Endothelial nitric oxide synthase of the bone marrow regulates myocardial hypertrophy, fibrosis, and angiogenesis

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Received 13 April 2011; revised 2 November 2011; accepted 11 November 2011; online publish-ahead-of-print 21 November 2011

Time for primary review: 26 days

Aims
The endothelial nitric oxide synthase (eNOS) regulates the mobilization and function of endothelial progenitor cells (EPC). We hypothesized that eNOS of the bone marrow (BM) affects cardiac remodelling during myocardial hypertrophy via the regulation of BM-derived vascular progenitor cells.

Methods and results
Ten-week-old male C57Bl6 wild-type (WT) and eNOS mice (eNOS-/-) were subjected to transverse aortic constriction (TAC, 360 μm, 35 days) or sham operation inducing cardiac hypertrophy and increasing the numbers of Ki67+ cardiomyocytes in both strains. Myocardial fibrosis was more pronounced in eNOS-/- TAC (3.4 ± 0.4 vs. 2.1 ± 0.2% in WT-TAC, P < 0.05). TAC up-regulated the number of EPCs in the peripheral blood and in the BM in WT but not in eNOS-/- . Baseline migratory capacity of EPCs was lower in eNOS-/- and was not raised by TAC in either strain. To test the role of eNOS in the BM during pressure overload, strain-mismatched (WT/eNOS-/-; eNOS-/-/WT) and strain-matched (WT/WT; eNOS-/-/eNOS-/-) BM transplantations (BMTs) were performed. Cardiac hypertrophy was most pronounced in WT/eNOS-/- TAC. Strain-mismatched BMT of eNOS-/- BM deteriorated and of WT BM ameliorated cardiac fibrosis, capillary density, the numbers of EPCs in the peripheral blood and in the BM, and their migratory capacity in pressure overload. Following transplantation of green fluorescent protein (GFP)-positive BM, TAC increased the number of BM-derived podocalyxin posGFPpos endothelial cells in both strains.

Conclusion
eNOS of the BM plays a key role for amelioration of cardiac hypertrophy, capillary density, and fibrosis during increased afterload.

Keywords
Endothelial nitric oxide synthase of the bone marrow • Pressure overload • Cardiac angiogenesis • Cardiac fibrosis • EPC

1. Introduction
Sustained pressure load occurring in pathological situations such as hypertension or aortic valve stenosis leads to maladaptive cardiac remodelling, which can progress to heart failure.1,2 Structural and functional changes of coronary microvasculature are important components of cardiac hypertrophy and fibrosis during pressure overload.3,4 Disruption of coordinated tissue growth and angiogenesis contributes to the transition from adaptive to maladaptive cardiac remodelling.5,6 Recent findings demonstrate that circulating bone marrow (BM)-derived endothelial progenitor cells (EPC) contribute to angiogenesis.7 These cells reside within the BM and can be mobilized and stimulated to perform their functions by different physical and chemical stimuli, among which are physical exercise, body weight reduction, hormones, angiogenesis-promoting growth factors, and pharmacological agents.8 – 11 EPCs are of interest as markers to assess the prognosis of cardiovascular disease and as a potential therapeutic target.12 Growing evidence suggests that both myocardial repair processes and EPC-mediated angiogenesis in pressure overload are dependent on nitric oxide (NO) production

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doi:10.1093/cvr/cvr305
by endothelial NO synthase (eNOS). However, it is not clear whether eNOS of the BM or extramedullary tissue is important for amelioration of cardiac remodelling and capillarization in increased cardiac afterload.

The aim of this study was to examine the role of eNOS in the BM and the myocardium for the regulation of myocardial hypertrophy under conditions of increased cardiac afterload.

