Fig. 5G, L). Furthermore, transplanted stem cells reduced the number of cardiac fibroblast identified by immunostaining for intracellular fibronectin and DDR2 in the pressure-overloaded LV (Fig. 5I, J, M, N). Transplantation of c-kit negative cardiac cells, similar to the injection of stem cell buffer, did not ameliorate cardiac fibrosis in pressure overload. Neither fractional area of collagen content, nor collagen Iα2 mRNA expression was reduced after c-kit$^-$ CC transplantation (Fig. 5E, F, H, L).

3.6. Amelioration of myocardial cell turnover and mRNA-expression of BNP and ANP in pressure-overloaded LV myocardium after c-kit$^+$ RCSC transplantation

TAC enhanced apoptosis of cardiomyocytes but not of non-cardiomyocytes. The extent of apoptosis caused by pressure overload was markedly decreased by c-kit$^+$ RCSC transplantation in cardiomyocytes and in non-cardiomyocytes (Fig. 6A–D).

Transplantation of c-kit$^+$ RCSC increased the percentage of cycling Ki67$^+$ cardiomyocytes in the SHAM + cells group and the percentage of cycling Ki67$^+$ non-cardiomyocytes in both experimental groups (Fig. 6E, F, H, I, J, Suppl. Fig. 5). GFP$^+$ Ki67$^+$ cells were detected in the LV myocardium of transplanted animals (Fig. 6K, Suppl. Fig. 5). Moreover, transplantation of c-kit$^+$ RCSC significantly increased the percentage of nestin$^+$ Ki67$^+$ non-cardiomyocytes in pressure-overloaded LV (Fig. 6G, L, Suppl. Fig. 5).

Myocardial content of natriuretic peptides is a marker of cardiac remodeling and progression to heart failure. TAC increased the myocardial content of BNP and ANP by five- to twenty-fold. The up-regulation of BNP and ANP was markedly diminished by c-kit$^+$ RCSC transplantation (Fig. 6M, N). Transplantation of c-kit$^+$ RCSC also reduced the concentration of BNP in the serum of TAC mice (Fig. 6O).

4. Discussion

The main finding of the study is the amelioration of pressure-overload induced left ventricular remodeling by intravenous infusion of adult c-kit$^+$ resident cardiac stem cells. We isolated adult c-kit$^+$ RCSC that expressed c-kit in the cell membrane and Oct3/4 in the nucleus (Lennartsson and Ronnstrand 2012; Liedtke et al. 2008). Specific transcription factors (MEF-2C, GATA-4, GATA-6, Nkx2.5, Ets1) regulate the differentiation of c-kit$^+$ RCSC into various cardiac cells both in vitro and in vivo (Beltrami et al. 2003; Ellison et al. 2013; Li et al. 2011; van Berlo et al. 2014). In agreement with these findings, the expanded c-kit$^+$ RCSC differentiated into the main three cardiac linages: cardiomyocyte, endothelial and smooth muscle cells. C-kit$^+$ RCSC cultivated in stem cell medium spontaneously differentiated primarily into endothelial cells explaining their high angiogenic activity (Russell and Brown 2014; Sandstedt et al. 2010). Transplantation of c-kit$^+$ RCSC markedly reduced fibrosis, fibroblast density, expression of CTGF and collagen Iα2, cardiomyocyte hypertrophy and increased cardiomyocyte density in the left ventricular myocardium. These ameliorative effects are at least in part explained by the observed reduction of apoptotic cell death and oxidative stress and the enhanced proliferation of cardiac cells.

