Preeclampsia-Like Symptoms Induced in Mice by Fetoplacental Expression of STOX1 Are Reversed by Aspirin Treatment


See Editorial Commentary, pp 561–563

Abstract—Preeclampsia (PE) is a common human-specific pregnancy disorder defined by hypertension and proteinuria during gestation and responsible for maternal and fetal morbimortality. STOX1, encoding a transcription factor, was the first gene associated with PE as identified by positional cloning approaches. Its overexpression in choriocarcinoma cells mimics the transcriptional consequences of PE in the human placenta. Here, we created transgenic mouse strains overexpressing human STOX1. Wild-type female mice crossed with transgenic male mice reproduce accurately the symptoms of severe PE: gestational hypertension, proteinuria, and elevated plasma levels of soluble fms-like tyrosine kinase 1 and soluble endoglin. Placental and kidney histology were altered. Symptoms were prevented or alleviated by aspirin treatment. STOX1-overexpressing mice constitute a unique model for studying PE, allow testing therapeutic approaches, and assessing the long-term effects of the preeclamptic syndrome. (Hypertension. 2013;61:662-668.) • Online Data Supplement

Key Words: hypertension ■ mouse model ■ preeclampsia ■ proteinuria ■ STOX1

Preeclampsia (PE) is a pervasive complication of human pregnancy characterized by a gestational hypertension associated with proteinuria occurring from midgestation.1 Worldwide, this syndrome affects 5% of pregnant women and is a leading cause of maternal mortality, especially in low- and middle-income countries;3-3; it is also a direct cause of iatrogenic prematurity,4 because the only definitive therapeutic act is the extraction of the fetoplacental unit.

STOX1 is a transcription factor belonging to the enlarged Forkhead Box gene family that has been associated with PE,5 able to modulate trophoblast proliferation and migration.6 STOX1 is maternally expressed in a specific cell type of the placenta, column extravillous trophoblasts.7 Overexpression of STOX1 in human choriocarcinoma cells induces transcription alterations that mimic those of preeclamptic placentas.8,9 Founds et al10 showed in a transcriptomic analysis that STOX1 is overexpressed (x2.1; P=0.013) during the first trimester in pregnancies that will have a preeclamptic outcome.

In the present work, we generated mice overexpressing human STOX1. Wild-type (WT) female mice crossed with transgenic males overexpressing STOX1 have a severe gestational hypertension, proteinuria, an increased plasma level of soluble angiogenic factors, as well as kidney and placenta histological alterations. We also demonstrate a beneficial effect of low-dose aspirin treatment on the maternal symptoms. Hence, STOX1-overexpressing mice constitute a unique model for studying severe PE.

Methods
More details of the methods are provided in the online-only Data Supplement.

Animals and Transgenesis and Aspirin Treatment
The complete open reading frame (ORF) of the human STOX1 (A isoform) cDNA was used to generate transgenic animals.11 The number of copies of the transgene was assessed by quantitative polymerase chain reaction (qPCR) from mouse DNA relative to a single-copy

Received July 26, 2012; first decision November 25, 2012; revision accepted January 5, 2013.
From INSERM U1016, Institut Cochin, Paris, France (L.D., C.M., V.R., S.B., A.D., F.M., M.B.-F.); CNRS UMR 8104, Paris, France (L.D., C.M., V.R., S.B., A.D., F.M., M.B.-F.); INSERM U970, Hôpital Georges Pompidou, Paris, France (V.B.); Institut national de la santé et de la recherche médicale (INSERM), UMR S702, Paris, France (A.-L.B., G.G.); Paris Centre de recherche Cardiovasculaire, INSERM U970, Hôpital Georges Pompidou, Paris, France (V.B.); Institut national de la santé et de la recherche médicale (INSERM), UMR S702, Paris, France (A.H.); Aix-Marseille Université, URMIT, Unité Mixte de Recherche 6236, Marseille, France (C.B.); APHM, Hôpital Conception, Laboratoire de Biologie Moléculaire, Marseille, France (C.B.); and APHM, Hôpital de la Conception, Service de Médecine Néonatale, Marseille, France (U.S.). The online-only Data Supplement is available with this article at http://hyper.ahajournals.orglookup/suppl/doi:10.1161/HYPERTENSIONAHA.111.202994/-/DC1.

