Molecular Human Reproduction, Vol.16, No.12 pp. 960–968, 2010
Advanced Access publication on July 19, 2010 doi:10.1093/molehr/gaq064

MHR Basic science of reproductive medicine

ORIGINAL RESEARCH

STOX2 but not *STOX1* is differentially expressed in decidua from pre-eclamptic women: data from the Second Nord-Trøndelag Health Study

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Submitted on May 27, 2010; resubmitted on July 9, 2010; accepted on July 14, 2010

ABSTRACT: Variation in the Storkhead box-1 (*STOX1*) gene has previously been associated with pre-eclampsia. In this study, we assess candidate single nucleotide polymorphisms (SNPs) in *STOX1* in an independent population cohort of pre-eclamptic ($n = 1.139$) and non-pre-eclamptic ($n = 2.269$) women (the HUNT2 study). We also compare gene expression levels of *STOX1* and its paralogue, Storkhead box-2 (*STOX2*) in decidual tissue from pregnancies complicated by pre-eclampsia and/or fetal growth restriction (FGR) ($n = 40$) to expression levels in decidual tissue from uncomplicated pregnancies ($n = 59$). We cannot confirm association of the candidate SNPs to pre-eclampsia ($P > 0.05$). For *STOX1*, no differential gene expression was observed in any of the case groups, whereas *STOX2* showed significantly lower expression in deciduas from pregnancies complicated by both pre-eclampsia and FGR as compared with controls ($P = 0.01$). We further report a strong correlation between transcriptional alterations reported previously in choriocarcinoma cells over expressing *STOX1A* and alterations observed in decidual tissue of pre-eclamptic women with FGR.

Key words: intrauterine growth restriction (IUGR) / decidua basalis / pre-eclampsia / HUNT2 / *STOX* genes

Introduction

Pre-eclampsia is a serious complication of pregnancy and a major cause of preterm intervention by Caesarean section, as delivery relieves symptoms. The condition presents with *de novo* elevated blood pressure and proteinuria after 20 weeks of pregnancy. Severe pre-eclampsia is not well defined, but assessment for severity includes both the mother (severe hypertension, end organ manifestations and preterm disease), and the fetus (intrauterine growth restriction, fetal movement assessment and oligohydramnios) (Gifford *et al.*, 2000). As defined in clinical practice, ~20% of pre-eclamptic cases are severe. Early identification, as well as more refined treatment options, is particularly important for these women, and this remains a major challenge in obstetric medicine. Thus, there is a rationale for concentrating research efforts on severe cases (Gifford *et al.*, 2000).

Pre-eclampsia is a complex disorder, including both placental and maternal components. Disease heritability is as high as 54% (Salonen *et al.*, 2000), but a distinct pattern of inheritance is not known. Whole genome linkage studies of families with an increased prevalence of pre-eclampsia have identified several genetic susceptibility loci for pre-eclampsia (Harrison *et al.*, 1997; Arngrimsson *et al.*, 1999; Moses *et al.*, 2000, 2006; Lachmeijer *et al.*, 2001; Laivuori *et al.*, 2003; Johnson *et al.*, 2007). Several studies have suggested the involvement of epigenetic mechanisms for the disease (Graves, 1998), and evidence for a maternal effect in one of the pre-

eclampsia susceptibility loci has been observed for a region of genes on chromosome 10q22 (Oudejans *et al*., 2004). The exons of 17 positional candidate genes in this region were sequenced in a Dutch cohort of families with two or more sibling pairs affected by pre-eclampsia (van Dijk *et al*., 2005). All families with increased prevalence of pre-eclampsia showed missense mutations within the Storkhead box-1 (*STOX1*, AK057891) gene on chromosome 10q22, identical between affected sisters (van Dijk *et al*., 2005).

The *STOX1* gene encodes a winged-helix domain-containing transcription factor, and is believed to play a role in the differentiation of trophoblast cells (van Dijk *et al*., 2005). The longest isoform of the STOX1 protein, *STOX1A* (NM_152709) exerts regulatory effects in several tissues (van Dijk *et al*., 2010a). Over expression of *STOX1A* in choriocarcinoma cells (JEG-3 cell line) was shown to be associated with transcriptional alterations similar to those observed in third-trimester pre-eclamptic placentas (Rigourd *et al*., 2008). The predominant variation co-segregating with pre-eclampsia disease status in the Dutch families (van Dijk *et al*., 2005), *STOX1A*-Y153H (rs1341667) is located in the DNA binding domain. Recently published data suggest that this variant may negatively regulate trophoblast invasion by up-regulation of the cell–cell adhesion protein a-T-catenin (CTNNA3) (van Dijk *et al*., 2010b).