Transplantation of c-kit$^+$ RCSC shifted the balance between pro- and anti-angiogenic proteins in the pressure-overloaded LV myocardium towards pro-angiogenic mediators and the myocardium of transplanted mice showed an increased density of endothelial (CD31$^+$) cells. In contrast, GFP$^+$ cardiomyocytes were only identified in a small minority of the animals despite very meticulous examination. Transplanted cells transdifferentiated partly into smooth muscle cells and CD31$^+$ endothelial cells and pressure overload further increased their percentage. The histological characterization of endothelial-transdifferentiating cells can be difficult because non-endothelial GFP$^+$ cells lay in the vicinity of endothelial cells. To overcome this issue, we used Tie-2-GFP mice as donor animals for additional transplantation studies. In these animals, cells are expressing GFP by the endothelial Tie-2-promoter and c-kit$^+$ RCSC in this model only express GFP after differentiating towards endothelial phenotype (Müller et al. 2009). The data therefore strongly suggest that differentiation took place. Reduction of endothelial cell number and number of capillaries during sustained pressure overload caused by decrease in myocardial content of Hif1α leads to progression of heart failure. (Kazakov et al. 2012; Müller et al. 2009; Müller et al. 2008; Sano et al. 2007). Hence, the increase of the numbers of CD31-positive endothelial cells and enhanced myocardial expression of Hif1α after transplantation of the adult stem cells represent additional protecting effects (Gyongyosi et al. 2010; Tillmanns et al. 2008). Circulating stem cells demonstrate increased intracellular expression of Hif1α under normoxic conditions induced by ligand of c-kit stem cell factor (Piccoli et al. 2007). Moreover, inhibition of c-kit abrogates Hif1α-mediated angiogenic activity of hematopoietic stem cells and cancer cells (Litz and Krystal 2006; Pedersen et al. 2008). These findings suggest that paracrine effects and differentiation into non-cardiomyocytes represent the primary underlying mechanisms of the cell therapy (Beltrami et al. 2003; Ellison et al. 2013; Elnakish et al. 2013; Li et al. 2011; Ramkisoensing et al. 2014; van Berlo et al. 2014).

Cardiac fibrosis is an important part of ventricular remodeling and an independent and predictive risk factor for heart failure (Burchfield et al. 2013; Segura et al. 2014). Pathological stress elicited by pressure