Correspondence to Daniel Vaiman, INSERM U1016-CNRS UMR8104, 24 rue du Fg St Jacques, Paris 75014, France. E-mail daniel.vaiman@inserm.fr

Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.111.202994

662
gene. Transmission analysis through generations showed that there was a single locus of insertion on the 3 strains that were analyzed.

Animals (FVB/N) were bred in the animal facility of INRA (Institut National de Recherche Agronomique, Jouy en Josas, France) in a controlled environment (light/dark cycle, temperature, free access to food and water). Blood collection and urine collection, blood pressure (BP) measurements kidney perfusion and aspirin supplementation in the drinking water were performed according to standardized protocols (see Methods in the online-only Data Supplement).

**RNA Extraction and Quantitative Reverse Transcriptase PCR Conditions**

Total RNA from collected tissues was extracted and quantified by spectrophotometry. Quality of the RNA was systematically verified by Bioanalyzer and RIN to be consistently >8. After reverse transcription, quantitative reverse transcriptase PCR was performed. The efficiency of the PCR was checked for every primer couple and estimated (Methods in the online-only Data Supplement). The Cts were normalized by the Ct values obtained for the murine succinate dehydrogenase subunit A (Sdha) used as a normalizing gene and shown previously to be stable and highly normalizing in the placenta. All primers are presented in Table S1 in the online-only Data Supplement.

**BP Measurements**

BP was measured repeatedly in the tail artery in mice before, during, and after pregnancy (for a total of ≈35 consecutive days). Measurements were performed using a computerized, noninvasive tail-cuff plethysmography method (Letica 5001; Biosys), using thermally statically warmed restrainers designed for mice and adapted to the size of the animal. This system uses volume-pressure recording technology to detect changes in tail volume that corresponds to systolic and diastolic pressures during each measurement cycle. Unanesthetized mice previously accustomed during 1 week to the manipulation (systolic BP stabilized) were placed in plastic holders. The protocol consisted of at least 6 satisfactory measurements daily, meaning without detectable movement of the mice. Thirty-six gestations were followed, which represents >5000 independent BP measurements by 2 independent researchers (systolic, diastolic, and median always displayed a highly similar profile). In addition, invasive measures were performed on 14 WT mice mated with WT or transgenic males at the end of gestation (14.5–17.5 days postcoitum [dpc]). The catheter was inserted into the left common carotid artery under xylazine/ketamine anesthesia. Mice were kept on a heating pad during experimentation. BP was recorded and analyzed from continuous measurements using the BIOPAC MP36 system. The extraction of the systolic BP started when cardiovascular parameters were stabilized.

**Assessment of Albumin/Creatinine Ratio**

Urine samples were collected daily during gestation and were pooled at early (1–5 dpc), middle (9–12 dpc), and late (15–18 dpc) gestation times separately for each mouse. Albumin/creatinine ratio was measured in these urine specimens using the ELISA kit of Exocell (Philadelphia, PA).

**sFlt1 and sEng Plasmatic Concentration Assessment**

ELISAs were used to determine the concentration of soluble fms-like tyrosine kinase 1 (sFlt1) and soluble endoglin (sEng) in mouse plasma with commercial kits (R&D Systems).

**Histological Analysis**

Placentas and kidneys were sampled from 10 transgenic and WT mice (after heart perfusion by 20 mL of PBS in the case of kidneys). Antifibrin immunostaining of slides from paraffin-embedded kidneys was performed using the anti-human fibrinogen at 1/400 from Dako (A0080) and the LSAB revelation system (Dako, K0679) according to the manufacturer’s instructions (standard conditions). Placental histology was performed after hematoxylin–eosin staining or periodic acid–schiff (PAS) staining. The number of vacuolated foci present in the labyrinth was counted after hematoxylin–eosin staining. A grade of 1 to 5 was given according to the level of PAS intensity, and the PAS-positive areas were measured in the labyrinth.

**Statistical Analysis**

During this study, parametric tests were used using ANOVA, followed by post hoc Dunnett’s tests for comparison with controls. The StatistXL add-in of Excel was systematically used. *P*<0.05 was considered significant.

**Results**

**Generation of STOX1 Transgenic Mice**

Three independent transgenic lines (called TgSTOX13, TgSTOX34, and TgSTOX42) were generated by introducing the human *STOX1A* ORF (complete isoformal) under the control of a ubiquitously expressed promoter (the cytomegalovirus promoter). TgSTOX13 and TgSTOX42 strains contain a unique copy that was transmitted in a Mendelian fashion. Because the expression level of TgSTOX34 was very similar to that of TgSTOX13, we focused our attention on the TgSTOX13 and TgSTOX42 strains.