2. Methods

2.1 Animals and transarctic constriction

The study was approved by the animal Ethics Committee of the Universität des Saarlandes and conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Pub. No. 85-23, revised 1996). Ten-week-old male C57Bl/6 (Charles River Laboratories, Sulzfeld, Germany) and eNOS−/− mice (B6129/P2-Nos3, Charles River Laboratories) were housed under standard conditions. For surgery, animals were intraperitoneal anaesthetized with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg). Anaesthetic monitoring was performed by testing of rear foot reflexes before and during the surgery, observation of respiratory pattern, mucous membrane colour, and responsiveness to manipulations throughout the procedure. After orotracheal intubation using a 20 G catheter, the tube was connected to a volume cycled rodent ventilator (Harvard Apparatus, Holliston, MA, USA) on supplemental oxygen with a tidal volume of 65–70%. Control mice underwent a sham operation. After 5 weeks, animals were intraperitoneal anaesthetized with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg) and a 1.4 Fr pressure-transducing catheter (Milko Tip Catheter, Millar instruments) was used for left ventricle (LV) pressure measurements. The adequacy of anaesthesia was evaluated as described above. After the LV pressure measurements, mice were sacrificed by intraperitoneal injection of ketamine (1 g/kg body weight) and xylazine (100 mg/kg) and hearts were rapidly excised. Hearts were partly snap frozen in liquid nitrogen and stored at −80°C and partly embedded in paraffin after fixation in PBS-buffered formalin (4%). Blood and BM were sampled, as well as the spleen for further preparation. Blood pressure was measured on the tail artery of mice 4 weeks and partly embedded in paraffin after fixation in PBS-buffered formalin (4%).

2.2 Bone marrow transplantation

Six-week-old C57Bl/6 wild-type (WT) mice, C57Bl/6-Tg(ACTbEGF-P)1Osb mice [Jackson Laboratory; expressing green fluorescent protein (GFP) ubiquitously], and eNOS−/− mice (B6129/P2-Nos3, Charles River Laboratories) were sacrificed 4 weeks after BMT and histological examination of BM sections was performed. To monitor reconstitution of the BM, three animals per group were sacrificed 4 weeks after BMT and histological examination of BM sections was performed. The detailed information is described in the Supplementary material online. Four weeks after BMT, TAC or sham operation was carried out as described above.

2.3 Fluorescence-activated cell sorter analysis

Blood and BM were analysed as described. The viable lymphocyte population was analysed for stem cell antigen 1 (Sca-1)-FITC (E13–161.7, Pharmingen, Germany) and vascular endothelial growth factor receptor 2 (VEGFR-2) (Flk-1; Avas12x1, Pharmingen) conjugated with the corresponding phycoerythrin-labelled secondary antibody (Sigma, Germany). Isotype-identical antibodies served as controls (Becton–Dickinson, Germany).

2.4 Culture of spleen-derived EPCs and migration assay

Spleen mononuclear cells were isolated and cultured in endothelial basal medium (Cell Systems, Germany) as described. Antibiotics, calf serum, and cell culture medium were obtained from Invitrogen. After 4 days in culture, 500 cells from each mouse were transferred to modified Boyden chambers (BD Bioscience, Germany) in 24-well plates filled with 750 μL. medium containing 10 μL SDF-1 (R&D Systems, Germany) to assess their migratory capacity. After 24 h of incubation, tetramethylrhodaminecarbocyanine-labelled acetylated low-density lipoprotein (DiLDL, 2.4 μg/mL; Cell Systems) was added to identify EPCs. Boyden chamber filters were cut out, placed on slides, and mounted with fluorescent mounting medium (Vectashield, Vector Laboratories, Burlingame, CA, USA) for fluorescence microscopic analysis. The whole filter was analysed using a Nikon E600 epifluorescence microscope (Nikon, Japan) with appropriate filters. Cells positive for red Dil-AC-LDL were judged to be EPCs and were counted.

2.5 Immunofluorescence analysis

To detect cardiomyocytes, capillaries, cycling cardiomyocytes, endothelial cells, fibroblasts, and GFP 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 first antibody and incubation with the appropriate secondary antibody at 37°C for 1 h. The detailed methods are described in the Supplementary material online.

2.6 Tissue morphometry

For morphometric analyses, LV tissue sections (3 μm) were examined. Numbers of cross-sectioned capillary and cardiomyocyte profiles (all cross-sectioned muscle fibres with and without nuclei) per mm² were determined in tissue sections double stained for podocalyxin and α-sarcemeric actin in 20 randomly chosen fields at ×1000 magnification. Two sections per each heart were analysed. Capillary density and the ratio of capillaries to cardiomyocytes were calculated. The cardiomyocyte short-axis diameter and the degree of cardiac fibrosis were evaluated as described.