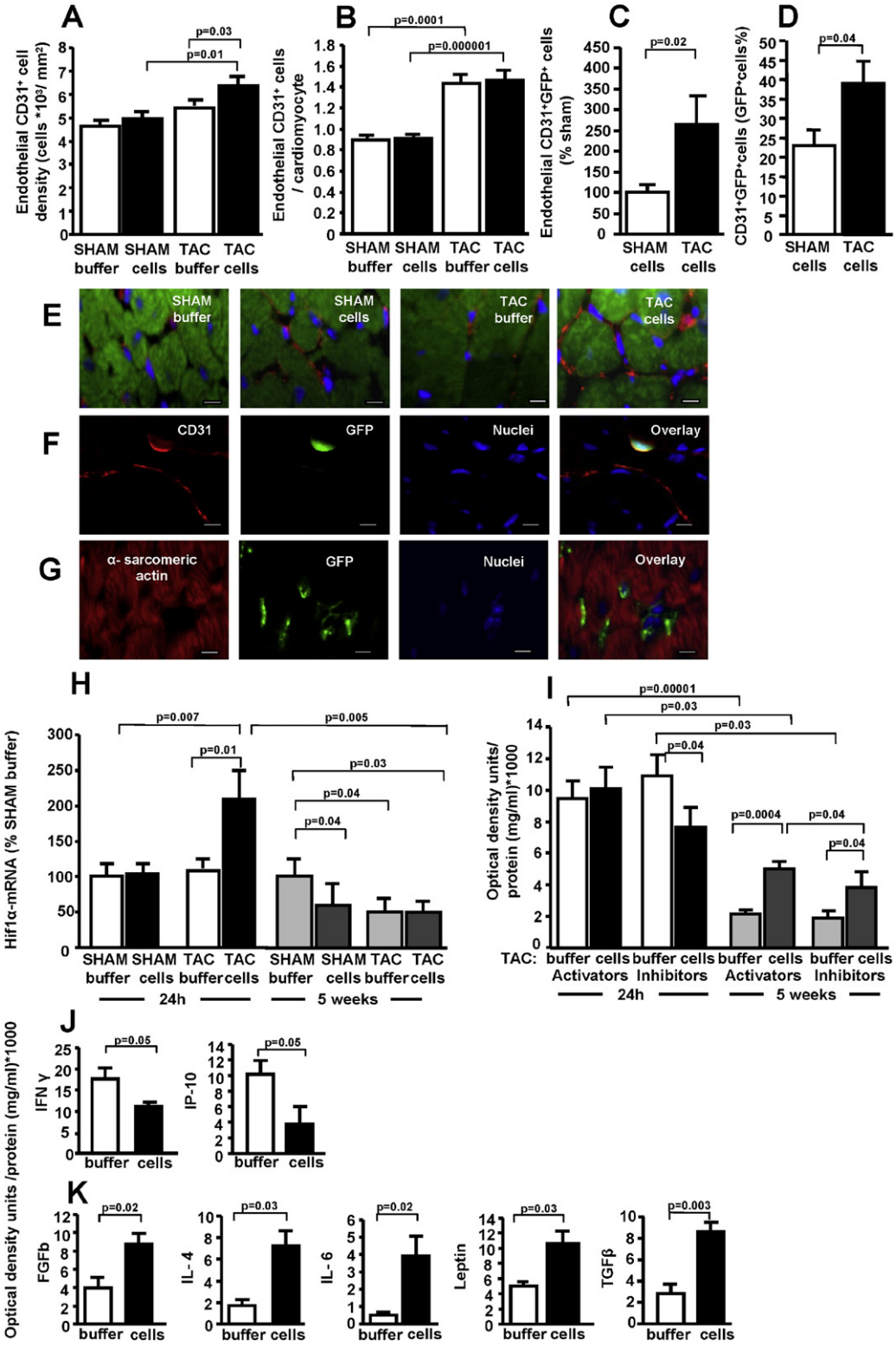

Fig. 4. Effect of intravenous transplantation of c-kit$^+$ resident cardiac stem cells on endothelial cells, Hif1α expression and expression of angiogenesis regulators in pressure-overloaded LV myocardium. Injection of c-kit$^+$ RCSC increases the number of CD31$^+$ endothelial cells (A), but not the ratio of CD31$^+$ endothelial cells to cardiomyocyte (B), n = 11–24 per group. Pressure overload increases the percentage of CD31$^+$ GFP$^+$ endothelial cells in the LV myocardium (C) and stimulate differentiation of c-kit$^+$ GFP$^+$ RCSC into endothelial cells (D), n = 11–24 per group. Hif1α mRNA level in the LV myocardium was upregulated 24 h after transplantation of c-kit$^+$ RCSC in the TAC + cell group and 35 days later only in the SHAM + buffer group (H), n = 4–5 per group. Myocardial expression of anti-angiogenic proteins was reduced 24 h and expression of pro-angiogenic proteins was increased 5 weeks after stem cell transplantation (I), n = 4 per group. Injection of c-kit$^+$ RCSC reduces expression of anti-angiogenic mediators IFN γ and IFN γ inducible protein (IP-) 10 in pressure-overloaded LV myocardium 24 h after transplantation (J), n = 4 per group. Enhanced expression of pro-angiogenic proteins (FGFb, IL-4, IL-6, leptin, and TGFβ) in pressure-overloaded LV myocardium 5 weeks after stem cell transplantation (K), n = 4 per group. Representative sections of the LV myocardium (E) stained with co-immunostaining for endothelial cell marker CD31 (red) and cardiomyocyte marker α-sarcomeric actin (green) respectively. A GFP$^+$ endothelial cell-overlay (F): CD31 (red) and GFP (green). C-kit$^+$ RCSC from Tie-2 GFP FVB/N mice migrate to the heart and transdifferentiate partly into endothelial cells as proved by green GFP expression driven by the endothelial cell promoter Tie-2. A Tie-2-GFP$^+$ endothelial cell-overlay (G): α-sarcomeric actin (red) and GFP (green). Nuclei are stained blue by DAPI. Bars = 10 μm.

