Published in final edited form as:

Genesis. 2012 September ; 50(9): 685–692. doi:10.1002/dvg.22026.

Generation of a Conditional Allele for the Mouse Endothelial Nitric Oxide Synthase Gene

Rosie Jiang^a, Suwan Wang^a, Keiko Takahashi^a, Hiroki Fujita^b, Christopher R. Fruci^a, Matthew D. Breyer^{a,c}, Raymond C Harris^a, and Takamune Takahashi^{a,1}

^aDivision of Nephrology, Vanderbilt University School of Medicine, Nashville, TN, USA

^bDivision of Endocrinology, Metabolism and Geriatric Medicine, Akita University Graduate School of Medicine, Akita, Japan

^cBiotechnology Discovery Research, Lilly Research Laboratories, Eli Lilly, and Company, Indianapolis, IN, USA

Abstract

Mice with endothelial nitric oxide synthase (eNOS) deletions have defined the crucial role of eNOS in vascular development, homeostasis, and pathology. However, cell specific eNOS function has not been determined, although an important role of eNOS has been suggested in multiple cell types. Here we have generated a floxed eNOS allele in which exons 9–12, encoding the sites essential to eNOS activity, are flanked with loxP sites. Mice homozygous for the floxed allele showed normal eNOS protein levels and no overt phenotype. Conversely, homozygous mice with Cre-deleted alleles displayed truncated eNOS protein, lack of vascular nitric oxide production, and exhibited similar phenotype to eNOS knockout mice, including hypertension, low heart rate, and focal renal scar. These findings demonstrate that the floxed allele is normal and it can be converted to a non-functional eNOS allele through Cre recombination. This mouse will allow time and cell specific eNOS deletion.

Keywords

Endothelium; Vasodilator; Gene Targeting; Conditional; Cre Recombinase

Nitric oxide (NO) is a gaseous signaling molecule playing a role in various biological functions. In vasculature, NO is constitutively produced by endothelial nitric oxide synthase (eNOS), which is encoded by the *Nos3* gene (Dudzinski *et al.*, 2006). In past decades, mice with global gene deletion of eNOS have defined an important role of eNOS in vascular development, homeostasis, and pathology. Mice, lacking eNOS, exhibit developmental vascular defects in certain organs, including heart, aorta, lung, and kidney (Feng *et al.*, 2002; Forbes *et al.*, 2007; Han *et al.*, 2004; Lee *et al.*, 2000). Further, a large number of studies have shown that eNOS deficiency causes various vascular disorders, including impaired vasodilatation and hypertension, and accelerates vascular diseases in mice (Albrecht *et al.*, 2003; Ortiz and Garvin, 2003). On the other hand, growing evidence demonstrates an important role of eNOS in non-endothelial sites, including cardiomyocytes, osteoblasts, adipocytes, renal tubules, and erythrocytes (Aguirre *et al.*, 2001; Herrera *et al.*, 2006; Kleinbongard *et al.*, 2006; Koh *et al.*, 2010; Lepic *et al.*, 2006). Thus, conditional gene

¹Corresponding author Takamune Takahashi MD, PhD., Address: Division of Nephrology and Hypertension, Vanderbilt University School of Medicine, S-3223, MCN, Nashville, TN 37232, USA., Phone: +1 615 343 4312, Fax: +1 615 343 7156, takamune.takahashi@vanderbilt.edu or raymond.harris@vanderbilt.edu.

targeting to determine cell specific eNOS function in developmental and adult tissues facilitates further understanding of the role of eNOS in tissues. Therefore, here we have generated a conditional allele of the mouse eNOS (*Nos3*) gene.