Storkhead box 2 (*STOX2*, AB037813) has been identified as the only known human paralogue to *STOX1* (van Dijk *et al*., 2005; Kivinen *et al*., 2007), but to our knowledge little is known of its function. In humans, there is evidence that *STOX2* is a component of a molecular profile unique and globally characteristic of uncommitted stem cells (Thomas *et al*., 2008). The *STOX2* transcript is also included in a transcriptional profile observed with increased inflammatory response to air pollutants, differing between pregnant and non-pregnant mice (Fedulov *et al*., 2008). The gene is situated at chromosome 4q35, near a replicated region of known, suggestive linkage to pre-eclampsia on chromosome 4q31-q32 (Harrison *et al*., 1997; Moses *et al*., 2000; Laivuori *et al*., 2003).

The involvement of *STOX1* in pre-eclampsia observed in the Dutch families (Oudejans *et al*., 2004; van Dijk *et al*., 2005) has not been confirmed in independent studies (Berends *et al*., 2007; Kivinen *et al*., 2007; Iglesias-Platas *et al*., 2007). It has therefore been hypothesized that *STOX1* is relevant to a clinically severe subgroup of women with early onset of the disease, and fetal growth restriction (FGR) (Oudejans *et al*., 2007; Oudejans and van Dijk, 2008; van Dijk *et al*., 2007).

In the present study, we investigated candidate functional single nucleotide polymorphisms (SNPs) within *STOX1* in an independent large population-based pre-eclampsia cohort. The proposed disease-causing *STOX1A*-Y153H (rs1341667) variant was tested for association with defined clinical subgroups of pre-eclamptic women. We also compared decidual gene expression of *STOX1* and *STOX2* in uncomplicated pregnancies and pregnancies complicated by pre-eclampsia, FGR or both. Furthermore, we compared transcriptional alterations in Rigourd's cultured trophoblast cells with increased expression of *STOX1A* (Rigourd *et al*., 2008), with transcriptional alterations seen in our clinically defined subgroups.

Materials and Methods

Candidate gene expression study

Human subjects

Women with pregnancies complicated by pre-eclampsia, FGR, pre-eclampsia with FGR and women with uncomplicated pregnancies were recruited at St Olavs Hospital, Trondheim, Norway and Haukeland University Hospital, Bergen, Norway from 2002 to 2006. Pre-eclampsia was defined as hypertension (blood pressure $\geq$140/90 mmHg) plus proteinuria ($\geq$0.3 g/24 h or $\geq$1 + according to a dipstick test) on at least two occasions, developing after 20 weeks of pregnancy (Gifford *et al*., 2000). FGR was defined by a stringent small for gestational age (SGA) definition (birthweight $\leq$2 SD below the expected weight for gestational age and sex, corresponding to the 2.5 percentile) (Marsal *et al*., 1996), confirmed by at least one of the following: reduced fundal height in serial measurements, serial ultrasound biometry identifying failure to grow along a consistent centile or abnormal umbilical artery wave form. Multiple pregnancies and pregnancies with chromosomal aberrations, fetal and placental structural abnormalities or suspected perinatal infections were excluded.

Decidual tissue collection

Decidua basalis tissue samples were collected by vacuum aspiration of the placental bed during Caesarean section as previously described (Staff *et al*., 1999; Harsem *et al*., 2004). Caesarean section in the control group was done for reasons considered irrelevant to the study hypotheses (breech presentation, cephalopelvic disproportion or maternal request). Samples were flushed with 500 ml sterile saline solution at room temperature to remove excess blood. The decidual tissue was immediately submerged in RNA-later (Ambion, Huntington, UK), incubated at 4°C and stored at −80°C for later RNA extraction. The quality of the decidual material was assessed by immune histochemistry, as described in Eide *et al*. (2006). Only specimens containing extravillous trophoblast (decidua basalis) were included in the study.

Total RNA processing

Decidua basalis tissue was disrupted in a 2:1 (w/v) trizol:sample tissue mix using a Polytron® PT 1300 D digital, handheld homogenizer with a PT-DA 1307/2EC generator (Kinematica Inc., Lucerne, Switzerland). Total RNA was isolated using a trizol extraction protocol with chloroform interphase separation; isopropanol precipitation and ethanol wash steps. Isolated total RNA was purified with an RNeasy Mini Kit using spin technology (Qiagen, Valencia, CA, USA). Spectrophotometric determination of purified total RNA yield (μg) was performed using the NanoDrop ND-1000 (Wilmington, DE, USA). Total RNA quality was measured using the total RNA Nano Series II kit on the Agilent BioAnalyzer 2100 using the 2100 Expert software (Agilent Technologies, Germany). Synthesis, amplification, purification and biotin labelled complementary RNA (cRNA) was produced from a total RNA template using the Illumina TotalPrep RNA Amplification Kit (Ambion, TX, USA), according to manufacturer's instructions. Purified cRNA yield was determined spectrophotometrically using the NanoDrop ND-1000.