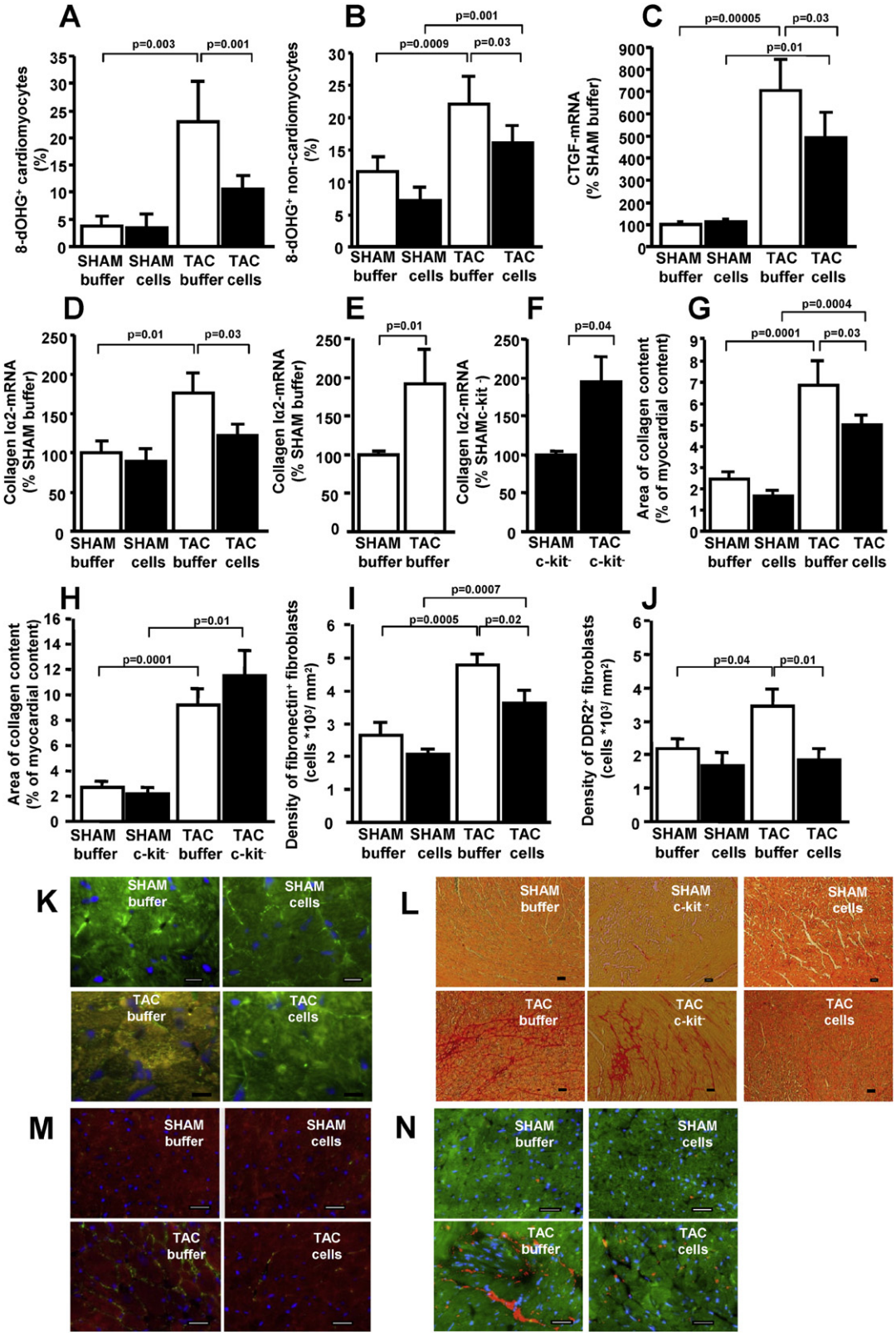

Fig. 5. Intravenous transplantation of c-kit$^+$ resident cardiac stem cells reduces oxidative stress and cardiac fibrosis in TAC. Injection of c-kit$^+$ RCSC reduces oxidative stress in cardiomyocytes (A) and in non-cardiomyocytes (B) in TAC, n = 11–24 per group. Transplantation of c-kit$^+$ RCSC reduces mRNA-expression of CTGF (C) and collagen Ia2 (D) 5 weeks after TAC, n = 11–24 per group. Both injections of stem cell buffer (E) and c-kit$^-$ cardiac cells (F) lead to a similar increase in mRNA-expression of collagen Ia2 during 5 weeks TAC, n = 3–5 per group. Injection of c-kit$^+$ RCSC reduces cardiac fibrosis (expressed as fractional area of collagen content in % of myocardial content) (G, L) and the number of fibronectin$^+$ (I, M) and DDR2$^+$ fibroblasts per mm^2 (J, N) n = 11–24 per group. Fractional area of collagen content is similarly increased after injection of stem cell buffer and c-kit$^-$ cardiac cells 5 weeks post-TAC (H, L), n = 3–5 per group. Representative sections of the LV myocardium (K) stained with co-immunostaining for marker of oxidative stress 8-hydroxy-guanosine (red) and cardiomyocyte marker α-sarcomeric actin (green) respectively. Representative sections of the LV myocardium stained with picrosirius red (L), co-immunostaining for α-sarcomeric actin (red) and the fibroblast marker intracellular fibronectin (green) (M), and co-immunostaining for α-sarcomeric actin (green) and the fibroblast marker DDR2 (red) (N). Nuclei are stained blue by DAPI. Bars = 30 μm.