2.7 Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Unpaired t-test, the Mann–Whitney test, the Kruskal–Wallis ANOVA, or two-way ANOVA with a Bonferroni post hoc test was used where applicable. Values of P < 0.05 were considered significant.
3. Results

3.1 eNOS-deficient mice show higher LV pressure and increased cardiac fibrosis compared with WT mice

eNOS−/− mice exhibited increased peripheral arterial blood pressure, LV systolic pressure (LVSP) and LV end-diastolic pressure (LVEDP) (Table 1). TAC (360 μm, 35 days) resulted in elevated LVSP, LVEDP, and dP/dtMax and decreased dP/dtMin and peripheral blood pressure in both eNOS−/− and WT mice, but these effects were more pronounced in the eNOS−/− TAC group. As expected, TAC reduced myocardial hypertrophy (increased ratio of heart weight to tibia length) both in eNOS−/− and WT mice. Cardiac fibrosis was quantified morphometrically as fractional area of collagen content in percent of myocardial content using picrosirius red staining. The eNOS−/− mice expressed almost three-fold more fibrosis than WT animals (WT sham 0.7 ± 0.2 vs. eNOS−/− sham 2.0 ± 0.3%, P < 0.01). TAC increased cardiac fibrosis in both groups; however, the up-regulation was greater in eNOS−/− mice (WT TAC 2.1 ± 0.2 vs. eNOS−/− TAC 3.4 ± 0.4%, P < 0.05) (Figure 1A).

3.2 Absence of eNOS does not alter the number of cycling cells in the pressure-overloaded LV myocardium

TAC led to a marked six- to eight-fold increase in the numbers of the cycling cardiomyocytes and of the cycling non-cardiomyocytes identified by expression of Ki67 compared with sham-operated mice. While eNOS−/− sham animals had higher levels of Ki67pos cardiomyocytes than the WT sham groups, the numbers of Ki67pos non-cardiomyocytes were similar (Figure 1B and C). Co-immunostainings with the endothelial cell marker podocalyxin and the fibroblast marker fibronectin were performed to further characterize the identity of the Ki67pos non-cardiomyocytes. There were no significant differences between the numbers of Ki67pos endothelial and fibroblast cells in the LVs of eNOS−/− and WT (Figure 1D–H).

3.3 Up-regulation of EPC by aortic constriction in WT but not in eNOS−/− mice

Aortic constriction increased the numbers of EPC both in the peripheral and in the BM of WT animals. eNOS−/− mice exhibited similar EPC numbers compared with WT mice in the sham group but—in contrast to the WT—TAC did not result in an up-regulation of EPC in the BM or the blood in eNOS−/− mice (Figure 2A and B). Boyden chamber assays showed that EPC of eNOS−/− mice were characterized by a markedly lower migratory capacity compared with WT mice, and migration of the WT EPC was two-fold higher than the eNOS−/− EPC (Figure 2C). TAC reduced migratory capacity of WT EPC but the effect was not significant; TAC had no effect on the migration of eNOS−/− EPC (Figure 2C). The capillary density was higher in eNOS−/− mice and the ratio of capillaries to cardiomyocytes was similar in eNOS−/− and WT mice (Figure 2D and E).

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Table 1: Anatomical and functional data

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LV, left ventricle; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; SABP, systolic arterial blood pressure; DABP, diastolic arterial blood pressure; TL, tibia length; CSAD, cardiomyocyte short-axis diameter. *P < 0.05 vs. corresponding Sham, †P < 0.05 vs. corresponding group in another strain with the other type of eNOS expression in the BM (WT vs. eNOS−/−; WT/TAC vs. WT/eNOS−/−; eNOS−/−/WT vs. eNOS−/−/eNOS−/−/WT), §P < 0.05 vs. corresponding group in another strain of BMT-mice with the other type of expression of extramedullary eNOS (WT/WT and WT/eNOS−/− vs. eNOS−/−/eNOS−/− and eNOS−/−/WT).
3.4 Strain-mismatched transplantations: importance of eNOS in the BM for myocardial remodelling and fibrosis

To assess the importance of eNOS in the BM for cardiac remodelling, both WT mice and eNOS$^{-/-}$ mice were transplanted with WT or eNOS$^{-/-}$ BM. These four groups were randomized to sham surgery or aortic ligation. Similar to the results of the mice without BM transplantation (BMT), aortic banding caused an elevation of LVSP, LVEDP, and $dP/dt_{\text{max}}$ that was significantly higher in all eNOS$^{-/-}$ mice compared with WT, and these findings were not affected by the strain-mismatched BMT (Table 1). Strain-mismatched transplantation of eNOS$^{-/-}$ BM increased and of WT BM decreased the ratio of lung fluid weight to tibia length in TAC groups, pointing out a negative effect of eNOS$^{-/-}$ BM for this symptom of heart failure (Table 1).