The expression levels of human and mouse *STOX1* were tested by quantitative reverse transcriptase PCR in the maternal kidney, liver, brain, and placenta of pregnant TgSTOX13 and TgSTOX42 females, as well as in the testis and spermatozoa of transgenic males of both strains, using species-specific primers (Table); *hSTOX1* expression was comparatively higher in placental tissue than in any other tissue or spermatozoa. Quantitative reverse transcriptase PCR efficiency was maximal for mouse and human STOX1, allowing to suggest that the placental expression level of the human *hSTOX1* was roughly similar to that of mSTOX1 in TgSTOX13 transgenic mice (Table) and =13-fold higher in TgSTOX42. In the other tissues tested (kidney, brain, testis, and liver), the transgene expression level was lower than that of the endogenous gene but always higher in TgSTOX42 than in TgSTOX13 (Table). Because there is a unique transgene insertion in both strains (as tested by quantitative PCR), we assume that the difference in the expression level was strictly due to the genomic position of this insertion. In transgenic placentas from WT×TgSTOX42 crosses, where

### Table. Expression of the Endogenous *Stox1* Gene and STOX1 Transgene in Mice in Various Tissues

<table>
<thead>
<tr>
<th>Mice</th>
<th>Placenta</th>
<th>Kidney</th>
<th>Liver</th>
<th>Brain</th>
<th>Testis</th>
<th>Spermatozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (endogenous <em>Stox1</em>)*</td>
<td>3000±57</td>
<td>178±4.3</td>
<td>92±2.1</td>
<td>1646±96</td>
<td>882±19</td>
<td>358±14</td>
</tr>
<tr>
<td>TgSTOX13*</td>
<td>2400±90</td>
<td>1.0±0.05</td>
<td>4.2±0.15</td>
<td>1.9±0.04</td>
<td>1646±140</td>
<td>66±4.7</td>
</tr>
<tr>
<td>TgSTOX42*</td>
<td>38 088±5540</td>
<td>12±4.6</td>
<td>38.2±11</td>
<td>1384±100</td>
<td>7304±2066</td>
<td>3755±1195</td>
</tr>
</tbody>
</table>

*WT indicates wild type.*

*Arbitrary units, the kidney expression in TgSTOX13 mice was set to 1 and served as a reference for the expression of the transgene. The values of the endogenous *Stox1* are corrected by the Ct values of the *sda* gene, giving a rough indication of the relative expression of the endogenous *Stox1* compared with the transgene.
the transgene expression is the highest, the endogenous gene was overexpressed 4-fold ($P=2\times10^{-11}$ by Student test compared with WT×WT crosses).

**STOX1 Transgenic Mice Develop a Strong Gestational Hypertension, Proteinuria, and Present an Increased Level of Circulating sFlt1 and sEng**

WT females were crossed with males of 3 genotypes (WT, TgSTOX13, or TgSTOX42) to ensure that the transgene expression would be restricted to the fetoplacental unit. BP was monitored daily in the various crosses (Figure 1A). An increase in BP during gestation was observed in all cases of crosses involving transgenic males. The BP curves reached a plateau in both transgenic crosses around embryonic day 10.5, until days 14.5 to 15.5, when the BP tends to increase again by an additional 15 to 20 mm Hg. The maximal increase reached ≈80 mm Hg from a normal pressure of ≈116 mm Hg (Figure 1A). BP returned to normal rapidly (in ≈48 hours) after parturition. In addition, we carried out measurements by catheterization of one of the carotids at late gestation times, and consistently with human BP data under anesthesia, the increase in mice carrying transgenic fetuses was present, albeit reduced; the BP was 12 mm Hg more elevated in the transgenic crosses ($P=0.008$; Figure S1).

