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ABSTRACT

Nitric oxide (NO) produced by endothelial nitric oxide synthase (eNOS) in the vessel wall regulates blood pressure and cardiovascular hemodynamics. In this study, we generated conditional eNOS knock out (KO) mice characterized by a duplicated/inverted exon 2 flanked with two pairs of loxP regions ($eNOS^{inv/inv}$); a Cre-recombinase activity induces cell-specific reactivation of eNOS, as a result of a flipping of the inverted exon 2 ($eNOS^{fl}$). This work aimed to test the efficiency of the Cre-mediated cell-specific recombination and the resulting eNOS expression/function. As proof of concept, we crossed $eNOS^{inv/inv}$ mice with $DeleterCre^{pos}$ ($DelCre^{pos}$) mice, expressing Cre recombinase in all cells. We generated heterozygous $eNOS^{fl/inv}$ or homozygous $eNOS^{fl/fl}$ mice, and $eNOS^{inv/inv}$ littermate mice. We found that both $eNOS^{fl/fl}$ and $eNOS^{fl/inv}$ mice express eNOS and the overall expression level depends on the number of mutated alleles, while $eNOS^{inv/inv}$ mice did not show any eNOS expression. Vascular endothelial function was restored in $eNOS^{fl/fl}$ and $eNOS^{fl/inv}$ mice, as determined by ACh-dependent vasodilation of aortic rings. Cre-dependent reactivation of eNOS in $eNOS^{fl/fl}$ and $eNOS^{fl/inv}$ mice rescued $eNOS^{inv/inv}$ (phenotypically global eNOS KO) mice from hypertension. These findings demonstrate that eNOS expression is restored in $eNOS^{fl/fl}$ mice at comparable physiological levels of WT mice, and its functional activity is independent on the number of the reactivated alleles. Therefore, $eNOS^{inv/inv}$ mice are a useful model for studying the effects of conditional reactivation of eNOS and gene dosage effects in specific cells for gain-of-function studies.

1. Introduction

By converting L-arginine to L-citrulline and nitric oxide (NO), the endothelial nitric oxide synthase (eNOS) (NOS3; EC: 1.14.13.39) expressed in the vessel endothelium plays a central role in the regulation of blood pressure and cardiovascular hemodynamics [1]. Although the eNOS isoform was initially isolated from endothelial cells (ECs), it was found to be expressed in other cells including red blood cells (RBCs), cardiomyocytes, renal epithelial cells, cardiac fibroblast and different subpopulations of leukocytes [2]. The role of eNOS in non-endothelial cells is still not fully understood.

To study the role of eNOS *in vivo,* four lines of global eNOS knock out (KO) mice have been generated so far [3–6]. Recently, also cell-specific eNOS KO and knock in (KI) mice models for both loss-of-function and gain-of-function models were generated by us [7,8], which allowed discriminating the function of eNOS in ECs as compared to RBCs. The

Abbreviations: ACh, acetylcholine; BSA, bovine serum albumin; DelCre, DeleterCre; DBP, diastolic blood pressure; ECs, endothelial cells; EDR, endothelium-dependent relaxation; eNOS, endothelial nitric oxide synthase; $eNOS^{inv/inv}$ duplicated/inverted exon 2, flanked by two independent LoxP pairs for recombination/inversion of exon 2; HR, heart rate; KI, knock in; KO, knock out; MAP, mean arterial pressure; NO, nitric oxide; PCR, polymerase chain reaction; PE, phenylephrine; RBCs, red blood cells; RT, reverse transcription; SBP, systolic blood pressure; SD, standard deviation; SNP, sodium nitroprusside; WT, wild type.

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eNOS^{flox} allele for the loss-of-function model consists of exon 2 flanked by a single pair of loxP sites, while the eNOS^{inv} allele for the gain-of-function model is characterized by a duplicated and inverted exon 2 flanked by two independent LoxP pairs for recombination/inversion of exon 2.

The aim of this work was to test the efficiency of the Cre-mediated cell-specific recombination, the resulting eNOS expression, and the effects of gene dosage on functional reactivation of eNOS for vascular reactivity and blood pressure regulation. Therefore, as a proof-of-concept we crossed an eNOS$^{inv/inv}$ mouse with a DeleterCre (DelCre) mouse, expressing Cre recombinase in all cells. We generated mice carrying eNOS in one allele only (eNOS$^{fl/inv}$) or in both alleles (eNOS$^{fl/fl}$), and also eNOS$^{inv/inv}$ littermate mice, which do not express eNOS in any cell. We measured eNOS expression, vascular endothelial function *ex vivo*, and blood pressure *in vivo*. The results of this study demonstrated that the Cre recombinase dependent reactivation of eNOS in eNOS$^{inv/inv}$ mice, generating eNOS$^{fl/fl}$ and eNOS$^{fl/inv}$ mice, restored eNOS expression and functional activity *ex vivo* and *in vivo*; interestingly, the levels of eNOS expression were comparable to WT mice and independent on the number of alleles carrying the active gene, as tested in homozygous and heterozygous mice. Therefore, eNOS$^{inv/inv}$ mice can be used for studying the effects of conditional reactivation of eNOS and gene dosage effects in specific cells for gain-of-function studies.

2. Material and methods

2.1. Animals

All experiments were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe Treaty Series No. 123). Animal care was provided following the institutional guidelines. DelCre (C57Bl/6.C-Tg(CMVCre)1Cgn/J) mice [9] expressing Cre recombinase in all tissues were kindly provided by Prof. Claus Pfeffer (Heinrich Heine University of Düsseldorf). eNOS$^{flox/flox}$ mice and eNOS$^{\Delta/\Delta}$ mice (obtained by crossing of eNOS$^{flox/flox}$ with DelCre^{pos} mice, followed by breeding the Cre recombinase out of the genetic background) were generated by us [7]. All mice were bred at the ZETT (Heinrich-Heine University). Experimental planning and execution followed the ARRIVE recommendations [10]. For experiments, 2–6 months old male mice up to 30 g were used. Mice of the same genotype and age were randomly assigned to experimental groups.