Aortic constriction increased the ratio of heart weight to tibia length in all groups of the BM transplanted animals. However, WT mice that received eNOS$^{-/-}$ BM showed increased cardiac hypertrophy compared with the other TAC groups (Table 1). Cardiac fibrosis was increased by TAC in all BM transplanted animals. Strain-mismatched transplantation of eNOS$^{-/-}$ BM into WT dramatically increased the extent of fibrosis which was increased by four-fold post-TAC surgery (Figure 3A). In contrast, transplantation of WT BM in eNOS$^{-/-}$ mice markedly reduced cardiac fibrosis in the sham mice, which showed only half of the fibrosis compared with eNOS$^{-/-}$ mice with eNOS$^{-/-}$ BM. Transplantation of WT BM in eNOS$^{-/-}$ mice potently prevented the cardiac fibrosis during increased afterload compared with eNOS$^{-/-}$/mice with eNOS$^{-/-}$ BM (Figures 3A and 4A). Taken together, these data show the importance of eNOS in BM cells for myocardial fibrosis.

There were no significant differences between the groups with regard to the percentages of proliferating cardiomyocytes and non-cardiomyocytes (Ki67$^{\text{pos}}$ cardiomyocytes/Ki67$^{\text{pos}}$ non-cardiomyocytes: WT/WT/sham $0.012 \pm 0.02/0.5 \pm 0.08\%$, WT/WT/TAC $0.09 \pm 0.03/0.9 \pm 0.3\%$, WT/eNOS$^{-/-}$/sham $0.014 \pm 0.01/0.4 \pm 0.07\%$, WT/eNOS$^{-/-}$/TAC $0.1 \pm 0.05/0.8 \pm 0.24\%$, eNOS$^{-/-}$/eNOS$^{-/-}$/sham $0.011 \pm 0.02/0.4 \pm 0.2\%$, eNOS$^{-/-}$/eNOS$^{-/-}$/TAC $0.09 \pm 0.03/0.77 \pm 0.25\%$; eNOS$^{-/-}$/WT/sham $0.016 \pm 0.01/0.36 \pm 0.1\%$, eNOS$^{-/-}$/WT/TAC $0.07 \pm 0.03/0.68 \pm 0.16\%$).
3.5 Decreased EPC and reduced capillary density in mice lacking eNOS in the BM

eNOS<sup>−/−</sup> mice transplanted with WT BM showed a marked increase in the numbers of EPC circulating in the blood and of the EPC in the BM (Figure 3B and C). TAC enhanced the number of EPCs in the blood and in the BM in WT/WT/TAC but did not increase EPC in mice that were deficient of eNOS either in the periphery or in the BM (Figure 3B and C).

Both TAC and sham mice lacking eNOS in the BM showed a reduced migratory capacity compared with mice with WT BM. Aortic constriction reduced migratory capacity in all groups with the exception of eNOS<sup>−/−</sup>/eNOS<sup>−/−</sup>/TAC (Figure 3D).

Mice lacking eNOS<sup>−/−</sup> both in the BM and in the extramedullary tissues showed reduced capillary density and a reduced ratio of capillaries to cardiomyocytes compared with corresponding WT/WT controls. Strain-mismatched transplantation of eNOS<sup>−/−</sup>/BM worsened both parameters in the TAC group. In contrast, transplantation of WT BM in eNOS<sup>−/−</sup> mice significantly ameliorated capillarization of the LV in both sham- and TAC-operated animals compared with corresponding eNOS<sup>−/−</sup>/eNOS<sup>−/−</sup>/TAC (Figures 3E and F).