A total of 1.5 μg purified cRNA per sample was hybridized onto Illumina's HumanWG-6 v2 Expression BeadChip® following Illumina's 6 × 2 BeadChip protocol (Illumina, San Diego, CA, USA). After hybridization, the 6 × 2 expression arrays were washed, blocked, stained with streptavidin-Cy3 (GE Healthcare, Buckinghamshire, UK) and dried prior to them being scanned on the Illumina BeadArrayer 500GX reader using Illumina BeadScan image data acquisition software (version 2.3.0.13). Illumina's BeadStudio Gene Expression software module

(version 3.2.7) was used to subtract background noise signals for each individual sample and generate an output file for statistical analysis. The control summary report generated by the Gene Expression software module was used to evaluate the performance of the built-in controls for each BeadChip. The control summary report summarizes signal intensity, hybridization signal, background signal and the background to noise level for all samples scanned in a particular batch.

Candidate gene association study

HUNT2 case/control subjects

The samples used for our candidate gene association study were retrospectively identified from the second Nord-Trøndelag Health Study in Norway (HUNT2). The HUNT2 study was conducted from 1995 to 1997. All residents above 19 years were invited and 75.5% of the invited women ($n = 35.280$) participated. This population is considered well suited for genetic studies as it is homogeneous (<3% non-Caucasians) and stable (net out migration of 0.3% each year). The HUNT2 survey included a questionnaire, a clinical examination and collection of biological samples as previously described (Holmen *et al*., 2003). Obstetrical data from these women were obtained by linking the HUNT2 data with the The Medical Birth Registry of Norway (MBRN). Physicians and midwives have been registering obstetrical data from all deliveries in Norway after 16 weeks of gestation in standard questionnaires since 1967. More than 1.8 million births are included. Each resident in Norway is registered with an 11-digit personal identification number, also used for all national registries such as the MBRN and HUNT. The women having experienced pre-eclampsia in one or more pregnancies, defined as new onset of hypertension (blood pressure ≥140/90 mmHg) and proteinuria (≥0.3 g/d or ≥1 + according to a dipstick test), developing after 20 weeks of pregnancy (Gifford *et al*., 2000) were identified using diagnosis codes ICD-8 (before 1998) and ICD-10 (after 1998). Pre-eclamptic women with multiple pregnancies were excluded. Two controls per case were identified at random among parous women in the HUNT2 cohort with no registered pre-eclamptic pregnancy in the MBRN. All personal identification numbers in the total data set were randomly replaced by a serial number in order to ensure patient anonymity.

Clinical characterization of the HUNT2 pre-eclampsia-cohort

Preterm delivery was defined as delivery before 37.0 weeks (Gifford *et al*., 2000), and FGR as delivery of a SGA infant (birthweight ≤2 SD below the expected weight for gestational age and sex, corresponding to the 2.5 percentile) (Marsal *et al*., 1996). As fasting blood glucose was not available for all the individuals in the study cohort, an International Diabetes Federation (IDF)-proxy definition [waist circumference ≥80 cm plus any two of the following: high-density lipoprotein (HDL) cholesterol <1.29 mmol/l, treatment for hypertension or blood pressure ≥130/85 mmHg, diabetes diagnosed after the age of 30] (Hildrum *et al*., 2007) was used for assessment of metabolic syndrome. This method has been evaluated in a cross-sectional analysis of 10.206 participants in HUNT2 and no differences in the prevalence of metabolic syndrome between fasting and non-fasting groups were shown using the IDF-proxy definition (Hildrum *et al*., 2007).

Genotyping

DNA from pre-eclamptic (case) and non-pre-eclamptic (control) pregnancies was extracted from peripheral blood leukocytes from blood clots or from EDTA blood stored in the HUNT biobank, using the PURGENE chemistry (Gentra Systems Inc, Minneapolis, MN, USA) for large volumes or the MagAttract DNA Blood M48 kit (Qiagen, Hilden, Germany) for low volumes (<400 μl) as previously described (Holmen *et al*., 2003; Moses *et al*., 2007).

The four *STOX1* missense SNPs described by van Dijk *et al*. (2005) plus two additional known *STOX1* missense SNPs were selected for genotyping in the HUNT2 case/control cohort. The additional *STOX1* SNP was selected from the NCBI SNP database (*Homo sapiens* NCBI Build 35; dbSNP build 124). The six *STOX1* SNPs were genotyped using Applied Biosystems' SNPlex™ Genotyping System and sample genotypes were interrogated using Applied Biosystems' GeneMapper (version 4.0) software as previously described (Moses *et al*., 2007; Roten *et al*., 2008).