The targeting vector was designed to allow conditional deletion of exons 9-12 of the mouse eNOS gene, removing the calmodulin-binding site and resulting in non-functional eNOS protein (Fig.1A). The exons 9-12 were flanked with a loxP sequence and a neomycin selection cassette (PGK-neo) was flanked with a FRT sequence (Fig.1A). The construct was electroporated into TL1 ES cells and G418 resistant clones were screened by Southern blots using 5'- and 3'-external probes (Fig.1B). Two independent ES clones (clones 1A9 and 6A9) bearing correctly targeted allele were injected into C57BL/6J blastocysts. Resulting chimera mice were mated to 129-FLPe mice, removing a PGK-neo cassette through FRT/FLPe recombination, and the floxed (flox) allele was generated (Fig.1A). Removal of the neo cassette was assessed in F₁ offspring by tail DNA PCR using Neo F1, F2, and R primers (Fig.1C, left panel). The neo-present targeted (flox-neo) allele was completely converted to flox allele in mice carrying FLPe transgene (Fig.1C, right panel). Heterozygous (WT/flox) F₁ mice were further intercrossed to generate F₂ offspring and the mice were genotyped by PCR of tail genomic DNA using Flox F and R primers. Analysis of 94 F₂ mice showed 18 (23%) of wild-type (WT/WT), 49 (50%) of heterozygous (WT/flox), and 27 (26%) of homozygous (flox/flox) mice, indicating transfer of flox allele with Mendelian frequencies. Both WT/flox and flox/flox mice showed normal appearance and fertility. The floxed mice that lack FLPe transgene were subjected to further characterization.

The floxed eNOS allele was characterized by the generation of global eNOS knockout (del/del) mice and their phenotyping. As illustrated in Fig.2A, the flox allele was globally converted to the del allele by mating floxed eNOS mice to Sox2-cre strain. This Cre transgenic mouse drives Cre recombinase in all epiblast cells at an early embryonic stage under the transcriptional control of SRY-box containing gene 2 (Sox2) (Hayashi *et al.*, 2002). Hence, this Cre deleter mouse eliminates floxed gene in all cells and creates a complete gene knockout. Deletion of exons 9-12 was assessed by PCR of tail genomic DNA using del F and R primers (Fig.2B). Successful recombination was determined by the presence of a 475bp band (Fig.2B). Homozygous and heterozygous del mice were differentiated by PCR using del F and flox R primers, where homozygous del mice are denoted by an absence of a 448bp wildtype band. The resulting homozygous del (del/del) mice were further characterized, comparing with wildtype (WT/WT) and homozygous floxed (flox/flox) mice. First, eNOS protein levels were assessed in WT/WT, flox/flox, and del/del mice for kidney and lung tissues by Western blot analysis. Shown in Fig. 2C, comparable levels of wildtype eNOS protein (135kDa) were expressed in WT/WT and flox/flox mice tissues, while the expected size (114 kDa) of truncated eNOS protein was expressed in del/del mouse tissues at low levels. Wildtype eNOS protein was not detected in the del/del mice tissues. Consistent with these findings, eNOS immunohistochemistry labeled kidney and lung vasculature in WT/WT and flox/flox mice in a similar pattern, while sections from del/del mice show faint staining (Fig.2D), confirming low levels of truncated eNOS expression in del/del mice. Although the mechanism of low level expression of truncated eNOS protein is currently unknown, this may be due to that deletion of exons 9-12 results in instable eNOS protein or that the region of exons 9-12 contains regulatory elements and its deletion reduces eNOS gene transcription. Further investigation would be required on this point. Lastly, eNOS activity was assessed for each genotype of mice using the 4,5-Diaminofluorescein diacetate (DAF-2) reagent, visualizing intracellular NO production. Kidneys were perfused with DAF-2+L-Arg solution and NO production in renal vasculature was examined by fluorescence microscopy. As shown in Fig.2E, renal vasculature, especially renal artery and glomerulus, was brightly labeled with DAF-2 reagent in wildtype and flox/flox mice, while NO production was not observed in renal