2.2. Generation of eNOS$^{inv/inv}$ mice and eNOS KI mice

The eNOS$^{inv/inv}$ mice were generated as described before [7]. Briefly, to generate the eNOS^{inv} allele, we generated first a LoxP-eNOS construct by simultaneously inserting an orphan loxP site and a FRT-neo-FRT-loxP resistance cassette into the *Nos3* genomic locus to target exon 2 of *Nos3* by Cre recombinase-mediated excision; then we inserted an inverted exon 2 of *Nos3* and two additional LoxP511 sites in the LoxP-eNOS construct to generate a LoxP-eNOS^{inv} construct. The plasmid was sequenced, linearized, and electroporated in A9 ES cells (hybrid C57/129), 300 clones picked, and positive clones were screened by Southern Blot at 5′ arm and by long-range polymerase chain reaction (PCR). We obtained heterozygous C57/129Sv chimera eNOS$^{wt/inv}$ mice from two independent clones, and two independent homozygous eNOS$^{inv/inv}$ mice lines were generated. Genotyping was carried out by PCR (Table 1).One line was selected by testing efficiency of breeding, Cre-mediated recombination, and blood pressure response. These were then backcrossed for 10 generations with C57BL/6J mice. Homozygous backcrossed eNOS$^{inv/inv}$ mice were then crossed with DelCre^{pos} mice to generate eNOS$^{fl/fl}$ Cre$^{pos/neg}$ mice (Table 2). After further crossing with eNOS$^{inv/inv}$ Cre^{neg} mice we generated eNOS$^{fl/inv}$ Cre^{neg} mice, which were used for experiments described here.

2.3. Analysis of DNA recombination

The Cre recombinase-dependent recombination of the *Nos3* DNA locus was determined by real-time PCR with specific primers and probes designed to recognize the floxed/inverted allele (eNOS^{inv}), the flipped allele (eNOS^{fl}), as well as the two intermediates of partial recombination (intermediate 1 and 2) (Transnetyx, Cordova, TN) (Table 1).

Table 1
Primer for DNA recombination. Specific primers and probes for real-time PCR analysis of the expression of each allele designed by Transnetyx (Cordova, TN).

Allele	Forward Primers	Reverse Primers	Probes
eNOS^{inv}	ACCTCCTAAGGCTGTTGTGAGA	CTCCTCTTCCTGACACTTTCTGT	CCCTCACTAAAGGGCG
eNOS^{fl}	CTCCTCTTCCTGACACTTTCTGT	GCTTGCTGCAATTGATAACTTCGTA	CAGCTCATAACTTCGTATAGCAT
Intermediate 1	AGGAAGGACCAGAGGGATCAAG	GGAACTTCAGATCTCCATAACTTCGT	GCAAGAACTTATAACTTCGTATAGTAT
Intermediated 2	GGAACTTCAGATCTCCATAACTTCGT	ACCCTCCTCTTCCTGACACTTT	CTGTCAGCTCATAACTTC
Cre recombinase	TTAATCCATATTGGCAGAACGAAAACG	CAGGCTAAGTGCCTTCTCTACA	CCTGCGGTGCTAACC

Table 2
Crossing strategy for generating heterozygous eNOS$^{fl/inv}$ and homozygous eNOS$^{fl/fl}$ mice from eNOS$^{inv/inv}$ mice. The founder eNOS$^{inv/inv}$ mice were crossed with DelCre^{pos} mice to generate heterozygous (eNOS$^{fl/inv}$) and homozygous (eNOS$^{fl/fl}$) eNOS KI mice (F4).

Pairing	Crossing	Progeny			Frequency
P1	eNOS$^{inv/inv}$ Cre^{neg} X eNOS$^{wt/wt}$ Cre^{pos}	F1	1	eNOS$^{inv/wt}$ Cre^{pos} → eNOS$^{fl/wt}$ Cre^{pos}	1/2
			2	eNOS$^{inv/wt}$ Cre^{neg}	1/2
P2	eNOS$^{inv/inv}$ Cre^{neg} X eNOS$^{fl/wt}$ Cre^{pos}	F2	1	eNOS$^{fl/inv}$ Cre^{pos} → **eNOS$^{fl/fl}$ Cre^{pos}**	1/6
			2	**eNOS$^{fl/inv}$ Cre^{neg}**	1/6
			3	eNOS$^{inv/wt}$ Cre^{pos} → eNOS$^{fl/wt}$ Cre^{pos}	1/6
			4	eNOS$^{inv/wt}$ Cre^{neg}	1/6
			5	eNOS$^{fl/inv}$ Cre^{pos} → **eNOS$^{fl/fl}$ Cre^{pos}**	1/6
			6	**eNOS$^{fl/inv}$ Cre^{neg}**	1/6
P3	eNOS$^{inv/inv}$ Cre^{neg} X eNOS$^{fl/fl}$ Cre^{pos}	F3	1	eNOS$^{fl/inv}$ Cre^{pos} → **eNOS$^{fl/fl}$ Cre^{pos}**	1/2
			2	**eNOS$^{fl/inv}$ Cre^{neg}**	1/2
P4	eNOS$^{fl/inv}$ Cre^{neg} X eNOS$^{fl/inv}$ Cre^{neg}	F4	1	**eNOS$^{fl/fl}$ Cre^{neg}**	1/3
			2	**eNOS$^{fl/inv}$ Cre^{neg}**	1/3
			3	**eNOS$^{inv/inv}$ Cre^{neg}**	1/3