Proteinuria was evaluated as a ratio between albumin and creatinine (Figure 1B). After individual normalization, we observed an increase in the albumin/creatinine ratio, which comprised between ≈1.7- and 1.9-fold at midgestation ($P=0.02$ and 0.03 for TgSTOX13 and TgSTOX42, respectively) and between ≈1.8- and 3.8-fold at late gestation points ($P=0.026$ and 0.002, respectively), in females crossed with transgenic males compared with control crosses.

sFlt1, considered as a biomarker for PE in the human disease, was monitored (Figure 2A). The average concentration in the plasma of pregnant mice was 2075 pg/mL when TgSTOX13 males have fathered and 2455 pg/mL when crossed with TgSTOX42 males, an increase of 1.9- and 2.3-fold compared with control (1076 pg/mL), respectively ($P=0.02$ and 0.03). sEng was also measured in the plasma (Figure 2B), and it was found to be increased in the crosses involving TgSTOX13 (×2.0; $P=0.04$) and TgSTOX42 males (×2.3; $P=0.01$) compared with crosses with WT males.

**Figure 1.** Hypertension, elevated albumin/creatinine ratio in wild-type (WT) females crossed with STOX1 transgenic males. A, Systolic arterial pressure is represented for pregnant mice after the indicated crosses, treated or not with aspirin (ASP). The values were obtained daily, and the curves were smoothened on 3 consecutive days. Mice overexpressing STOX1 in the fetoplacental units display a strong gestational hypertension, increased by up to 80 mm Hg in the TgSTOX42 strain. Two or 3 days postpartum, blood pressure goes back to normal. B, Albumin/creatinine ratio (ACR) in the various crosses with or without ASP treatment. The values were normalized for each mouse to the ACR value measured at the beginning of gestation; the value obtained for WT mice was taken as a reference and set to 1. The ACR of females crossed with transgenic males was significantly different from the ACR of females crossed with WT at midgestation and late gestation times, whereas ASP treatment brought back the ACR to values not significantly different from those of controls. Significant comparisons with the control after ANOVA and Dunnett test.
WT Females Carrying Transgenic Fetuses Have Kidney Anomalies That Are Characteristic of PE

In the kidneys of females crossed with TgSTOX13 mice, glomerular changes were observed: first, a glomerular tuft swelling with expansion of the capillary wall, up to the point that the tuft could occasionally make contact with the Bowman’s capsule; second, we observed a slight increase in the glomerular cellularity (42.3±10.1 in TgSTOX13 and 48.1±11.9 in TgSTOX42 versus 34.0±11.8 in WT; Figure 3A; P=3.4×10^{-3} and P=6.4×10^{-7}, respectively) attributable to a mild mesangial cell proliferation. Immunohistochemistry revealed subendothelial fibrin deposition, with a predominant peripheral pattern. These changes are strongly reminiscent of the renal pathology observed in human PE17–19 and could not be observed in the kidneys of WT pregnant mice crossed with WT males (Figure 3B, top and middle photographs). No major abnormality was found in the tubulointerstitial compartment, or in the larger vessels, in either group.

Aspirin Treatment Efficiently Improves the Maternal Phenotype

WT females crossed with TgSTOX13 or TgSTOX42 males were treated with aspirin at low doses in the drinking water during the whole gestation. As a control, WT pregnant females received aspirin all along their pregnancy after a cross with WT males. Aspirin completely prevented the onset of hypertension and brought the albumin/creatinine ratio to normal values at midgestation and late gestation times (Figures 1A and 1B). Fibrin immunolabeling of the kidneys showed that the deposit was considerably reduced in aspirin-treated WT females crossed with WT males (Figure 3B, top and middle photographs). No major abnormality was found in the tubulointerstitial compartment, or in the larger vessels, in either group.

STOX1 Transgenic Placentas Present Structural Alterations

The anatomic alterations of hemizygous TgSTOX13 and TgSTOX42 (validated as transgenic by PCR) placentas were studied by histology (Figure S2). No difference was observed in gross morphology at 16.5 dpc, such as in the ratio between the labyrinth and the junctional zone. Because deregulated imprint gene expression is known to alter glycogen trophoblast location, we counted the number of vacuolated foci, graded the PAS staining, specific of glycoproteins, and measured the surface of PAS area in the labyrinth. Although in hemizygous TgSTOX13 placentas no significant difference was detected compared with measures in WT placentas, in hemizygous TgSTOX42 placentas a significant diminution of vacuolated foci in the labyrinth, as well as PAS grade and PAS area (Figure 4A–4C), was consistently observed.