vasculature of del/del mice. Intensity of glomerular NO fluorescence was comparable between wildtype and flox/flox mice, while it was reduced in del/del mice to the level of eNOS^{-/-} mice (Fig.2F), indicating the lack of eNOS activity in these mice. Further, wildtype and flox/flox mice showed normal kidney and lung histology, while del/del mice exhibited characteristic renal lesions in eNOS^{-/-} mice (Forbes *et al.*, 2007) (Fig.2G), including thrombotic glomerular lesion and focal renal scar containing crowded atubular glomeruli. These renal lesions were observed with the following frequency: 1) focal renal scar, WT/WT 0% (n=10), flox/flox 0% (n=10), del/del 77% (n=9) [Both kidneys were evaluated in each mouse]. 2) thrombotic/mesangiolytic glomeruli, WT/WT 0% (n=10), flox/flox 0% (n=10), del/del 2.4% (n=9) [50 glomeruli were observed in each mouse]. Lung histology was normal in del/del mice as in eNOS^{-/-} mice (Balasubramaniam *et al.*, 2006) (Fig.2G).

The WT/WT, flox/flox, and del/del mice were further characterized by phenotypic analysis. Shown in Fig.3, systolic blood pressure levels were comparable in WT/WT and flox/flox mice (WT/WT: 123.9 ± 9.9 mmHg, n=14, flox/flox: 122.5 ± 11.5 mmHg, n=14, P=0.85), while it was significantly elevated in del/del mice to the levels comparable to those in eNOS^{-/-} mice (Shesely *et al.*, 1996) (141.6 ± 7.4 mmHg, n=14, P=0.009 vs. WT/WT, P=0.01 vs. flox/flox). The mean blood pressure was also significantly elevated in del/del mice (121.3 ± 10.5 , n=10) as compared with WT/WT (100.3 ± 11.7 , n=9, P=0.0007) or flox/flox (99.6 ± 11.0 , n=9, P=0.0004) mice. Further, del/del mice showed significantly lower heart rate (455 ± 66 , n=10) than WT/WT (559 ± 64 , n=9, P=0.002) or flox/flox (609 ± 72 , n=9, P=0.0002) mice, as has been demonstrated for eNOS^{-/-} mice (Kojda *et al.*, 1999; Yang *et al.*, 1999). Table1 shows body and organ weight in each genotype mice at 16–19 weeks of age. Significant difference was not observed in body weight, and heart, lung, and liver weight to body weight ratios between WT/WT, flox/flox, and del/del mice. Consistent with the renal lesions, del/del mice showed reduced kidney weight (for both right and left kidneys), when compared to WT/WT and flox/flox mice. Table2 shows blood parameters in each genotype mice at 16–19 weeks of age. No significant difference was observed between WT/WT and flox/flox mice, while del/del males showed increased plasma triglyceride levels and del/del females showed higher blood glucose levels, when compared to WT/WT and flox/flox counterparts. Given the fact that eNOS^{-/-} mice show insulin resistance and hyperlipidemia (Duplain *et al.*, 2001), it is probable that lack of eNOS activity resulted in these changes in del/del mice. Lastly, survival rate was assessed in each genotype mice by following 25 mice for over 10 months (from 3 weeks of age). All mice were survived until 10 months of age and there was no difference in survival rate between WT/WT, flox/flox, and del/del mice, which are comparable results to those with eNOS^{-/-} mice (Li *et al.*, 2004). Phenotypic difference was not observed in flox/flox and del/del mice generated from 1A6 and 6A9 ES clones.

In summary, these findings demonstrate that the floxed eNOS allele behaves as wild type allele and it can be converted to deleted allele through Cre-loxP recombination, abolishing eNOS activity. Thus, conditional targeting of eNOS gene can be achieved in this floxed eNOS mouse. The mouse should provide a useful reagent for elucidating time or cell specific eNOS function.

Materials and Methods

Construction of the targeting vector

eNOS genome fragments were prepared by PCR using 129s7/AB2.2 bMQ-BAC clone (SourceBioscience LifeSciences) as a template, and inserted into the FRT.LoxP vector (provided by Dr. Magnuson at Vanderbilt University) (Jones *et al.*, 2005). A 2kb HindIII fragment including eNOS exon 9-12 was inserted to the HindIII site and a 2.5kb fragment

including exon13 was ligated to the BamHI site in the vector. Finally, a 2.3kb, 5' fragment was inserted to SalI-ClaI site in the FRT.LoxP vector. The targeting vector includes eNOS exon 9-12 flanked with a loxP sequence followed by a neomycin selection cassette (PGK-neo) flanked with a FRT sequence. The accuracy of the targeting vector, LoxFRT-eNOS, was confirmed by DNA sequencing.