2.4. Analysis of eNOS protein expression by Western blot analysis

Western blot analysis was carried out as described [11]. Briefly, aortas were lysed in RIPA buffer (0.5 % sodium deoxycholate, 0.1 % SDS, 1 % NP40 in phosphate-buffer saline (PBS), pH 7.4) containing a cOmplete EDTA-free protease inhibitor cocktail (Roche, Applied Science, Indianapolis, IN, USA) and homogenized by using TissueRuptor (Qiagen, Hilden, Germany) on ice. The lysates were sonicated for 1 min and centrifuged at 10,000 g for 10 min at 4 °C. The total concentration of protein in the supernatant was determined by Lowry assay (BioRad, Feldkirchen, Germany). The samples underwent to electrophoresis on 10 % Bis-Tris gel (CarlRoth, Karlsruhe, Germany) and transferred to a nitrocellulose membrane (Amersham Biosciences, Munich, Germany). The membranes were blocked with 5 % milk (Merck, Darmstadt, Germany) in T-TBS (10 mM Tris, 100 mM NaCl, 0.1 % Tween) for 1 h at room temperature, followed by incubation with a mouse anti-eNOS (1:100) in 5 % bovine serum albumin (BSA) in T-TBS (BD Transduction Laboratories, Heidelberg, Germany) overnight at 4 °C, or anti-mouse anti-β-actin (1:1000) (Merck, Darmstadt, Germany) for 1 h at room temperature in 5 % milk in T-TBS. The 4 to 6 consecutive washings steps were done by using T-TBS for 1 h in total, followed by detection using West Pico and West Femto Chemiluminescence Detection Reagents (Thermo Fisher Scientific, Darmstadt, Germany) in an iBright FL1000 (Thermo Fisher Scientific). Band intensity of eNOS was quantified by using ImageJ (National Institutes of Health, USA).

2.5. Analysis of vascular endothelial reactivity ex vivo

Functional activity of thoracic aorta was analyzed in an organ bath as described [12]. Briefly, after a 60-min equilibration phase, the aortic rings were treated with 80 mM KCl. The vasoconstriction that developed during the last KCl application was taken as the maximal receptor-independent vasoconstriction. Aortic rings were treated with cumulative concentrations of acetylcholine (ACh) (0.1 nM - 10 μM), followed by treatment with phenylephrine (PE) (0.1 nM - 10 μM) and, subsequently, with sodium nitroprusside (SNP) (0.1 nM - 10 μM).

2.6. Invasive measurement of blood pressure by Millar cathether

Invasive blood pressure measurements were performed by using a 1.4F Millar pressure-conductance catheter (SPR-839, Millar Instrument, Houston, TX, USA) placed into the left ventricle through the right carotid artery according to the closed chest method as described [11]. The pressure data were recorded by a Millar Box and analyzed with LabChart 7 (AD Instruments, Oxford, UK).

2.7. Statistical analysis

Unless otherwise specified, all results are presented as means ± standard deviation (SD). Statistical analysis were carried out with GraphPad 10 for Windows (Version 10.1.2 (324)). As indicated, unpaired Student's t-test with Welch's correction was used to determine statistical significance between two independent groups. Comparisons among multiple groups were performed using two-way ANOVA followed by Tukey post hoc analysis or using unpaired *t*-test with Welch's corrections. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Gene-targeting strategy for the generation of $eNOS^{inv/inv}$, $eNOS^{fl/inv}$ and $eNOS^{fl/fl}$ mice

We aimed to generate mice for conditional Cre recombinase-dependent activation of eNOS in any cell of interest that constitutively express eNOS. To achieve this, we designed a gene-targeting construct

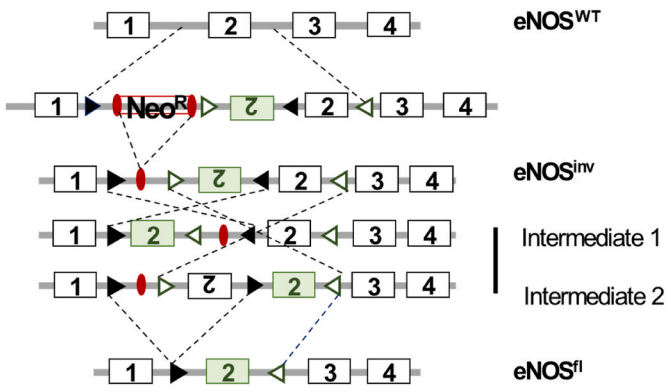

Fig. 1. Scheme describing the gene-targeting strategy for the generation of $eNOS^{inv/inv}$ and $eNOS^{fl/fl}$ mice. The founder $eNOS^{inv/inv}$ mice were generated by gene targeting recombination using a construct characterized by an inverted (or "flipped") exon 2 (indicated in green) followed by a second copy of exon 2 (in black), both flanked by two pairs of LoxP (black) and LoxP511 (white) sequences. The neomycin cassette was removed by using restriction enzymes and the $eNOS^{inv}$ construct was used to generate $eNOS^{inv/inv}$ mice. In the presence of a Cre recombinase, two intermediates can occur depending on which couple of LoxP sites will be recognized from the enzyme. In the intermediate 1, the Cre recombinase recognized the LoxP sites (black), which are oriented head-to-head and therefore the inverted exon 2 (green) is "flipped"; in the intermediate 2, the Cre recombinase recognized the LoxP511 (white) sites, which are also oriented head-to-head leading to the flipping of the whole sequence including both copies of exon 2; in this way the inverted exon 2 (green) will be found in the right orientation. In the last step of the recombination, the sequence floxed by two LoxP sites oriented to the same direction (head-to-tail) is excised and the other remains. Both intermediates leads to a final construct where just the newly inserted exon 2 is oriented in the right position and codify for a full functional *Nos3* locus under the control of its own promoter.

with an inverted (or "flipped") exon 2 followed by a second copy of exon 2, both flanked by two pairs of LoxP and LoxP511 sequences (Fig. 1; Construct). After removal of the neomycin resistance cassette, this plasmid construct was used to generate $eNOS^{inv/wt}$ mice by gene targeting replacement. The generated mice were backcrossed for 10 generations with C57BL/6J mice and finally crossed to generate $eNOS^{inv/inv}$ mice.