Statistical analysis

Gene expression analysis

Decidual gene expression profiles were analysed using SOLAR (Almasy and Blangero, 1998) as previously described (Goring *et al*., 2007). All raw gene expression signals were initially log-transformed. To identify significantly expressed genes in decidua, the distribution of expression values for a given gene across all samples was compared with the distribution of the expression values of the control targets implemented in each expression array. For each gene, we performed a χ^2 tail test using a false discovery rate (Hochberg and Benjamini, 1990) of 0.01 to determine whether there was a significant excess of samples with expression values above the 95th percentile of the control null distribution (Goring *et al*., 2007). The pre-processing of gene expression signals produces a sub-set of significantly expressed (detected) genes from the total number of targets synthesized onto each expression array. Within each sample, the mean log expression signal for each detected gene was ranked and grouped into bin sets of 2.000 genes. Each bin set was then *z*-scored independently. We applied a direct normalization procedure using an inverse Gaussian transformation for each detected same gene across all samples. This conservative procedure results in normalized expression phenotypes that are comparable between individuals and across all genes (Goring *et al*., 2007). To evaluate the magnitude of differential gene expression between case (pre-eclampsia and/or FGR) and control women, we measured the displacement of each detected gene's mean expression value between the two groups. We performed a standard regression analysis on the case group to test whether the mean gene expression level is significantly different (up- or down-regulated) in the case group compared with the control group.

When evaluating the correlation between transcriptional alterations in Rigourd's data set (Rigourd *et al*., 2008) and our data set (E-TABM-682), we performed a Spearman rank test, as the beta-values and the fold change values generated from these two studies are not directly comparable.

The microarray data have been reported to ArrayExpress according to MIAME standards with accession number E-TABM-682.

SNP association analysis

SNP association analyses of all selected SNPs were performed for the total case/control cohort using Pearson's χ^2 statistic in the software package SPSS 16.0 for Windows. The Y153H variation was analyzed separately for subgroups of pre-eclamptic women and control women (FGR, recurrence) for both a dominant (CC + CT genotype frequency versus TT genotype frequency) and additive (C allele frequency versus T allele frequency) genetic model. Multivariate logistic regression was used to model pre-eclampsia as the (dichotomous) dependent variable against maternal age. Odds ratios (OR) with 95% confidence intervals were calculated. Concordance with Hardy–Weinberg proportions was tested using a χ^2 goodness-of-fit statistic. A threshold of $\alpha = 0.05$ was set for statistical significance of all computed analyses.

Clinical characterization

Descriptive statistics means and standard deviations were computed in the software package SPSS 16.0 for Windows. *P*-values were computed based on *t*-test statistics. Recurrent and non-recurrent pre-eclamptic cases were

Table I Clinical characteristics of the cohort used in the gene expression analysis.

	Pre-eclampsia ($n = 13$)	FGR[a] ($n = 9$)	Pre-eclampsia with FGR ($n = 24$)	Controls ($n = 58$)
Maternal age (years)	30 ± 5*	34 ± 4	31 ± 5	31 ± 5
Gestational age (weeks)	35 ± 3**	32 ± 5**	30 ± 4**	39 ± 1
Systolic blood pressure (mmHg)	153 ± 18**	128 ± 15	151 ± 16**	116 ± 11
Diastolic blood pressure (mmHg)	95 ± 9**	74 ± 8	97 ± 12*	70 ± 9
Birthweight (g)	2364 ± 510**	1225 ± 21**	1118 ± 470**	3619 ± 469

Data are presented as mean ± SD. All case groups were compared with controls and *P*-values were computed based on *t*-test statistics.
[a]≤2 SD of expected weight, confirmed by at least one of the following: reduced fundal height in serial measurements, serial ultrasound biometry identifying failure to grow along a consistent centile or abnormal umbilical artery wave form.
*$P < 0.05$; **$P < 0.001$.

analyzed separately. Each pre-eclamptic group (recurrent and non-recurrent) was compared with the non-pre-eclamptic group. Multivariate logistic regression was used to model pre-eclampsia as the (dichotomous) dependent variable against maternal age. A threshold of $\alpha = 0.05$ was set for statistical significance of all computed analyses.

Ethical approval

Informed written consent was obtained from all participants in this study. The study was approved by the Regional Committees for Medical Research Ethics, the National Data Inspectorate and The Directorate of Health and Social Welfare, Norway. Ethical approval for genotyping the Norwegian case/control cohort (HUNT2), decidual RNA processing and statistical analysis of decidual RNA was obtained from The University of Texas Health Science Center at San Antonio, Institutional Review Board.