Generation of mice carrying floxed and deleted eNOS alleles

NotI-linearized targeting vector was electroporated into TL1 embryonic stem (ES) cells (Zhao *et al.*, 1996). After positive selection with G418, correctly targeted ES cell clones were screened by Southern blot analysis using 5' - (381bp) and 3' - (363bp) external probes. With the 5'-probe and BamHI digestion, wild-type allele generates a 5.1kb fragment and targeted allele gives a 7.1 kb fragment. With the 3'-probe and SacI digestion, wild-type allele generates a 7.6kb fragment and targeted allele gives a 9.6 kb fragment. Chimera mice were generated from two correctly targeted ES clones (clones 1A6 and 6A9) by microinjection into C57BL/6J blastocysts. Once germline transmission was confirmed by breeding to C57BL/6J females, the chimera males were bred to 129-FLPe strain (129S4/SvJaeSor-Gt(ROSA)26Sor<tm1(FLP1)Dym>/J, Stock 3946, The Jackson Laboratory). Removal of PGK-neo cassette was determined by genomic PCR using three Neo-primers (Neo F1. 5'-GTGTGAAGGCAACCATTCTG-5', Neo F2. 5'-CGGTGGATGTGGAATGTG-3', and Neo R. 5'-CGCTGAATCCTTGTAGACTG-3'). Subsequent genotyping of the floxed eNOS mice was carried out by PCR to detect the inclusion of loxP site (primers: flox F. 5'-GGAGCTTGTGAAGGATAG-3', flox R. 5'-CTGGGTCAAGTTGAAGAG-3'). Global eNOS knockout (del/del) mice were generated by crossing floxed eNOS mice with Sox2-cre strain (B6.Cg-Tg(Sox2-cre)1Amc/J, Stock 8454, The Jackson Laboratory). First, WT/del(F1) and WT/flox(F1) mice were generated by cross breeding of WT/flox mice and Sox2-cre mice. Then, del/del, flox/flox, and WT/WT mice were prepared by cross breeding of the F1 mice [WT/del(F1) × WT/del(F1), WT/flox(F1) × WT/flox(F1)]. These mice were subjected to phenotypic analysis. Deletion of exons 9-12 was assessed by genomic PCR using del primers (del F. 5'-GGAGCTTGTGAAGGATAG-3', del R. 5'-CGCTGAATCCTTGTAGACTG-3'). Genomic PCRs were carried out on DNAs isolated from tail tissues. All animal work was carried out in accordance with Institutional Animal Care and Use Committee (IACUC). The eNOS flox line will be made available to the research community after publication.

Western blot analysis

Kidney and lung tissues were homogenized on ice in RIPA buffer with protease inhibitor cocktail tablets from Roche (150mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris-HCl pH 8.0). Following centrifugations at 1700 × g for 15min at 4°C, clarified tissue lysates (kidney: 150µg, lung: 115µg) were separated on 8% SDS-polyacrylamide gel under denaturing condition and transferred to polyvinylidene fluoride membrane (Milipore). The membrane was blocked with 3% nonfat milk in TBST (50mM Tris, 150mM NaCl, 0.05% Tween 20) for 3hrs at room temperature, then incubated with anti-eNOS antibody (sc-654, Santa Cruz) overnight at 4°C. After washes in TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare UK Limited) for 1hr at room temperature. The immunoreactions were visualized using chemiluminescence detection system (ThermoScientific).