After crossing $eNOS^{inv/inv}$ mice with $DelCre^{pos}$ mice, the Cre recombinase activity resulted in two possible recombination reactions, which depend on which couple of LoxP sites are targeted by the Cre recombinase. One reaction leads to the "flipping" of the inverted exon 2 (intermediate 1) (green), followed by the removal of the second exon 2 (black). The second type of reaction leads to the flipping of the whole sequence containing both the first and the second copy of exon 2 (intermediate 2) followed by removal of the flipped exon (black). Both reactions lead to a final construct where only one exon 2 oriented in the correct direction remains, resulting in the "reactivation" of the gene (Fig. 1).

Thus, in the presence of the Cre recombinase the DNA recombination reaction results in the transformation of $eNOS^{inv}$ allele into the $eNOS^{fl}$ allele. We found that in very few cases the recombination was stuck at intermediate 1 or intermediate 2. These mice were easily recognized as they resulted positive for the Cre recombinase and for the $eNOS^{inv}$ allele at the same time, and the DNA locus corresponding to intermediate 1 or intermediate 2 was detectable by using specific primers by real-time PCR (Table 1).

Next we aimed to generate $eNOS^{fl/fl}$ Cre^{neg} and $eNOS^{fl/inv}$ Cre^{neg} mice. As shown in Table 2, by crossing $eNOS^{inv/inv}$ Cre^{neg} mice with WT ($eNOS^{wt/wt}$) Cre^{pos} mice, the first generation (F1) are heterozygous $eNOS^{wt/inv}$ Cre^{pos} or $eNOS^{wt/inv}$ Cre^{neg} mice. Only when the Cre recombinase is present, the $eNOS^{inv}$ allele is flipped, resulting in $eNOS^{fl/wt}$ Cre^{pos} mice and $eNOS^{inv/wt}$ Cre^{neg} mice. The F2 was generated by

crossing eNOS$^{inv/inv}$ Cre^{neg} mice with eNOS$^{fl/wt}$ Cre^{pos} mice (both from F1). In this case, four different results are possible: eNOS$^{fl/inv}$ Cre^{pos} or Cre^{neg} mice, and eNOS$^{inv/wt}$ Cre^{pos} or Cre^{neg} mice. Again, when the Cre recombinase is expressed, the eNOS^{inv} allele is "flipped" leading to a F2 characterized by: eNOS$^{fl/fl}$ Cre^{pos} mice, eNOS$^{fl/inv}$ Cre^{neg} mice, eNOS$^{fl/wt}$ Cre^{pos} mice and eNOS$^{inv/wt}$ Cre^{neg} mice. At this point (F3), eNOS$^{fl/fl}$ Cre^{pos} mice or eNOS$^{fl/inv}$ Cre^{neg} mice can be crossed between them or with eNOS$^{inv/inv}$ Cre^{neg} mice to obtain the mice needed for the experiments, i.e. eNOS$^{fl/fl}$ Cre^{neg} mice, eNOS$^{fl/inv}$ Cre^{neg} mice and eNOS$^{inv/inv}$ Cre^{neg} mice. Mice carrying these three genotypes were used for the following experiments, and are phenotypically homozygous eNOS KI mice (i.e. phenotypically a WT mice with reactivation of eNOS in both alleles), heterozygous eNOS KI mice (i.e. with eNOS reactivation in one allele and lacking eNOS in the other allele) and global eNOS KO mice (i.e. lacking eNOS in both alleles) (Table 2).

3.2. Characterization of eNOS$^{inv/inv}$, eNOS$^{fl/inv}$ and eNOS$^{fl/fl}$ mice

The recombination of the *Nos3* DNA locus was analyzed in ear tissue from eNOS$^{inv/inv}$, eNOS$^{fl/inv}$ and eNOS$^{fl/fl}$ mice by real-time PCR using specific primers recognising the eNOS^{inv} and the eNOS^{fl} alleles (Fig. 2A). This analysis demonstrated the presence of the recombined eNOS^{fl} DNA locus only in samples from the eNOS$^{fl/inv}$ and eNOS$^{fl/fl}$ mice, and its absence in the eNOS$^{inv/inv}$ mice (Fig. 2A). Vice versa, eNOS^{inv} allele was present in eNOS$^{fl/inv}$ and eNOS$^{inv/inv}$ mice, and not in the eNOS$^{fl/fl}$ mice.

Further characterization was done by immunoblotting of eNOS in aorta lysates from eNOS$^{fl/fl}$, eNOS$^{fl/inv}$, eNOS$^{inv/inv}$ mice and WT mice (Fig. 2B–D). We found that both eNOS$^{fl/fl}$ and eNOS$^{fl/inv}$ mice expressed eNOS in the aorta, while eNOS$^{inv/inv}$ mice did not show any eNOS expression in aorta (or any other organ as shown elsewhere [7,8]) (Fig. 2C, D representative Western blots). The eNOS expression was at the same level in eNOS$^{fl/fl}$ and eNOS$^{fl/inv}$ mice as compared to the WT controls (Fig. 2B, Semiquantitative analysis of independent mice cohorts and gels, each dot indicate a sample collected from a different mouse). Taken together, these results showed that eNOS was successfully reactivated in the aorta of the eNOS$^{fl/fl}$ and eNOS$^{fl/inv}$ models and that the presence of one allele can compensate for lack of eNOS in the other allele by up-regulation of eNOS expression.