Transplantation of GFP-positive BM cells was used to study the importance of extramedullar eNOS for the integration and transdifferentiation of BM-derived cells in the pressure-overloaded heart. Both chimeric mice had eNOS in their BM since the transplanted GFP-positive BM contained eNOS. Aortic banding increased the percentage of podocalyxin<sup>POS</sup>GFP<sup>POS</sup> cells in the LV myocardium of both chimeric groups compared with sham (Figure 5, WT/GFP/sham 2.5 ± 0.6%, WT/GFP/TAC 13 ± 3.4%, P < 0.05; eNOS<sup>−/−</sup>/GFP/sham 4.8 ± 1.6%, eNOS<sup>−/−</sup>/GFP/TAC 14.6 ± 3.5%, P < 0.05). In some animals from the WT/GFP/TAC group, large cardiomyocytes co-staining with α-sarcomeric actin and GFP were found.

4. Discussion

The main finding of the study is the importance of eNOS of the BM for cardiac remodelling during increased cardiac afterload. Pressure overload by TAC up-regulated the number of BM-derived progenitor cells in the peripheral blood and in the BM in WT but not in eNOS<sup>−/−</sup> animals. Baseline migratory capacity of EPCs was lower in eNOS<sup>−/−</sup> mice. The absence of eNOS in the BM of WT mice enhanced cardiac hypertrophy and fibrosis post-TAC and diminished myocardial capillarization. Furthermore, eNOS-deficient BM reduced the production and mobilization of EPCs and their migratory capacity, compared with WT/WT TAC mice. In contrast, eNOS<sup>−/−</sup> mice transplanted with WT BM showed less cardiac fibrosis, better myocardial capillarization, increased levels of EPCs in the peripheral blood and in the BM, and enhanced migratory capacity relative to eNOS<sup>−/−</sup>/eNOS<sup>−/−</sup>/TAC and WT/eNOS<sup>−/−</sup>/TAC mice. TAC similarly raised the number of BM-derived podocalyxin<sup>POS</sup>GFP<sup>POS</sup> endothelial cells in both strains with eNOS-positive BM.

Our findings are in agreement with previous investigations demonstrating the importance of eNOS for EPC-mediated angiogenesis, for
Figure 3 Role of eNOS deficiency in the BM and of loss of extramedullar eNOS for cardiac fibrosis and EPC. Wild-type mice (WT) received transplantation of WT BM (host/BM: WT/WT) or eNOS−/− BM (host/BM: WT/eNOS−/−). eNOS−/− mice received transplantation of eNOS−/− BM (host/BM: eNOS−/−/eNOS−/−) or WT BM (host/BM: eNOS−/−/WT). Animals underwent TAC or sham surgery (Sham). Depicted are the effects on cardiac fibrosis (A), on the numbers of Sca-1/VEGFR2-positive EPC in the peripheral blood (B) and in the BM (C), on EPC migration (D), on the number of podocalyxin-stained capillaries per mm² (E), and in relation to the number of cardiomyocytes (F). *P < 0.05 compared with corresponding Sham.
EPC residing in BM as well as mobilization and maintenance of their function. In particular, Aicher et al. demonstrated the pivotal role of eNOS in BM microenvironment for recruitment of stem and progenitor cells. Masaaki et al. revealed that eNOS from the BM cells is important for cardioprotective effects of myocardial ischaemic preconditioning. You et al. reported that BM cells from eNOS-knockout mice transplanted in WT animals were unable to induce neovascularization in a model of hindlimb ischaemia. Furthermore, ex vivo pre-treatment of BM mononuclear cells with an eNOS enhancer ameliorates their angiogenic potential. Moreover, amelioration of cardiac remodelling by exercise training depends on the level of eNOS in the cardiovascular system. Here, we extend these findings with the observation that eNOS of the BM plays a central role during myocardial remodelling in increased cardiac afterload.

Aortic ligation for 5 weeks led to significant elevation of LV systolic and end-diastolic pressures in both strains, more pronounced in eNOS animals where these parameters were increased also in the sham-operated group. TAC induced myocardial and cardiomyocyte hypertrophy, elicited cardiac fibrosis, and stimulated proliferation of cardiomyocytes and non-cardiomyocytes in eNOS and WT mice. The absence of eNOS did not increase the number of cycling cells in the pressure-overloaded LV. The most prominent effect of systemic eNOS deficiency was the marked increase in myocardial fibrosis compared with WT animals. The increase in cardiac fibrosis in the eNOS is consistent with induction of endothelial–mesenchymal transformation, activation of matrix metalloproteinases causing remodelling of extracellular matrix, and loss of endothelial cells.