Immunohistochemistry and histology

eNOS immunohistochemistry was carried out on the tissues perfused with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (Ricca Chemical Company) overnight at 4°C. De-waxed paraffin sections (3µm) were heated in 0.01M citric buffer (pH 6.0) for 15 min at 97°C using a microwave oven, then cooled for at least 2hrs. Sections were

stained using anti-eNOS antibody (BD Transduction Laboratories, Cat: 610297) and BioGenex Mouse-on-Mouse Iso-IHC Kit, according to the manufacture's instruction. Briefly, slides were blocked in 0.3% H₂O₂ for 5min, washed three times in PBS for 5min, and blocked in a universal protein blocking reagent of casein and 15mM sodium azide for 3min. eNOS antibody was labeled with biotin for 1hr at room temperature as per instructions of the kit. Tissue sections were incubated with the primary antibody overnight at 4°C followed by triplicate washes in PBS. Slides were incubated in a Streptavidin-Horseradish peroxidase conjugate and developed with the kit chromogen. For histological assessment, tissues were fixed overnight in 10% buffered formalin and stained with periodic acid-Schiff (PAS) and hematoxylin and eosin (H&E) using standard procedures.

***In situ* detection of nitric oxide(NO) production**

NO production in renal vasculature was assessed using 4,5-diaminofluorescein (DAF2, Alexis Biochemicals) as previously described(Kanetsuna *et al.*, 2007). In brief, mice were anesthetized and the kidneys were perfused with PBS (37°C) through the left ventricle using an infusion pump (flow rate, 5 ml/minute). After blood had been removed, the mice were perfused with PBS containing 0.01 mmol/L DAF2, 0.1 mmol/L L-Arg, and 2 mmol/L CaCl₂ for an additional 10 minutes at a flow rate of 1 ml/minute. Unreacted DAF2 was removed by postperfusion with PBS for 10 minutes. Lastly, kidneys were perfused with 4% paraformaldehyde, removed, sliced or cryo-sectioned, and fluorescence images were taken using a fluorescence microscopy (Zeiss Axioskop 40) with excitation at 495 nm and emission at 515 nm. The intensity of glomerular NO fluorescence (total 100 cortical glomeruli from five mice in each group) was analyzed on cryosections using Image J software (National Institutes of Health). The eNOS ^{-/-} mice with C57BL/6J background (The Jackson Laboratory) were used as a control.

Blood pressure measurement

Systolic blood pressure, mean blood pressure, and heart rate were measured in conscious trained mice at room temperature using a tail-cuff monitor (Visitech BP-2000 Series II Blood Pressure Analysis System).

Blood parameter measurements

Blood glucose was measured on sample obtained after a 6-h daytime fast using glucometer (Accu-chek, Advantage Meter System). Plasma blood urea nitrogen (BUN) was assayed using QuantiChrom™ Urea Assay Kit (Clonagen). Fasting (6hrs) plasma triglyceride and cholesterol were measured using the Raichem Triglyceride GPO Reagent and Raichem Cholesterol Reagent (Cliniq), respectively.

Statistical analysis

Data are expressed as means ± SEM. Single-factor analysis of variance (ANOVA) was used to evaluate the significance of difference between the groups. P<0.05 was considered significant.

Acknowledgments

We wish to thank Drs. Racheal Wallace and Edward H. Leiter at The Jackson Laboratory for excellent assistance (supported by NIDDK-75000). We also thank Dr. Qingping Feng for technical help with eNOS immunohistochemistry. This study was facilitated by the Vanderbilt Mouse Metabolic Phenotyping Center, O'Brien Mouse Kidney Physiology and Disease Center, and Pathology Core.

Grant sponsor: National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), DK61018 to R.C.H., M.D.B. and T.T.