3.3. Vascular endothelial function is restored in eNOS$^{fl/inv}$ and eNOS$^{fl/fl}$ mice

Next, we aimed to investigate the vascular endothelial function after the Cre recombinase induced reactivation of eNOS from the eNOS$^{inv/inv}$ mice. We found that aortic rings from eNOS$^{fl/fl}$ and eNOS$^{fl/inv}$ mice showed a fully restored endothelium-dependent relaxation in response to ACh as compared to eNOS$^{inv/inv}$ mice, which instead showed a lack of ACh-induced vasodilation (Fig. 3A–B). The contractile response to PE is decreased in eNOS$^{fl/fl}$ and eNOS$^{fl/inv}$ mice as compared to eNOS$^{inv/inv}$ mice (Fig. 3C). The vasodilatory response to the NO donor SNP is increased in eNOS$^{fl/fl}$ and eNOS$^{fl/inv}$ mice as compared to eNOS$^{inv/inv}$ mice. This is also visible from the shift of the curves to the right (Fig. 3D). The ACh-induced relaxation, PE-induced constriction, and SNP-induced relaxation were not different in in eNOS$^{fl/fl}$ as compared to WT (eNOS$^{flox/flox}$) mice (Fig. S1). These findings indicate that the vascular endothelial function is fully restored in eNOS$^{fl/fl}$ and eNOS$^{fl/inv}$ mice.

3.4. Hypertension is fully rescued after reactivation of eNOS in eNOS$^{fl/inv}$ and eNOS$^{fl/fl}$

To verify if the Cre recombinase induced reactivation of eNOS may also rescue the hypertensive phenotype of global eNOS KO mice [3–5,7], blood pressure and cardiac performance were determined invasively in eNOS$^{fl/inv}$, eNOS$^{fl/fl}$ and eNOS$^{inv/inv}$ mice by using a Millar catheter. For comparison, we here report also data from WT (eNOS$^{flox/flox}$ mice) and global eNOS KO (eNOS$^{\Delta/\Delta}$ mice), which were previously published (Fig. 4; Table 3) [7].

Interestingly, both eNOS$^{fl/inv}$ and eNOS$^{fl/fl}$ mice showed a significant decrease in systolic blood pressure (SBP), as compared to eNOS$^{inv/inv}$ (Fig. 4A; Table 3). eNOS$^{inv/inv}$ mice showed increased SBP and no changes in diastolic blood pressure (DBP) as compared to WT mice (eNOS$^{flox/flox}$) (Fig. 4). The levels of blood pressure are comparable with data obtained in global eNOS KO (eNOS$^{\Delta/\Delta}$) mice as also published before [7]. Also in this case the values are comparable to the values of WT mice (eNOS$^{flo/flox}$) [7]. Taken together, these results show that Cre-induced reactivation of eNOS in global eNOS KO mice rescues the mice from hypertension.

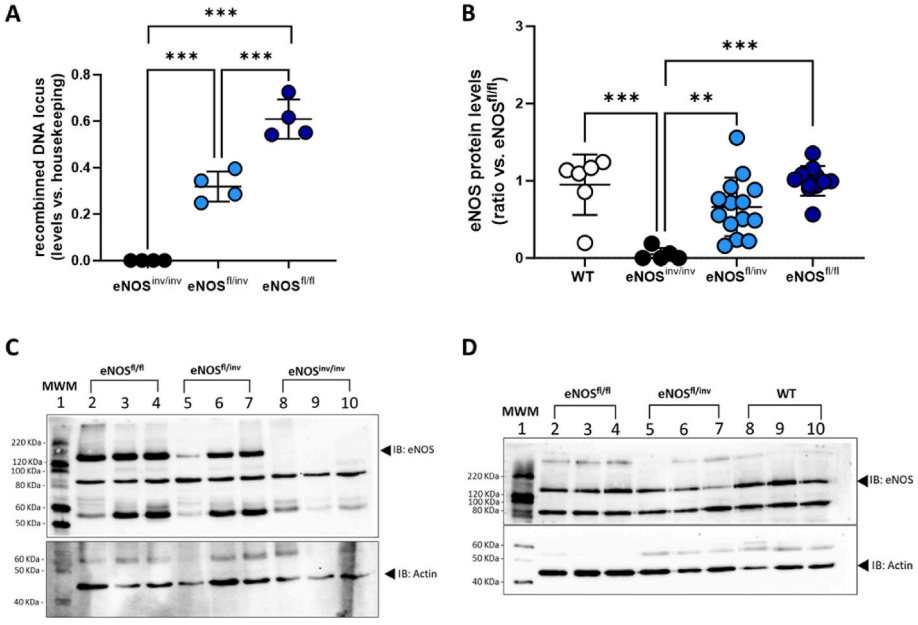

Fig. 2. Characterization of eNOS$^{inv/inv}$, eNOS$^{fl/fl}$ and eNOS$^{fl/inv}$ mice. DNA recombination was detected by (A) real-time PCR on DNA extracted from ear tissue, showing that DNA recombination occurred in both eNOS$^{fl/inv}$ and eNOS$^{fl/fl}$ mice, while the recombination did not occur in the eNOS$^{inv/inv}$ mice. (B–D) Western blot analysis of eNOS expression show eNOS in the aorta of eNOS$^{fl/fl}$, eNOS$^{fl/inv}$ mice and in the WT controls, while eNOS was not detected in eNOS$^{inv/inv}$ mice. One-way ANOVA; post hoc Tukey; *p < 0.05; **p < 0.01, ***p < 0.001.