Litter Size Is Affected by the Transgene

Litter size seemed to be linked to the genotypes of the males used in crosses (Figure S3; P=0.0016 by 1-factor ANOVA), with 8.4 (±2.0) pups on average in WT crosses. In crosses with TgSTOX13 males, the litter size was reduced to 6.9 (±3.4; P=0.048). Consistent with the higher transgene expression in TgSTOX42, the litter size was reduced to 6.0 (±2.6, P=0.001) in crosses involving a TgSTOX42 male. When aspirin was given to females during gestation, the litter size was 7.8 (±2.4) and 9.3 (±3.1) in crosses involving TgSTOX13 and TgSTOX42 males, respectively. In the latter case, this was significantly different from nontreated crosses (P=0.022), showing that the drug was able to correct the reduced litter size phenotype.

Discussion

Herein, we show that mice overexpressing STOX1 in the embryo–fetoplacental unit reproduce in extenso and without further manipulation the major features of PE: proteinuria and hypertension. A peculiarity of the present mouse model of PE is the intensity of the gestational hypertension, which reaches values corresponding to severe human PE at the end of gestation. The lower difference in BP observed under anesthesia compared with vigil animals may presumably be explained by a relaxing effect of the anesthesia, moderating as well the BP level. We also observed an elevation of both sFlt1 and sEng, key antiangiogenic factors of PE. These mice also harbored PE-specific kidney alterations.

In summary, a unique transcription factor (STOX1) expressed in the fetoplacental unit is sufficient to trigger PE-like symptoms in WT females in a dose-dependent manner (illustrated by the more or less severe phenotype observed in crosses involving TgSTOX13 or TgSTOX42 males). Although the involvement of STOX1 in human PE was debated after its discovery,20 the present study confirms a growing corpus of evidence indicating an actual contribution of STOX1 to the preeclamptic phenotype.
Rodent models of PE have been considered very important for studying PE pathophysiological mechanisms. Various models of reduced uterine perfusion were generated by inducing placental ischemia, administration of angiotensin antagonists, proinflammatory drugs, or vasoconstrictive agents or by using the BPH/5 hypertensive mouse strain and crosses between the CBxAxDBA strains. Gene manipulations have also been used, such as gene invalidation experiments for p57Kip2 or Comt\(^{23,24}\) additive transgenesis of antiangiogenic factors, such as s-flt1,\(^{25}\) or transgenesis leading to manipulation of the renin/angiotensin system.\(^{26}\) In the handful of existing mouse models of PE, gestational hypertension is mild,\(^{27}\) far below the one of our model and far below what is observed in severe PE in women. Furthermore, we observed that the principal maternal symptom, gestational hypertension, seems very early, from 3.5 dpc. In humans, it has been shown that PE can exceptionally occur very early with a complete phenotypic profile before 15 weeks of gestation,\(^{28}\) whereas in general symptoms are reported to appear from midgestation (21–22 weeks) at the earliest. The occurrence of the maternal hypertension at the very beginning of gestation in our model suggests that soluble substances produced by the blastocyst, the embryo, or even from the ejaculate may initiate the maternal preeclamptic phenotype. It is interesting to compare hypertension curves obtained here with those of most studies\(^{23,25}\) that show a raise during the last 5 days of gestation or so. This increase is also visible in our model, but is superimposed on a highly elevated BP triggered by gestation. In sum, our model cumulates presumably the effects of an early message from the embryo–fetoplacental unit to the mother systems controlling BP (endothelial system and kidney) and the effect of an increased placental/fetal demand occurring at the end of gestation, when the rhythm of weight gain is the highest for the fetuses, this effect being the one reproduced in most mouse models of PE.

Under anesthesia, the difference in BP measurement between the control pregnant mice and the ones crossed with transgenic males was present, albeit less pronounced than on vigil animals. This is completely consistent with recent human data showing that under anesthesia there is a steep drop in maternal BP but much more elevated in preeclamptic women than normotensive pregnant women (−10% in normotensive versus −30% in preeclamptic women).\(^{13}\) This suggests that BP variations in our model are very close to what is observed in human clinics.