Literature cited

- Aguirre J, Buttery L, O'Shaughnessy M, Afzal F, Fernandez de Marticorena I, Hukkanen M, Huang P, MacIntyre I, Polak J. Endothelial nitric oxide synthase gene-deficient mice demonstrate marked retardation in postnatal bone formation, reduced bone volume, and defects in osteoblast maturation and activity. *Am J Pathol.* 2001; 158:247–257. [PubMed: 11141498]
- Albrecht EW, Stegeman CA, Heeringa P, Henning RH, van Goor H. Protective role of endothelial nitric oxide synthase. *J Pathol.* 2003; 199:8–17. [PubMed: 12474221]
- Balasubramaniam V, Maxey AM, Morgan DB, Markham NE, Abman SH. Inhaled NO restores lung structure in eNOS-deficient mice recovering from neonatal hypoxia. *Am J Physiol Lung Cell Mol Physiol.* 2006; 291:L119–127. [PubMed: 16443642]
- Dudzinski DM, Igarashi J, Greif D, Michel T. The regulation and pharmacology of endothelial nitric oxide synthase. *Annu Rev Pharmacol Toxicol.* 2006; 46:235–76. [PubMed: 16402905]
- Duplain H, Burcelin R, Sartori C, Cook S, Egli M, Lepori M, Vollenweider P, Pedrazzini T, Nicod P, Thorens B, Scherrer U. Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase. *Circulation.* 2001; 104:342–345. [PubMed: 11457755]
- Feng Q, Song W, Lu X, Hamilton JA, Lei M, Peng T, Yee SP. Development of heart failure and congenital septal defects in mice lacking endothelial nitric oxide synthase. *Circulation.* 2002; 106:873–879. [PubMed: 12176963]
- Forbes MS, Thornhill BA, Park MH, Chevalier RL. Lack of endothelial nitric-oxide synthase leads to progressive focal renal injury. *Am J Pathol.* 2007; 170:87–99. [PubMed: 17200185]
- Han RN, Babaei S, Robb M, Lee T, Ridsdale R, Ackerley C, Post M, Stewart DJ. Defective lung vascular development and fatal respiratory distress in endothelial NO synthase-deficient mice: a model of alveolar capillary dysplasia? *Circ Res.* 2004; 94:1115–1123. [PubMed: 15016731]
- Hayashi S, Lewis P, Pevny L, McMahon AP. Efficient gene modulation in mouse epiblast using a Sox2Cre transgenic mouse strain. *Mech Dev.* 2002; 119(Suppl 1):S97–S101. [PubMed: 14516668]
- Herrera M, Ortiz PA, Garvin JL. Regulation of thick ascending limb transport: role of nitric oxide. *Am J Physiol Renal Physiol.* 2006; 290:F1279–1284. [PubMed: 16682483]
- Jones JR, Shelton KD, Magnuson MA. Strategies for the use of site-specific recombinases in genome engineering. *Methods Mol Med.* 2005; 103:245–257. [PubMed: 15542911]
- Kanetsuna Y, Takahashi K, Nagata M, Gannon MA, Breyer MD, Harris RC, Takahashi T. Deficiency of endothelial nitric-oxide synthase confers susceptibility to diabetic nephropathy in nephropathy-resistant inbred mice. *Am J Pathol.* 2007; 170:1473–1484. [PubMed: 17456755]
- Kleinbongard P, Schulz R, Rassaf T, Lauer T, Dejam A, Jax T, Kumara I, Gharini P, Kabanova S, Ozuyaman B, Schnurch HG, Godecke A, Weber AA, Robenek M, Robenek H, Bloch W, Rosen P, Kelm M. Red blood cells express a functional endothelial nitric oxide synthase. *Blood.* 2006; 107:2943–2951. [PubMed: 16368881]
- Koh EH, Kim M, Ranjan KC, Kim HS, Park HS, Oh KS, Park IS, Lee WJ, Kim MS, Park JY, Yoon JH, Lee KU. eNOS plays a major role in adiponectin synthesis in adipocytes. *Am J Physiol Endocrinol Metab.* 2010; 298:E846–853. [PubMed: 20124507]
- Kojda G, Laursen JB, Ramasamy S, Kent JD, Kurz S, Burchfield J, Shesely EG, Harrison DG. Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial cell nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control. *Cardiovasc Res.* 1999; 42:206–213. [PubMed: 10435012]
- Lee TC, Zhao YD, Courtman DW, Stewart DJ. Abnormal aortic valve development in mice lacking endothelial nitric oxide synthase. *Circulation.* 2000; 101:2345–2348. [PubMed: 10821808]
- Lepic E, Burger D, Lu X, Song W, Feng Q. Lack of endothelial nitric oxide synthase decreases cardiomyocyte proliferation and delays cardiac maturation. *Am J Physiol Cell Physiol.* 2006; 291:C1240–1246. [PubMed: 16822949]
- Li W, Mital S, Ojaimi C, Csiszar A, Kaley G, Hintze TH. Premature death and age-related cardiac dysfunction in male eNOS-knockout mice. *J Mol Cell Cardiol.* 2004; 37:671–680. [PubMed: 15350840]
- Ortiz PA, Garvin JL. Cardiovascular and renal control in NOS-deficient mouse models. *Am J Physiol Regul Integr Comp Physiol.* 2003; 284:R628–R638. [PubMed: 12571071]