Results

Decidual tissue candidate gene expression analysis

Decidua basalis tissue was vacuum aspirated from the placental bed of 14 women with pre-eclampsia, 9 non-pre-eclamptic women with FGR neonates, 29 women with both pre-clampsia and FGR (pre-eclampsia + FGR) and 59 normal pregnancies. Seven samples of low RNA quality were excluded (assessed by the RIN value and visual evaluation of digital electrophoretic gel pictures). We therefore processed a total of 104 samples. Clinical characteristics of the study groups are presented in Table I.

Both the *STOX1* (NM_152709.3) and *STOX2* (NM_020225.1) targets were represented on each expression array as a single known transcript, respectively and were annotated from NCBI's Human Genome Build 36.2, RefSeq content release 17. The expression of both the *STOX1* and *STOX2* targets were significantly detected in decidual tissue using a conservative false detection rate of 1%. To evaluate the magnitude of differential gene expression, we measured the displacement of the mean *STOX1* or *STOX2* expression signals in the case group from the control group. We performed this measurement on the three different sample groups and found no significant difference in *STOX1* gene expression levels ($P > 0.05$) (Table II). The expression of *STOX2* however, was significantly lower in the group of pre-eclamptic women delivering FGR neonates compared with the control group ($P = 0.01$) (Table II).

Table II Decidua basalis differential *STOX1* and *STOX2* expression analysis.

Sample group comparison	Gene			
	STOX1		*STOX2*	
	β*	*P*-value	β*	*P*-value
Pre-eclampsia versus control	0.39	0.19	0.07	0.81
FGR versus control	0.49	0.18	−0.17	0.61
Pre-eclampsia with FGR versus control	0.04	0.86	**−0.59**	**0.01**

*The β (beta) value is the measure of displacement between the case and control group mean expression signals and is expressed in standard deviation units. A positive β implies a higher mean transcript expression signal in the case group compared with the control group and is analogous to a gene/transcript being up-regulated. A negative β implies a lower mean transcript expression signal in the case group compared with the control group and is analogous to a gene/transcript being down-regulated. Values significant at the 1% level are outlined in bold.

Table III Correlation between transcriptional alterations seen in JEG-3 cells over expressing *STOX1A* and alterations observed in decidua basalis tissue from pre-eclamptic and/or FGR pregnancies.

	Correlation coefficient[2] (Spearman *r*)	*P*-value
Pre-eclampsia	0.16	0.004*
Pre-eclampsia with FGR[1]	0.23	0.00008*
FGR	0.09	0.17

[1]≤2 SD of expected weight, confirmed by at least one of the following: reduced fundal height in serial measurements, serial ultrasound biometry identifying failure to grow along a consistent centile or abnormal umbilical artery wave form.
[2]correlation between the degrees of up/down-regulation of 259 pre-eclampsia related genes from the study of Rigourd *et al.* (comparing JEG-3 cells over expressing STOX1 with mock-transfected JEG-3 cells) with the present study (comparing decidua basalis samples from patients with pre-eclampsia and/or FGR with control decidua basalis samples).
*$P < 0.01$.

In the study by Rigourd *et al.* (2008), 259 of the 500 most up/down-regulated genes in pre-eclamptic placentas had detectable transcript levels in the microarray experiment comparing JEG-3 cells over

expressing *STOX1A* with mock-transfected JEG-3 cells. Of these 259 genes, 242 were found to have transcripts expressed above the background in the transcriptomic data from our material (E-TABM-682). We observed a strong correlation (Spearman $r = 0.23$, $P = 0.00008$) between the beta-values (ratio of up/down-regulation compared with controls) for these genes in our subgroup of women experiencing pre-eclampsia with FGR and the ratio of up/down-regulation in the study by Rigourd *et al*. (2008). The beta-values for the women with pre-eclampsia only, showed a weaker correlation (Spearman $r = 0.16$, $P = 0.004$), and there was no correlation to the beta-values for the non-pre-eclamptic women delivering FGR neonates (Spearman $r = 0.09$, $P = 0.17$) (Table III).

The HUNT2 case/control cohort

DNA samples were available for 1139 women registered with pre-eclamptic pregnancies (cases) and 2269 non-pre-eclamptic women (controls) (Moses *et al*., 2007). Of the available cases, 1003 women were registered with one and 136 women with more than one pre-eclamptic pregnancy. Mean follow-up time from diagnosis in the MBRN to inclusion in the present study was 25 ± 10 years. As expected, gestational age and birthweight differed between the neonates in pre-eclamptic and non-pre-eclamptic pregnancies; the pre-eclamptic women had a higher risk of delivering preterm, and of delivering a FGR neonate (Table IV, $P < 0.001$). The metabolic syndrome, evaluated by data from the HUNT2 study, was also higher in the case groups as compared with controls (Table IV, $P < 0.001$). After adjusting for maternal age, the differences in clinical phenotype between case and control groups remained significant at $\alpha < 0.001$ level (Table IV).