To the best of our knowledge, we demonstrate here for the first time, in a mouse model, that an aspirin treatment at low doses administered in drinking water from the beginning of gestation is efficient for preventing the maternal syndrome. In humans, it seems that a major issue is the timing of aspirin administration: after 16 weeks of gestation it has no effect at all.\(^{29}\) A consensus tends nevertheless to emerge, with the idea that aspirin is quite efficient when administered early (before the end of the first trimester of pregnancy).\(^{29–33}\) Aspirin at low doses may change the hemodynamic properties of the blood thus preventing blood clotting, an issue that is sometimes associated with disseminated intravascular coagulation in severe PE with HELLP (hemolysis, elevated liver enzyme, low platelet count) syndrome.\(^{34}\)

Unexpectedly, mild anomalies of mature placenta (decrease of glycogen trophoblast surface) were only observed in crosses with TgSTOX42 males. Although we did not perform earlier histological examination of developing placenta, focusing on spiral artery invasion for instance, the observation that hypertension occurs before the formation of a placenta indicates that the mechanisms underlying the observed phenotype cannot be
fully explained by placentation defects. This opens the possibility that different causes of PE may explain the grade of severity of PE symptoms in humans, as well as the early or late onset of the disease.

Imprinted genes and, among them, the paradigmatic Igf2, are known to have a crucial role in placental and fetal growth.35 In particular, Igf2 invalidation leads to a lack of glycogen cells.36 The observed defaults in glycogen trophoblasts in hemizygous TgSTOX42 placentas may be related to the fact that STOX1 is an imprinted gene in humans and could thus regulate the other members of the imprinted gene network.37

Perspectives

The mouse model presented here can help in the understanding of severe PE, for which until now very few experimental alternatives to the direct study of the patients are available. These mice provide the community working on PE with a tool applicable for cognitive (understanding PE pathogenesis) as well as applied (drug screening) usage, and even potentially applicable for cognitive (understanding PE pathogenesis) as well as applied (drug screening) usage, and even potentially

Acknowledgments

Dr Guillermina Girardi is warmly acknowledged for her help and advices for studying kidney histology and preparing the slides. Emma Walton is acknowledged for her advice concerning the manuscript.

Sources of Funding

This work was supported by INSERM.

Disclosures

None.

References

Preeclampsia-Like Symptoms Induced in Mice by Fetoplacental Expression of STOX1 Are Reversed by Aspirin Treatment


Hypertension. 2013;61:662-668; originally published online January 28, 2013; doi: 10.1161/HYPERTENSIONAHA.111.202994

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/61/3/662

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2013/01/28/HYPERTENSIONAHA.111.202994.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Preeclampsia-like symptoms induced in mice by feto-placental expression of STOX1 are reversed by aspirin treatment.

STOX1 mice develop preeclampsia symptoms

Ludivine Dorido1,2,3, Bruno Passet4, Céline Méhats1,2,3, Virginie Rigourd1,2,3,5, Sandrine Barbaux1,2,3, Aurélien Ducat1,2,3, Françoise Mondon1,2,3, Marthe Villette4, Johann Castille4, Michelle Breuiller-Fouche1,2,3, Nathalie Daniel6, Fabienne le Provost4, Anne-Laure Baucher7, Véronique Baudrie8, Alexandre Hertig9, Christophe Buffat10,11, Umberto Simeoni12, Guy Germain7, Jean-Luc Villette4, Daniel Vaiman1,2,3

1 INSERM U1016, Institut Cochin, Paris, France.
2 CNRS UMRS 8104, Paris, France.
3 Université Paris Descartes, Paris, France.
4 INRA UMR1313, Génétique Animale et Biologie Intégrative, Jouy-en-Josas, France.
5 Lactarium d’Ile de France, Institut de Puériculture et de Périmatologie, Paris, France
6 INRA, UMR 1198 Biologie du Développement et Reproduction, Jouy en Josas, France
7 CEA, I2BM, MIRCen, Fontenay aux Roses, France.
8 Paris-Centre de recherche Cardiovasculaire, INSERM U970, Hôpital Georges Pompidou, Paris, France.
9 Institut national de la santé et de la recherche médicale (INSERM), UMR S702, Paris, France.
10 Aix-Marseille Université, URMIT, Unité Mixte de Recherche 6236, 13005 Marseille, France.
11 APHM, Hôpital Conception, Laboratoire de Biologie Moléculaire, 13385 Marseille 05, France.
12 APHM, Hôpital de la Conception, Service de Médecine Néonatale, 13385 Marseille 05, France.