- Shesely EG, Maeda N, Kim HS, Desai KM, Kregge JH, Laubach VE, Sherman PA, Sessa WC, Smithies O. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc Natl Acad Sci USA*. 1996; 93:13176–13181. [PubMed: 8917564]
- Yang XP, Liu YH, Shesely EG, Bulagannawar M, Liu F, Carretero OA. Endothelial nitric oxide gene knockout mice: cardiac phenotypes and the effect of angiotensin-converting enzyme inhibitor on myocardial ischemia/reperfusion injury. *Hypertension*. 1999; 34:24–30. [PubMed: 10406819]
- Zhao GQ, Deng K, Labosky PA, Liaw L, Hogan BL. The gene encoding bone morphogenetic protein 8B is required for the initiation and maintenance of spermatogenesis in the mouse. *Genes Dev*. 1996; 10:1657–1669. [PubMed: 8682296]

Fig. 1.

Generation of floxed eNOS mice. (A) Schema of targeting strategy. Targeting vector carries 4.5kb and 2.5kb homologous sequences in the left and right arm. Exons 9-12, sites essential to eNOS activity, were flanked with a loxP sequence (orange arrowhead) and a PGK-neo selection cassette (Neo) was flanked with a FRT sequence (purple arrowhead). Recombinant (floxed-Neo) allele was yielded by insertion of the targeting vector through homologous recombination. Neo cassette was removed from the recombinant allele using 129-FLPe strain, resulting in floxed (floxed) allele. Exons 9-12 can be removed from the floxed allele by Cre recombinase, yielding the deleted (del) allele. (B) Southern hybridization of targeted ES cells. Correct targeting event was confirmed by Southern blots using 5'- and 3'-external probes (green bars in panel A), displaying 7.1kb and 9.6kb recombinant allele. Clone 1A6 is shown. (C) Genomic PCR confirming Neo-removed floxed allele. Chimera mice were crossed with 129-FLPe strain and offspring were genotyped. Two forward primers (Neo F1, Neo F2) and a reverse primer (Neo R) were designed (left panel), amplifying 278 bp (floxed-Neo allele), 380 bp (WT allele), and 546 bp (floxed allele) products (right panel).

Fig. 2.

Characterization of floxed and Cre-deleted eNOS alleles. (A) Schema of breeding to generate global eNOS knockout (del/del) mice. The floxed allele was globally converted to del allele using Sox2-cre mouse. (B) Genomic PCR to assess deletion of exons 9-12. The del primers (del F. and del R.) amplify a 475bp product from del allele, while no product is amplified from wildtype allele. The flox R. primer, detecting and differentiating wildtype (448bp) and flox alleles (546 bp), was used to determine the presence of wild type allele. The loxP sites (white arrows) were also indicated on wildtype allele. (C) eNOS Western blot using lung and kidney tissue lysates. Arrow denotes a truncated eNOS protein in del/del mice. Equal loading was assessed by reprobing for β -actin. (D) eNOS immunohistochemistry of lung and kidney tissues. eNOS expression is observed in renal and lung vasculature in WT/WT and flox/flox mice, including renal artery, glomerular and peritubular capillaries, and pulmonary artery and microvessels, whereas eNOS immunoreactivity is faint in del/del mouse vasculature. Black arrow indicates renal glomerulus and green arrow indicates pulmonary vessel. Original magnification: 100x. (E) *In situ* detection of renal nitric oxide production using DAF2 reagent. Renal glomerulus (yellow arrow and G) and artery (white arrow) are prominently labeled with DAF2 reagent in WT/WT and flox/flox mice, while nitric oxide production is not observed in renal vasculature of del/del mice. Left: 1mm tissue slice; original magnification: 4.5x, 10x. Right: 5μm tissue section; original magnification: 200x. (F) Glomerular NO production was evaluated by the fluorescent intensity of DAF2. Data are means $\pm$ SEM of 20 cortical glomeruli from five mice in each group (total 100 glomeruli). (G) Renal and pulmonary histopathology in adult male mice. Glomerular lesion (black arrow) and renal scar containing crowded small glomeruli (blue arrows) are observed in del/del mice. Original magnification: 200x. PAS stain.