To specifically investigate the role of eNOS in the BM for cardiac remodelling and capillarization during increased cardiac afterload and to exclude the compensatory angiogenic mechanisms in the myocardium, BMTs were performed. In strain-matched BM transplanted animals, cardiac fibrosis was more pronounced in eNOS-eNOS/TAC mice. Aortic banding reduced myocardial capillarization in eNOS-eNOS/TAC compared with WT/WT/TAC. This reduction is likely due to an enhancement of the anti-angiogenic effects of eNOS-deficiency by ionizing radiation accelerating senescence of mouse tissues. Strain-mismatched transplantation of WT BM reduced pulmonary congestion and ameliorated cardiac fibrosis and capillarization, despite increased LV systolic and end-diastolic pressures in aortic-ligated mice. This ameliorative effect of WT BM on cardiac remodelling was observed parallel to the augmented myocardial angiogenesis associated with high levels of EPCs in the BM and in the peripheral blood and their increased migratory activity. In contrast, aortic-ligated WT mice lacking eNOS in the BM demonstrated marked pulmonary congestion, increased cardiac hypertrophy and fibrosis, reduced myocardial capillarization, diminished levels of

Figure 4 Effects of eNOS deficiency in the BM and extramedullar eNOS deficiency for cardiac fibrosis and capillary density. Representative sections of the LV myocardium stained with picrosirius red (A) and co-immunostaining for myocytic α-sarcomeric actin (green) and the capillary marker podocalyxin (red) (B). Bars = 30 μm.
EPCs in the peripheral blood and in the BM, and deteriorated EPC migration.

The presence of EPC mobilization only in WT/WT/TAC mice and reduction in their migratory capacity by TAC demonstrate the importance of eNOS both in the EPCs and in the local microenvironment of the BM for EPC mobilization and maintenance of their functions in pressure overload. Therefore, eNOS in EPCs and BM is important for cardiac angiogenesis during pressure overload. Moreover, reduced cardiac fibrosis associated with increased myocardial capillarization in aortic-ligated eNOS−/− mice supplemented with eNOS-positive BM compared with eNOS+/−/eNOS−/−/TAC and WT/eNOS+/−/TAC demonstrates the key role of eNOS in the BM-derived endothelial cells but not in cardiomyocyte for its reduction in pressure overload. Thus, beneficial effects of eNOS during cardiac remodelling depend not only on the availability of eNOS in the cardiovascular system but also on intact eNOS activity in the BM. This may be of importance during interventions that increase eNOS activity such as physical exercise. Indeed, the effects of exercise training in pathological hypertrophy and dysfunction may depend on intact eNOS activity in BM-derived cells.

This concept of BM-derived cells contributing to myocardial remodelling was further supported by results of experiments with transplantation of GFP-positive BM cells in WT and eNOS−/− animals. Congenital eNOS deficiency in extramedullary tissues did not influence the number of BM-derived endothelial podocalyxinGFPpos cells in the myocardium showing that renewal of chronic dysfunctional cardiac endothelium is primarily mediated by resident cardiac stem cells and proliferation of mature endothelial cells. In contrast, the number of podocalyxinGFPpos cells was similarly elevated in the myocardium of both strains under conditions of increased cardiac afterload, demonstrating the importance of medullary eNOS for the integration of BM-derived cells in the pressure-overloaded heart.

In summary, the data show that the BM is an important organ participating in the regulation of myocardial remodelling. Specifically, eNOS in the BM was identified as a regulator of myocardial angiogenesis and fibrosis in pressure-induced cardiac hypertrophy. This finding may lead to intriguing perspectives for the development of novel treatment strategies of pressure-induced cardiac hypertrophy and enhancing of myocardial capillarization to eventually delay transition to maladaptive cardiac remodelling.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

We thank E. Becker and S. Jäger for their excellent technical assistance.

Conflict of interest: none declared.

Funding

This work was supported by the Hans & Gertie Fischer-Stiftung to P.M., the Deutsche Gesellschaft für Kardiologie to A.K. and by the Deutsche Forschungsgemeinschaft (KFO 196) to P.M., A.K., M.B., and U.L.

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