Word count of the manuscript: ~6900

Word count of abstract: 160

Number of figures: 5

Corresponding Author:
Daniel Vaiman,
phone: +33 01 44 41 23 01
fax: +33 01 44 41 23 02
@: daniel.vaiman@inserm.fr
Supplementary Documents

1. Supplementary methods

Animals and Trangenesis

The complete ORF of the human STOX1 (A isoform) cDNA was cloned in the pCMX vector as described previously. The vector-free insert used for micro-injection was gel-purified after enzymatic digestion by SpeI and SfiI, according to classical protocols. The preparation was diluted in Tris-HCl pH 7.9-EDTA (10-0.1) medium at a final concentration of 2 ng/µl and microinjected into the pronuclei of FVB/N mouse eggs. Transgenic mice were identified by PCR of their genomic DNA extracted from tail biopsies, as previously described. Transgene-specific amplification was performed using the GoTaq Flexi DNA polymerase kit (Promega), an hybridization temperature of 62°C, an elongation time of 30s and 40 amplification cycles (see Supplemental Table S1 for primers). The number of copies of the transgene was assessed by qPCR from the mouse DNA relative to a single copy gene.

Animals (FVB/N) were bred in the animal facility of INRA (Jouy en Josas, France) in a controlled environment (light/dark cycle, temperature, free access to food and water). After mating with males of specific genotypes, females were inspected daily for vaginal plugs; detection of a vaginal plug in the morning was designated as day 0.5 of pregnancy (E0.5 or 0.5 dpc). Litter sizes were systematically recorded immediately after birth. Some mice (16 with placentas of different genotypes, either all WT, or some transgenic, or all transgenic) were sacrificed at E16.5 and different tissues (placenta, kidneys, liver) were collected for RNA extraction and/or histological analysis according to classical protocols. Some males were also sacrificed; testis and spermatozoa were used for RNA extraction. For kidney histology, the animals were perfused with ~20ml of PBS injected in the heart under ketamine/xylasin anesthesia, in order to remove the excess of red blood cells before recovering the kidneys. The number of foetuses, as well as placental and foetal weights, was also recorded in these cases.
RNA extraction and Quantitative RT-PCR conditions

Total RNA from collected tissues was extracted using TRIzol Reagent (Invitrogen) in accordance with the manufacturer’s instructions, treated with RNase-free DNase, and quantified by spectrophotometry. Reverse transcription (RT) was carried out according to a standardized protocol. Briefly, 4 µg of total DNase-treated RNA was reverse transcribed in a volume of 25 µL at 39°C using the M-MLV Reverse Transcriptase (Invitrogen) and random primers during 1 hour. Quantitative RT-PCR was carried out using the LightCycler® 480 SYBR Green I Master Mix (Roche Applied Science) in accordance with the manufacturer’s instructions. The reaction was performed in a Light-Cycler 480 Thermocycler (Roche Applied Science). Primers were designed for the coding sequences of the different genes using the PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3) and aligned with basic local alignment search tool software (BLAST) to avoid nonspecific annealing. The couple of primers for mouse Stox1 were chosen to recover only the long murine isoform (which contains all the active domains, and corresponds to the human hSTOX1A isoform used for transgenesis). Samples were submitted to cycling according to the following PCR program: 95°C for 5 min followed by 45 cycles of 3 temperature steps (94°C for 10 s, 58°C for 15 s, and 72°C for 15 s). Finally, samples were submitted to a progressive temperature elevation (from 65 to 99°C at 0.1°C/s), resulting in a melting curve, enabling to check the PCR products homogeneity. In addition, amplification products were systematically controlled by agarose gel electrophoresis. The threshold cycle number (Ct) values were collected with the LightCycler480 software (Roche Applied Science) in the exponential phase of the PCR reaction. These Cts were normalized by the Ct values obtained for the murine succinate dehydrogenase subunit A (Sdha) used as a reporter gene and shown previously to be stable and highly expressed in the placenta. The efficiency of the qPCR was calculated with the software proposed by Roche, with a standard of four points where the cDNAs were diluted ½, 1/10, 1/50 and 1/500. The efficiencies are 2.00 for mm-STOX1, 2.05 (rounded to 2.00) for hSTOX1A and 1.96 for mm-sdha. The efficiencies of these PCR reactions allows to compare approximately the level of expression of mouse and human STOX1 in the different organs.
Invasive Blood pressure measurement

In addition, invasive measures were performed on 2 WT mice mated with transgenic TgSTOX13 male mice and 2 mated with WT mice at 14.5 dpc. The measures were performed by indwelling a catheter in one of the carotids under xylazine/ketamine anesthesia.