Fig. 3. Systolic blood pressure in 16-19 week-old male mice. The flox/flox mice show blood pressure levels comparable to those of WT/WT mice. Significant difference is observed between del/del mice and both WT/WT and flox/flox mice. Data are presented as means $\pm$ SEM. * $p < 0.05$ vs. WT/WT, flox/flox mice.

Table 1

Body and Organ Weight

	Body Weight (g)	Heart/BW Ratio (mg/g)	Lung/BW Ratio (mg/g)	Liver/BW Ratio (mg/g)	R. Kidney/BW Ratio (mg/g)	L. Kidney/BW Ratio (mg/g)
Male WT/WT	28.4±2.4 (10)	6.1±0.5 (10)	7.5±0.8 (10)	47.4±6.0 (10)	8.4±0.5 (10)	8.9±0.7 (10)
Female WT/WT	24.4±0.8 (13)	6.2±0.7 (10)	7.6±1.0 (10)	46.9±4.9 (10)	7.5±1.4 (10)	8.0±1.6 (10)
Male flox/flox	26.2±2.2 (10)	5.9±0.5 (11)	7.7±1.2 (11)	47.9±5.4 (11)	8.6±0.9 (11)	9.1±1.0 (11)
Female flox/flox	26.5±3.8 (16)	5.7±0.8 (10)	7.8±0.9 (10)	46.7±6.6 (10)	7.3±0.6 (10)	7.9±1.2 (10)
Male del/del	27.3±0.5 (11)	5.8±0.5 (10)	6.8±0.6 (10)	42.6±4.5 (10)	6.8±0.7 (10)*	7.1±0.5 (10)*
Female del/del	23.8±1.5 (10)	5.8±0.3 (12)	8.7±1.8 (12)	48.9±5.7 (12)	6.3±0.8 (12)	6.7±0.8 (12)

Body weight (BW) and organ to BW ratio were measured at 16–19 weeks of age. Data are presented as means ± SEM. Numbers of mice are in parentheses.

* denotes significant difference between del/del and both WT/WT and flox/flox counterparts for p<0.05.

Table 2

Blood Parameters

	Blood Glucose (mg/dL)	BUN (mg/dL)	Cholesterol (mg/dL)	Triglyceride (mg/dL)
Male WT/WT	117.5±12.0 (10)	26.1±2.3 (12)	105.3±16.7 (13)	81.8±17.6 (10)
Female WT/WT	109.5±14.5 (13)	24.0±4.2 (10)	116.4±16.0 (10)	101.4±14.4 (10)
Male flox/flox	116.3±11.5 (10)	28.9±3.5 (14)	116.4±15.7 (12)	95.4±19.0 (9)
Female flox/flox	119.5±11.6 (16)	22.9±2.3 (18)	115.0±15.1 (16)	102.6±23.3 (10)
Male del/del	111.7±12.1 (10)	28.7±3.5 (15)	127.5±13.4 (14)	111.9±12.6 (10) [‡]
Female del/del	148.4±9.4 (10) [*]	24.9±2.6 (9)	117.9±13.9 (11)	86.8±10.7 (11)

Blood parameters were measured at 16–19 weeks of age. Data are presented as means ± SEM. Numbers of mice are in parentheses.

^{*} denotes significant difference between del/del and both WT/WT and flox/floxfemales for p<0.05.

[‡] denotes significant difference between WT/WT and del/del males for p<0.05. BUN, blood urea nitrogen.