We also observed clinical differences between the recurrent and non-recurrent pre-eclamptic groups (Table IV). The women in the recurrent group delivered earlier ($P = 0.018$) and the neonates had a lower birthweight (adjusted for gestational age, $P = 0.055$). The prevalence of preterm birth was higher in the recurrent group (22%) compared with the non-recurrent group (14%) ($P < 0.01$), but the seemingly different prevalence of FGR (20 versus 15%) was not statistically significant ($P = 0.2$). The *P*-values are adjusted for maternal age. Metabolic syndrome at inclusion in the HUNT2 study was more prevalent in the recurrent group compared with the non-recurrent when adjusting for age at inclusion ($P = 0.019$).

Statistical power analysis for the HUNT2 case/control cohort

A priori power calculations *ad modum* Lalouel and Rohrwasser (2002) for the *STOX1A* Y153H (rs1341667) variant demonstrated 90% power to detect an effect size (OR) difference of 1.3 for the total case/control (HUNT2) cohort and 1.9 for the recurrent pre-eclampsia subgroup ($n = 136$).

STOX1 *genotyping and association analysis*

The R18P *STOX1* SNP failed the SNPlex assay design due to high homology and another two *STOX1* SNPs were non-polymorphic. There were no significant differences in allele frequencies between the case and control groups for the genotyped SNPs (Table V). There was a high average sample genotype success rate of 87% (range 84–88%) and all SNPs conformed to Hardy–Weinberg proportions ($P > 0.05$).

Assuming a dominant genetic model for the Y153H variant, it is proposed that this variant becomes mutagenic through mechanisms of imprinting either of *STOX1* itself, or of downstream proteins (van Dijk *et al*., 2005). Under this analysis model, the pre-eclampsia + FGR subgroup did not show any association with the *STOX1A*-Y153H variant when compared with the control group (Table VI). However, the recurrent pre-eclampsia subgroup showed a tendency towards higher incidence of the C-genotype ($P = 0.09$) (Table VI).

Discussion

In this study, we demonstrate reduced decidual gene expression of *STOX2* in pre-eclamptic women delivering FGR neonates. To our

Table IV Clinical characteristics of the HUNT2 case/control cohort.

	Pre-eclampsia (recurrent[1], *n* = 136)	Pre-eclampsia (non-recurrent, *n* = 1.003)	Control (*n* = 2.269)
Maternal age at index pregnancy (years)	25 ± 5	27 ± 6*	25 ± 5
Gestational age (days)	271 ± 20*	275 ± 22*	282 ± 18
Birthweight (g)	3.040 ± 846*	3.238 ± 837*	3.483 ± 592
FGR[2]	26 (20)*	147 (15)*	87 (4)
Preterm birth[3]	29 (22)*	132 (14)*	114 (5)
Maternal age at inclusion in HUNT2	37 ± 9*	40 ± 11	40 ± 11
Metabolic syndrome[4]	30 (22)*	163 (16)*	212 (9)

Data presented as mean ± SD or number (percentage). *P*-values are computed based on *t*-test statistics, each pre-eclamptic group is compared with the non-pre-eclamptic group. IDF, the International Diabetes Federation; HDL, high-density lipoprotein; CI, confidence interval.
[1]More than one pre-eclamptic pregnancy.
[2]≤2 SD of expected weight.
[3]Delivery before week 37.
[4]IDF-proxy definition; waist circumference ≥80 cm plus any two of the following: (HDL cholesterol <1.29 mmol/l, treatment for hypertension or blood pressure ≥130/85 mmHg, diabetes diagnosed after age of 30 or fasting plasma glucose ≥5.6 mmol/l) [43].
*$P < 0.001$.

Table V Genotyped SNPs within *STOX1*.

SNP	Genotype (NN), Allele (N)	Pre-eclampsia (proportion of total)	Control (proportion of total)	*P*-value
R18P		Failed assay design		
rs1341667 (Y153H)	CC	412 (0.418)	840 (0.416)	0.9
	CT	450 (0.456)	931 (0.462)	
	TT	124 (0.126)	245 (0.122)	
	C	1274 (0.646)	2611 (0.648)	
	T	698 (0.354)	1421 (0.352)	
rs41278530 (L582F)		Non-polymorphic		
rs10509305 (E608D)	AA	583 (0.591)	1206 (0.597)	0.5
	AC	346 (0.351)	716 (0.355)	
	CC	57 (0.058)	97 (0.048)	
	A	1512 (0.767)	3128 (0.775)	
	C	460 (0.233)	910 (0.225)	
rs41278532 (N825I)	AA	953 (0.968)	1827 (0.972)	0.4
	AT	28 (0.028)	50 (0.027)	
	TT	3 (0.004)	2 (0.001)	
	A	1934 (0.983)	3704 (0.986)	
	T	34 (0.017)	54 (0.014)	
rs7904300 (A865T)		Non-polymorphic		

P-values were computed using Pearson's χ^2 statistic.