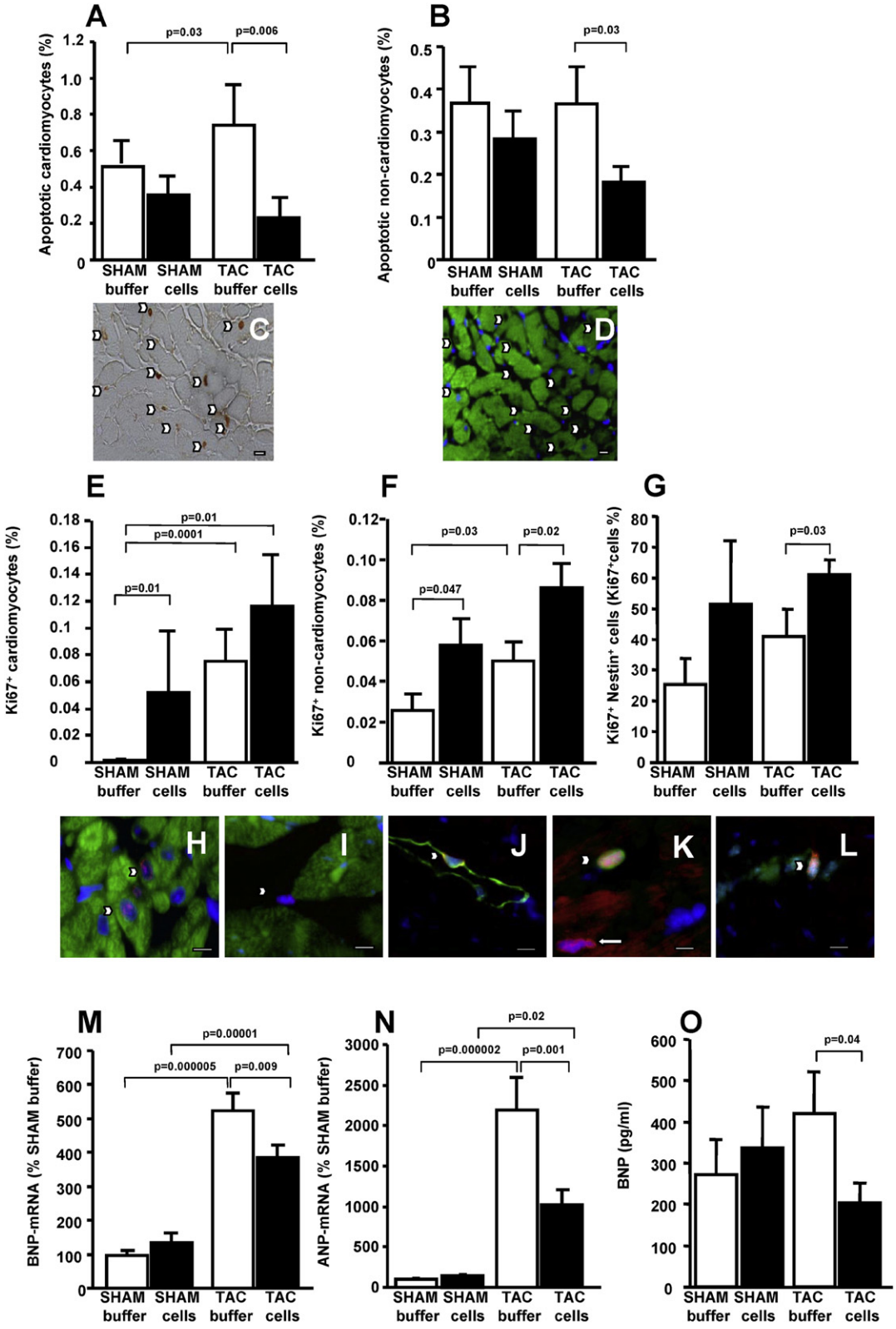

Fig. 6. Intravenous transplantation of c-kit$^+$ resident cardiac stem cells ameliorates cell turnover and mRNA-expression of BNP and ANP in pressure-overloaded LV myocardium. Transplantation of c-kit$^+$ RCSC reduces the percentage of apoptotic cardiomyocytes (A) and non-cardiomyocytes (B), n = 11–24 per group. (C) and (D) show the same section of the LV myocardium from a TAC + buffer mouse. (C) shows light microscopic staining for apoptosis (brown). (D) shows fluorescence microscopy for the myocyte marker α-sarcomeric actin (green). Apoptotic nuclei are marked by arrowheads. Nuclei are stained blue by DAPI. Transplantation of c-kit$^+$ RCSC significantly increases the percentage of Ki67$^+$ cycling cardiomyocytes in the SHAM + cell group (E), the percentage of Ki67$^+$ non-cardiomyocytes in the experimental groups (F) and the percentage of cycling Ki67-positive nestin-positive GFP-negative non-cardiomyocytes in TAC + cell group (G), n = 11–24 per group. (H), (I), (J), (K) and (L) show examples of LV sections from TAC mice co-immunostained for the proliferation marker Ki67 (red) and the following cell specific markers (in green): the myocyte marker α-sarcomeric actin (H) and (I), the endothelial cell marker podocalyxin (J), GFP (K) and nestin (L). Cycling cardiomyocyte (H), non-cardiomyocyte (I) endothelial cell (J), GFP$^+$ cell (K) and nestin$^+$ cell (L) nuclei are marked by arrowheads. Arrow marks cycling a Ki67$^+$ GFP$^-$ non-cardiomyocyte (K). Nuclei are stained blue by DAPI. Bars = 10 μm. mRNA-expression of BNP (M) and ANP (N) in the LV myocardium and BNP protein level in the peripheral blood (O), n = 11–24 per group.