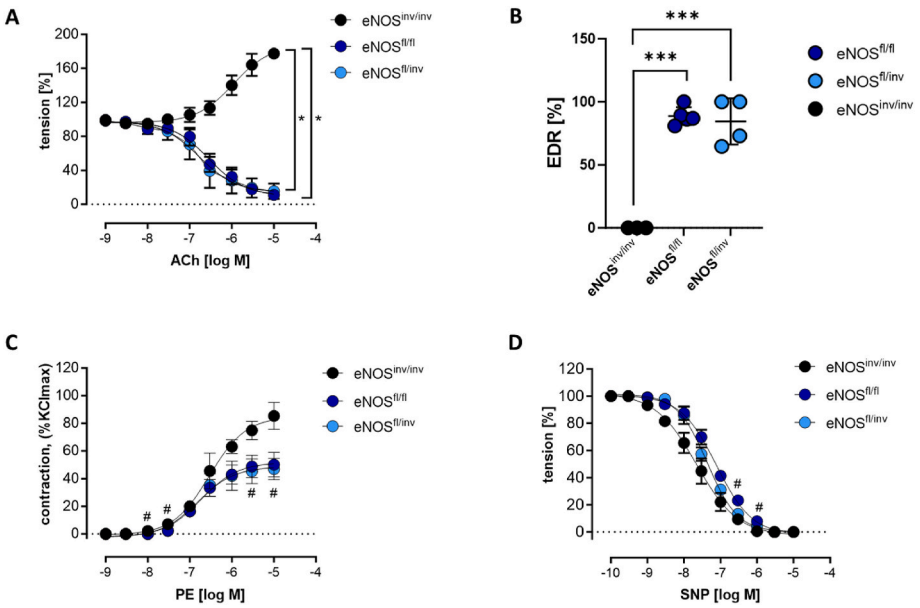

Fig. 3. Vascular endothelial function in aortic rings of eNOS$^{fl/fl}$, eNOS$^{fl/inv}$ and eNOS$^{inl/inv}$ mice. (A) eNOS$^{fl/fl}$ and eNOS$^{fl/inv}$ mice showed a fully preserved endothelium-dependent relaxation (EDR) in response to ACh as compared to eNOS$^{inv/inv}$ mice. Two-way ANOVA concentration p = 0.0003, genotype p = 0.0003; post hoc Tukey *p < 0.05 vs. eNOS$^{inv/inv}$. (B) EDR in response to ACh (calculated as the percentage of the maximal ACh response) is fully preserved in eNOS$^{fl/fl}$ and eNOS$^{fl/inv}$ mice. One-way ANOVA; post hoc Tukey; ***p < 0.001. (C) The contractile response to PE is decreased in eNOS$^{fl/fl}$ and eNOS$^{fl/inv}$ mice as compared to eNOS$^{inv/inv}$ mice. (D) The vasodilatatory response to SNP is increased in eNOS$^{fl/fl}$ and eNOS$^{fl/inv}$ mice as compared to eNOS$^{inv/inv}$ mice as shown in the shift of the curves of eNOS$^{fl/fl}$ and eNOS$^{fl/inv}$ mice to the right. Unpaired *t*-test with Welch's correction; no correction for multiple comparison; #p < 0.05. ACh, acetylcholine; PE, phenylephrine; SNP, sodium nitroprusside.

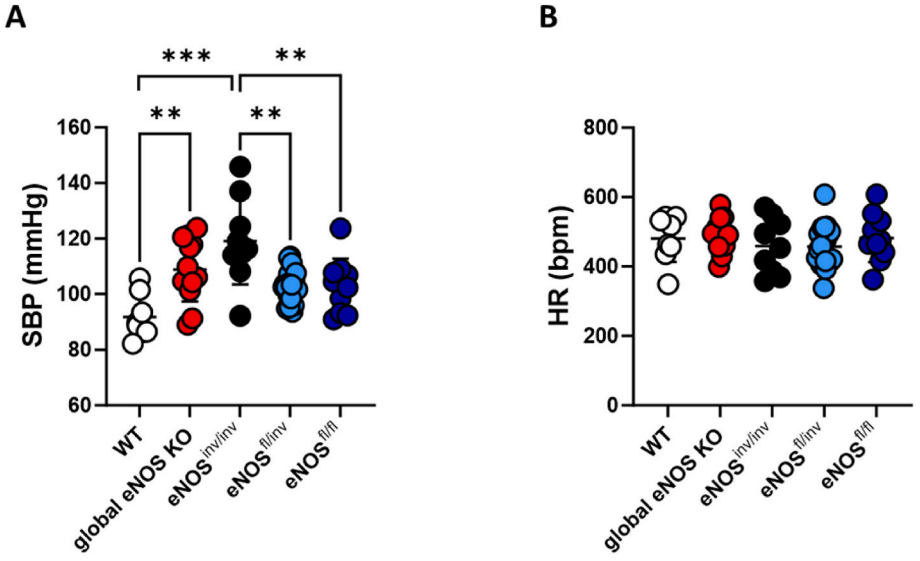

Fig. 4. Systolic blood pressure (SBP) of eNOS$^{inv/inv}$, eNOS$^{fl/inv}$ and eNOS$^{fl/fl}$ mice. (A) SBP measurements were assessed in anesthetized eNOS$^{inv/inv}$, eNOS$^{fl/inv}$, eNOS$^{fl/fl}$ mice as well as in WT and global eNOS KO mice by using a Millar catheter; both eNOS$^{fl/inv}$ and eNOS$^{fl/fl}$ mice showed a decrease in SBP as compared to eNOS$^{inv/inv}$ mice (black). Blood pressure data of WT (eNOS$^{flox/flox}$) and global eNOS KO (eNOS$^{\Delta/\Delta}$) mice were published in Leo et al., Circulation 2021, and reported here for comparison. The data show that eNOS$^{inv/inv}$ showed high SBP as compared to WT mice, and no differences in SBP as compared to eNOS$^{fl/inv}$ and eNOS$^{fl/fl}$ mice. (B) No differences in HR were shown among eNOS$^{inv/inv}$, eNOS$^{fl/inv}$, eNOS$^{fl/fl}$, WT and global eNOS KO mice). One-way ANOVA; post hoc Tukey; **p < 0.01; ***p < 0.001. SBP, systolic blood pressure; HR, heart rate.