Table VI The *STOX1A*-Y153H variation in severe subgroups of pre-eclamptic women.

Y153H variation	Pre-eclampsia + FGR[a]	Recurrent[b] pre-eclampsia	Control
Number of individuals genotyped	151	115	2010
Dominant model frequency CC + TC	0.881	0.930	0.878
OR* (CI)	1.0 (0.6–1.7)	1.9 (0.9–3.9)	
P-value	0.92	0.09	

[a]FGR, fetal growth restriction measured by birthweight below 2 SD for gestational age.
[b]More than one pre-eclamptic pregnancy.
*OR for each of the subgroups of pre-eclamptic women compared with controls with 95% confidence intervals (CIS) using χ^2 statistics.

knowledge, this is a novel finding. We also elaborate the results of Rigourd *et al*. showing correlation between transcriptional alterations of pre-eclamptic placentas (relative to controls) and JEG-3 cells over expressing *STOX1A* (relative to mock-transfected JEG-3 cells) (Rigourd *et al*., 2008). Transcriptional alterations in our decidua basalis tissue (E-TABM-682) show the strongest correlation to Rigourd's data set in pre-eclamptic pregnancies complicated by FGR, but not in non-pre-eclamptic pregnancies with FGR. Furthermore, in a population- and registry-based cohort, we find that women experiencing pre-eclampsia more than once are at a higher risk of complications and co-morbidity (preterm birth, lower birthweight and development of metabolic syndrome) compared with those experiencing pre-eclampsia once. There is also a tendency towards higher frequency of the C genotypes for the previously reported *STOX1A*-Y153H variation (van Dijk *et al*., 2005) in this group of women.

Reduced placental perfusion due to impairment of trophoblast invasion and failed spiral artery remodelling are proposed to be pathogenic features of both fetal growth restriction and pre-eclampsia (Brosens *et al*., 1972, 1977). We believe the present study of decidua basalis tissue, where these disease processes are taking place, to be an important supplement to previous reports of *STOX1* gene expression in placental tissues (van Dijk *et al*., 2005; Iglesias-Platas *et al*., 2007; Kivinen *et al*., 2007) and cultured trophoblast cells (van Dijk *et al*., 2005, 2010; Rigourd *et al*., 2009). We show novel evidence that the *STOX2* gene is down-regulated in term decidua basalis from pre-eclamptic women delivering FGR neonates. *STOX2* resides on chromosome 4q35, near a replicated region of suggestive linkage to pre-eclampsia on chromosome 4q31-q32 (Harrison *et al*., 1997; Moses *et al*., 2000; Laivuori *et al*., 2003). To our knowledge, the role of *STOX2* in the maternal–fetal interface is not known. Gene duplication is an important evolutionary mechanism, and is a continuous matter of research in evolutionary systems biology (Hughes, 2005; Hittinger and Carroll, 2007). Studies of paralogous genes show that duplication does not create genes with novel functions, but rather daughter genes performing specialized sub functions of the ancestral gene (Jensen, 1976; Conant and Wolfe, 2008). *STOX2* has a high sequence similarity to *STOX1* (van Dijk *et al*., 2005; Kivinen *et al*., 2007), and it is reasonable to hypothesize that it is involved in some of the same biological processes. Therefore, the role of *STOX2* in

normal placental development as well as in the pathogenesis of pre-eclampsia with FGR warrants further investigation. A comprehensive assessment of genetic regulatory variation as well as a molecular functional evaluation is required to confirm the biological importance of our observation.

Rigourd *et al.* recently showed a strong correlation between transcriptional alterations in trophoblast cell-lines over expressing *STOX1A* and transcriptional alterations shown in term pre-eclamptic placentas (Rigourd *et al.*, 2008). This supports the observation made by others, that the possible disease-causing effects of *STOX1* dysregulation are mediated through aberrant transcriptional regulation of trophoblasts (van Dijk *et al.*, 2005, 2010). The dysregulation of *STOX1* can potentially have deleterious effects as it affects the transcription level of many other genes shown to be important in the development of pre-eclampsia (such as Endoglidin, human Chorionic Gonadotrophin, and Glial Cell Missing Homolog (Rigourd *et al.*, 2009). Furthermore, a direct effect of *STOX1* on *CTNNA3* expression was recently confirmed, affecting trophoblast differentiation and growth (van Dijk *et al.*, 2010b). When comparing the transcriptional alterations of our defined subgroups of women with the transcriptional alterations in Rigourd's trophoblast cell line over expressing *STOX1A*, we confirm their results from placental tissue in decidua basalis. Furthermore, the observed correlation is strongest in the group of women with both pre-eclampsia and FGR. Our findings suggest that the *STOX* genes are associated with a severe fetal phenotype (FGR), interestingly though, only in pre-eclamptic patients, and not in non-pre-eclamptic women delivering FGR neonates. This supports the opinion that FGR in pre-eclampsia and FGR alone represent different pathogenic conditions, as discussed by others (Ness and Sibai, 2006; Huppertz, 2008; Srinivas *et al.*, 2009).