overload induces fibroblast proliferation, migration of fibrocytes to the heart increasing production of extracellular matrix proteins such as collagen I, collagen III and fibronectin (Kazakov et al. 2013; Kazakov et al. 2012; Müller et al. 2009; Müller et al. 2008). In our study transplantation of cardiac stem cells decreased the number of fibronectin-positive fibroblasts in LV and the production of the main component of extracellular matrix, collagen I. Furthermore, cardiac fibroblasts respond to pressure overload by increase in production of specific cytokines and growth factors (Segura et al. 2014). Connective tissue growth factor plays a pivotal role in development of cardiac fibrosis and hypertrophy (Daniels et al. 2009; Szabo et al. 2014; Yoon et al. 2010). Our findings are consistent with previous investigations demonstrating reduction of cardiac fibrosis and cardiomyocyte hypertrophy after diminishing CTGF-level in pressure-overloaded LV myocardium (Szabo et al. 2014; Yoon et al. 2010). Increased oxidative stress during pressure overload has been shown to elevate fibroblast numbers and the production of extracellular matrix in the left ventricular myocardium (Segura et al. 2014). Immunostaining for oxidative stress marker 8-hydroxy-guanosine revealed significant amelioration of oxidative stress both in cardiomyocytes and in non-cardiomyocytes after stem cell transplantation. Transplantation of c-kit$^+$ RCSC but not of c-kit$^-$ cardiac cells reduces cardiac fibrosis in pressure-overloaded myocardium demonstrating specificity of fibrosis reduction after c-kit$^+$ stem cell transplantation. Therefore, intravenous transplantation of c-kit$^+$ RCSC reduces cardiac fibrosis acting on several of the main pathogenetic mechanisms of fibrogenesis.