4. Discussion

In this study, we describe the generation and characterization of conditional eNOS KO mice (eNOS$^{inv/inv}$), which can be applied in gain-of-function studies for a Cre recombinase-induced reactivation of eNOS expression in the cell/tissue of interest. The reactivation of eNOS can be easily achieved by crossing eNOS$^{inv/inv}$ mice with any cell-specific Cre recombinase expressing mouse of choice.

As a proof-of-concept, we crossed eNOS$^{inv/inv}$ mice with DelCre^{pos} mice, expressing Cre recombinase in all cells and tested the efficiency of the DNA recombination. We found that the expression of eNOS in eNOS$^{fl/fl}$ mice is comparable to WT mice. Specifically, the quantification of protein expression of eNOS by immunoblotting showed that eNOS$^{fl/inv}$ and eNOS$^{fl/fl}$ mice exhibit comparable levels of eNOS expression to WT mice.

Table 3
Systemic hemodynamics and cardiac function as assessed by the Millar catheter. Data are reported as mean ± SD; n = number of analyzed mice. Differences between each group were calculated by one-way ANOVA, *P < 0.05, **P < 0.01, ***P < 0.001. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate.

Parameter	Unit	$eNOS^{inv/inv}$	$eNOS^{fl/fl}$	$eNOS^{fl/inv}$	p		
					$eNOS^{inv/inv}$ vs. $eNOS^{fl/fl}$	$eNOS^{inv/inv}$ vs. $eNOS^{fl/inv}$	$eNOS^{fl/fl}$ vs. $eNOS^{fl/inv}$
n		9	10	20			
HR	bpm	458.1 ± 80.0	482.3 ± 70.3	457.0 ± 59.3	0.7146	0.9990	0.5969
SBP	mmHg	119.1 ± 15.7	102.9 ± 9.9	103.1 ± 5.7	0.0027**	0.0007***	0.9993
DBP	mmHg	77.2 ± 16.3	67.2 ± 6.8	70.0 ± 6.5	0.0747	0.1687	0.7260
MAP	mmHg	91.2 ± 15.5	79.1 ± 7.7	81.0 ± 5.5	0.0185*	0.0245*	0.8478
n		3	4	4			
dP/dt_{max}	mmHg/s	7211.0 ± 1349.0	8327.0 ± 1251.0	7977.0 ± 984.7	0.5383	0.7363	0.9061
dP/dt_{min}	mmHg/s	−8185.0 ± 1785.0	−8034.0 ± 1660.0	−7827.0 ± 1050.0	0.9922	0.9567	0.9779

In this study, we showed that $eNOS^{fl/fl}$ and $eNOS^{fl/inv}$ mice have a fully preserved endothelium-dependent relaxation in response to ACh and SNP, as well as a decreased contractile response to PE. This is consistent to previous reports showing that eNOS KO mice exhibited no response to ACh-treatment in aortic rings [3], as well as no dilatory response to shear stress following occlusion of the iliac artery [11]. These studies suggest that eNOS plays a central role on vascular function regulation. Similarly, in a previous work we demonstrated that EC eNOS KO mice lacked ACh-mediated vasodilation of aortic rings as well as flow-mediated dilatory response after occlusion of the iliac artery. On the other hand, the specific reactivation of eNOS in EC eNOS KI mice fully restored vascular endothelial dilator function. Taken together reactivation of eNOS in all body (as shown here) or in vascular ECs fully rescues vascular function [7].

The rescue of eNOS function after the reactivation in the whole body is similarly evident in the regulation of the blood pressure levels. Indeed, while the founder line $eNOS^{inv/inv}$ mice are hypertensive, the $eNOS^{fl/fl}$ mice show normotension and preserved eNOS expression in the vessels. We here also report that even the reactivation of eNOS in a single allele is sufficient to completely rescue the hypertensive phenotype, as the blood pressure levels of the $eNOS^{fl/inv}$ heterozygous mice are not different from the homozygous $eNOS^{fl/fl}$ or the WT mice. These findings are supported by previous studies demonstrating that under basal conditions, heterozygous eNOS KO mice have a blood pressure similar to those of WT mice, as well as identical endothelial-dependent vasodilation and vasoconstriction responses to serotonin and PE [13,14]. The same group demonstrated that under physiological shear stress such as induced by exercise training, heterozygous eNOS KO mice showed altered vascular endothelial function as assessed by an accumulation of aortic cGMP after ACh stimulation. In this particular study, exercise training increased eNOS expression up to 3-fold in the aorta of WT mice, but this effect was absent in heterozygous eNOS KO mice, indicating that the presence of eNOS in only one allele may impair its expression under shear stress conditions.

A limitation of this study is that we tested vascular endothelial function in aorta only, and not in other vessel beds. It is important to point out that the aorta does not play any role in blood pressure regulation, but it is here used only as an indication of the presence of eNOS in the endothelium of the vessels. It would be more beneficial to investigate other kind of vessels such as small arteries, and resistance arterioles which contribute to the regulation of the peripheral vascular resistance and therefore to the control of blood pressure.

The use of the Cre/LoxP recombination system to reactivate a gene in gain-of-function studies was tested for the first time in a mouse genome in 2002 [15]. In this study, the authors successfully introduced point mutations in the CREB-binding domain, which led to a Cre-mediated inversion of exon 5 in bacteria and in embryonic stem cells with an efficiency of 100 % and 64–100 % respectively [15]. As reported in the literature, gene reactivation by the Cre/loxP system for this purpose not always led to successful results. For example, the generation of two conditional KO mice for the *Impad1* and *Clcn7* genes resulted in severe letal phenotype in *Impad1* CondKO mice, which was related to the synthesis of non-functional proteins and not to the introduction of missense mutations [16].