Assessment of Albumin/creatinine ratio (ACR)

Urine samples were collected daily during gestation and were pooled at early (1-5 dpc), middle (9-12 dpc) and late (15-18 dpc) gestation times separately for each mice. Albumin-to-creatinine ratio in these urine specimens (accepted alternative to 24-hour urine collections) was used to monitor kidney function. Urinary albumin was determined by ELISA (Exocell, Philadelphia, PA). Creatinine in urine was quantified with the Creatinine Companion kit (Exocell, Philadelphia, PA), based upon the Jaffe’s reaction of alkaline picrate with creatinine. The obtained values for mid and late gestation were normalized to the one of early gestation for each mice, in order to suppress individual variations, and then a second normalization to control mice was done for the graphical representation.

sFlt1 plasmatic concentration assessment

Enzyme-linked immunosorbent assays were used to determine the concentration of sFlt1 in mouse plasma (diluted 1/10) with commercial kits (R&D Systems).

Histological analysis

Placentas and kidneys were sampled from 10 transgenic and WT mice (after heart perfusion by 20 ml of PBS in the case of the kidneys), trimmed with one longitudinal section, fixed in a 4% PFA solution during 24h, before being transferred in 70% ethanol. The fixed placentas were shortly processed using a vacuum paraffin inclusion processor and then included into paraffin blocks. Five micrometer-thick sections were cut from these blocks for the placentas (ten micrometers for the kidneys) and the obtained sections were stained with hematoxylin-eosin, and for the placental slides with Masson’s trichrome and periodic acid-Schiff stains. Anti-fibrin immunostaining of slides from paraffin-embedded kidneys was performed using the LSAB revelation system (Dako, K0679)
according to the manufacturer’s instructions (standard conditions). For detecting fibrin/fibrinogen, the slides were incubated overnight with 400-fold diluted polyclonal rabbit anti-mouse fibrin antibody (Dako North America Inc., Carpinteria, CA). Sections were counterstained with hematoxylin and examined by light microscopy.

**Aspirin treatment**

Aspirin treatment was administered as described by Bulckaen and co-workers. Briefly, aspirin was added in the drinking water (30 µg/ml, Lysine acetylsalicylate Aspegic™), from E0,5 to E16,5 (the treatment was stopped before delivery in order to avoid abnormal bleeding during labor). The drinking water was replaced every day, and showed that the transgenic mice drank the same quantity of water than the controls. This low quantity was administered to obtain an amount similar to that prescribed in clinical practice, that is around 200 mg/day, see for instance. Eleven wild type females were used, two crossed with wild-type males, seven with TgSTOX13 male and two with TgSTOX42 males.
2. Supplementary Tables

**Supplemental Table S1 : Primers for qPCR analysis**

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>sdha (murine)</td>
<td>CTTGAATGAGGCTGACTGTG</td>
<td>ATCACATAAGCTGGTGCCTGT</td>
</tr>
<tr>
<td>STOX1 (human)</td>
<td>TCCAGTGCAAATGAATCCAA</td>
<td>TCTTTATCAGCTCCAGA</td>
</tr>
<tr>
<td>Stox1 (murine)</td>
<td>AACACCTTGAGGGAGAGAA</td>
<td>ATCCAAATGGCGGAATTAGTA</td>
</tr>
</tbody>
</table>
3. Supplementary Figures

Supplementary Figure S1: Invasive measurement of blood pressure under anesthesia. The mice at the end of gestation (14.5 to 17.5 dpc) were anesthetized, kept on a heating pad, and a catheter was inserted into the carotid as shown in the picture. Blood Pressure was recorded and analyzed from continuous measurements using BIOPAC MP36 system. The extraction of the Systolic Blood Pressure started when cardiovascular parameters were stable, and appeared more elevated in mice crosses with transgenic males.
Figure S2: Placental phenotype

Placental histology analysed by hematoxylin–eosin (HE) and Periodic Acid–Schiff stainings. These pictures are representative of the analyzed placentas (4-5 placentas from 2-3 independent crosses).
Supplementary Figure S3: Litter size is reduced in crosses involving transgenic males. When aspirin was given to the mothers during gestation (hatched bars), the litter size was closer to controls, with a significant p value for the crosses involving TgSTOX42 males (p = 0.035 between with and without treatment, represented by a # symbol). * for p < 0.05 and ** for p < 0.01.
References