Progression of maladaptive LV remodeling, increased wall stress and transition to heart failure are associated with an enhanced cardiac production of natriuretic peptides (Hohl et al. 2013; van Veldhuisen et al. 2013). TAC led to a marked five- to twenty-fold increase of myocardial BNP and ANP expression. The observed decrease of myocardial ANP and BNP expression and of serum BNP confirms a functional effect of the cell therapy in addition to the reduction of cardiac fibrosis.

The findings are in agreement with previous investigations demonstrating participation of c-kit$^+$ RCSC in myocardial adaptation to physiological and pathological stimuli (Beltrami et al. 2003; Ellison et al. 2013; Li et al. 2011; van Berlo et al. 2014). Transplanted c-kit$^+$ RCSC improved parameters of LV performance in different experimental models (Beltrami et al. 2003; Ellison et al. 2013; Li et al. 2011). Recently, Ellison et al. (Ellison et al. 2013) and van Berlo et al. (van Berlo et al. 2014) demonstrated active differentiation of c-kit$^+$ RCSC into cardiomyocytes, smooth muscle cells, endothelial cells and cardiac fibroblasts under physiologic conditions, in a model of isoproterenol-induced severe myocardial damage with preserved coronary circulation (Ellison et al. 2013) and in a model of myocardial infarction (van Berlo et al. 2014). Stem cell factor/c-kit signaling mediates the migration of c-kit$^+$ RCSC to the damaged heart (Kuang et al. 2008). Both our study and the study of Ellison et al. (Ellison et al. 2013) show a tropism of the intravenously injected c-kit$^+$ RCSC expressing CXCR4 to the myocardium and their engrafting in extracardiac tissues. In our study pressure overload during 3 h increased recruitment of injected cells to the LV myocardium by two-fold. However, the contribution of c-kit$^+$ RCSC to the differentiation into cardiomyocytes in the isoproterenol-induced damage model was more pronounced compared to myocardial infarction (Ellison et al. 2013; van Berlo et al. 2014). Despite careful examination, GFP$^+$ cardiomyocytes were only found in ~10% of the TAC + cell mice in our study. The discrepancy may be explained by different experimental conditions and different participation of c-kit$^+$ RCSC in various experimental models. Furthermore, the decrease of cardiomyocyte loss after stem cell transplantation can diminish cardiomyogenesis and can be caused by paracrine effects of c-kit$^+$ RCSC (Kawaguchi et al. 2010). Transplantation of c-kit$^+$ RCSC increased the percentage of GFP-negative nestin-positive cardiomyocytes and percentage of cycling Ki67-positive nestin-positive GFP-negative non-cardiomyocytes 5 weeks post-TAC suggesting proliferation and cardiomyogenic transdifferentiation of endogenous cardiac stem cells (Calderone 2012; Hatzistergos et al. 2010). The new-formed small cardiomyocyte from endogenous resident cardiac stem cells might contribute to increased cardiomyocyte density and reduced cardiomyocyte cross-sectional area.

Our study has limitations: we did not study effects of other types of resident cardiac stem cells (Dey et al. 2013), different cell culture conditions (Yang et al. 2013), and the effect of different doses of transplanted stem cells (Richardson et al. 2013) on LV remodeling in TAC. The data support a role of HIF-1 alpha as a mediator of the positive effects after c-kit + stem cell transplantation. However, further studies are needed to further proof and characterize this concept. Although GFP$^+$ cardiomyocytes were mononucleated, we cannot exclude the role of cell fusion in their origin. However, our data set the stage for future studies to address these issues.

In conclusion, the data show an ameliorative effect of the intravenously transplanted c-kit positive resident cardiac stem cells on left ventricular fibrosis independently of the changes in afterload induced by aortic ligation. Transplantation of ex-vivo expanded c-kit$^+$ RCSC reduces cardiac fibrosis and stimulates expression of pro-angiogenic proteins and endothelial density in pressure-overloaded LV myocardium. Importantly, apoptosis and markers of oxidative stress are reduced. These findings may provide new perspectives for the understanding of the importance of adult c-kit$^+$ cells for cardiac remodeling and heart failure.

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Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.10.017.

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