To the best of our knowledge, this is the first time that a conditional eNOS KO ($eNOS^{inv/inv}$) mouse model was generated targeting exon 2 of the eNOS gene. In the past, other global eNOS KO mice were generated by gene targeting replacement of other exons [3,4]. In the Huang's strain, the fragment Hind Ill to Sa/I containing the exons encoding the NADPH ribose and adenine binding sites were replaced [3]; in the Shesely's strain (now available by Jackson Laboratory, JAX stock #002684) the exon 12 of eNOS gene was targeted leading to the disruption of the calmodulin binding site [4]; in the strain generated by A. Gödecke, the sequences encoding for the essential NADPH binding site (exons 24 and 25) were deleted [5]. Last, in the Morishita's strain, all the three isoforms of NOS were disrupted; for the generation of this line, the Huang's strain was crossed with $nNOS^{-/-}$ and $iNOS^{-/-}$ mice [6]. The $eNOS^{flox/flox}$ mice and the $eNOS^{inv/inv}$ mice generated by us [7] have the advantage that by targeting exon 2, the transcription of the $eNOS^{\Delta}$ (obtained after Cre recombinase induced deletion of $eNOS^{flox}$) and $eNOS^{inv}$ allele does not form any coding mRNA and consequently any truncated protein. For example, formation of a ca. 70 kDa truncated eNOS protein is observed in the commercially available eNOS KO strain from Jackson Lab. Truncated proteins may result in unspecific phenotypes due to for example of negative dominant formation of non-functional complexes with protein targeting the N-terminus sequence of eNOS.

The data presented here with global eNOS KI mice ($eNOS^{fl/fl}$) and elsewhere for EC eNOS KI mice [7] show that eNOS expression is restored at comparable levels of WT mice. This is particularly relevant, as in this mouse model the expression of eNOS is under the control of its own constitutive promoter. Therefore, the reactivation of eNOS expression does not result in major changes in basal levels of expression. This means also that by crossing conditional eNOS KO mice with any cell-specific Cre recombinase expressing mice will not result in eNOS expression in cells that do not express eNOS under physiological condition. Thus, this model allows the analysis of the physiological role of eNOS after its reactivation in specific cells.

Previously, models of cell-restricted expression of eNOS resulted in a 5-fold overexpression of the enzyme [17,18]. On the other hand, $eNOS^{inv/inv}$ can be applied strategically to effectively trace the physiological expression of eNOS in cells and tissues, i.e. by crossing $eNOS^{inv/inv}$ mice with mice expressing Cre recombinase under the control of the promoter of interest and measuring eNOS expression by single cell sequencing or other techniques. In this context, the absence of eNOS in all other cells avoid the issue of measuring eNOS expression from other cross contaminating cells, as all other cells are eNOS KO. By using this model for example we were not able to measure any expression of eNOS in mouse cardiomyocytes [8] but we confirmed its presence in RBCs [7]. Previusoly, purified ventricular myocytes from neonatal and adult rats

were shown to constitutively express eNOS as assessed by immunocytochemistry and by measuring responses to carbachol in neonatal cardiac cells preparations [19,20], where eNOS was proposed to modulate cardiac contractility in response to adrenergic and cholinergic stimuli [19]. By using co-immunoprecipitation it was shown that in mouse cardiomyocytes eNOS is mainly found in caveolae in association with caveolin-3 [21]. Human cardiomyocytes were shown to express eNOS in heart tissue as measured in heart biopsies of patients with cardiac failure [22]. Moreover, in mouse cardiac fibroblasts eNOS expression was detected by Western blotting analysis and NOS activity by citrullin assay, and it was proposed that eNOS from cardiac fibroblasts had a protective role against the development of cardiac fibrosis [23,24]. Our group showed that in cardiomyocytes preparations from hearts of WT or EC eNOS KO mice, eNOS expression was very low or undetectable under the conditions applied. Moreover, in whole heart tissue, eNOS expression was not detectable by immunoblotting and showed very low levels by ELISA [8]. Therefore, more data are necessary to understand the role of non-endothelial eNOS in the mouse heart, in particular in fibroblast and cardiomyocytes, and its relevance as compared to the human situation.

Although the expression of eNOS in the kidney is mostly in ECs, its expression was found also in epithelial cells of the inner medullary collecting duct and the thick ascending limb of the loop of Henle [25–27] where it plays a role in sodium handling and therefore in the long-term blood pressure regulation [28]. In kidney tissue lysates from EC eNOS KO mice we found a very low, but detectable expression of eNOS, indicating its presence in non ECs (likely renal epithelial cells) [7]. We found similar results also in kidney lysates [7]. Further studies are needed to clearly distinguish the role of eNOS in these different compartments.

In summary, in this paper we describe and characterize a conditional eNOS KO model, which allows the reactivation of eNOS in any specific cell type at a physiological level under the control of the endogenous eNOS promoter. By crossing $eNOS^{inv/inv}$ mice with $DelCre^{pos}$ mice, we demonstrated that the reactivation of eNOS restored vascular function and blood pressure. The ability to selectively reactivate eNOS expression in specific cell types by using $eNOS^{inv/inv}$ mice models offers a powerful tool for manipulating the eNOS expression in cell-specific compartments and investigating the cell-specific role of eNOS-derived NO production in health and diseases. Thus, it may help to identify novel therapeutic targets and develop more effective treatments for conditions such as hypertension and endothelial dysfunction.

CRediT authorship contribution statement

Anthea LoBue: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization, Data curation, Project administration. **Zhixin Li:** Writing – review & editing, Investigation. **Sophia K. Heuser:** Writing – review & editing, Investigation. **Junjie Li:** Writing – review & editing, Visualization, Data curation. **Francesca Leo:** Investigation. **Lukas Vornholz:** Investigation. **Luke S. Dunaway:** Investigation. **Tatsiana Suvorava:** Writing – review & editing, Data curation, Investigation, Supervision. **Brant E. Isakson:** Writing – review & editing, Formal analysis, Supervision. **Miriam M. Cortese-Krott:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization, Formal analysis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2024.10.009.

Data availability

Data will be made available on request.

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