The analysis of gene expression in whole tissue harvested from patients with manifest disease benefits from describing the *in vivo* situation. However, interesting differences will potentially be masked due to the heterogeneity of the material. Approximately 40% of cells in decidua basalis are maternal leukocytes, 20% are extravillous trophoblast and 30% are decidual stromal cells (Benirschke *et al.*, 2006). This is a possible confounding factor; however, our results were interpreted in relation to, and are consistent with, earlier observations (Rigourd *et al.*, 2009; van Dijk *et al.*, 2010b). Our findings are limited to describing gene expression in decidua basalis samples collected at birth; we do not report expression throughout the pregnancy. The strength of this study, however, is a thorough monitoring by an obstetrician; multiple blood pressure and proteinuria measurements, prenatal ultrasound measures and birthweight confirmation, providing a sound diagnostic basis (Eide *et al.*, 2006).

There were no significant differences in allele frequencies between the case and control groups in the population-based cohort for the genotyped candidate SNPs within *STOX1*. This is in agreement with smaller population samples included in other studies (Berends *et al.*, 2007; Kivinen *et al.*, 2007).

Severe pre-eclampsia is diagnosed by assessment of both maternal and fetal phenotypes (Gifford *et al.*, 2000). It has been shown that mothers developing early onset pre-eclampsia have a higher prevalence of fetal growth restriction, as well as increased maternal morbidity and cardiovascular risk later in life (Ness and Roberts, 1996; Mostello *et al.*, 2002; Brown *et al.*, 2007). The MBRN did not include information about onset of disease until 1998, and we are therefore not able to include this parameter in our analyses. It has been shown, however, that women developing severe pre-eclampsia in the second trimester are at high risk of recurrent pre-eclampsia as well as later life chronic hypertension and increased maternal morbidity and mortality (Sibai *et al.*, 1986, 1991; Odegard *et al.*, 2000). Therefore, research on multiparous pre-eclamptic women is encouraged as a tool of better understanding of disposition to, and development of strategies for treatment and prevention of pre-eclampsia (Gifford *et al.*, 2000). Findings in our cohort are consistent with earlier studies, as the recurrent group shows the highest risk of preterm labour, of low fetal birthweight and the highest later life cardiovascular risk, assessed as metabolic syndrome. We do find a tendency towards higher incidence of the C genotypes of the Y153H mutation in the recurrent group under a dominant genetic model. This finding is however, not significant at the 5% level. Assessment of the Y153H mutation in pre-eclamptic women delivering FGR neonates did not show association. Thus, we cannot confirm the hypothesis that this variant is linked to severe pre-eclampsia, or pre-eclampsia with FGR in the Norwegian population.

In the population-based part of the study, we are using registry data, and some misclassification can be expected. This will limit the power to detect a true association. The prevalence and recurrence rates of pre-eclampsia in MBRN are, however comparable to those reported in other Nordic countries (Trogstad *et al.*, 2001). Being born SGA does not necessarily imply FGR, but can also reflect e.g. different ethnicity, constitutional smallness and chromosomal aberrations. However, the growth curves used in this study are based on ultrasonically estimated fetal weights, appropriate for the population (which is homogeneous) and considers the sex of the fetus (Marsal *et al.*, 1996). Furthermore, a stringent SGA definition of ± 2 SD is used, which is more likely to identify the true FGR cases within the SGA group (ACOG, 2002; Sheridan, 2005).This leads us to believe that SGA is a good approximation to FGR in our study. Also, the possible maternal imprinting effect on *STOX1* is a matter of debate (Berends *et al.*, 2007; Iglesias-Platas *et al.*, 2007; van Dijk *et al.*, 2007). We are not able to evaluate this in a case–control cohort, and epigenetic effects will lessen our power to detect a true association. However, the strength of this study is a large sample size, and the extensive clinical information available.

In conclusion, we present novel observations suggesting involvement of *STOX2* in the pathogenesis of pre-eclampsia with FGR. We confirm Rigourd's findings in decidual tissue, and link the transcriptional alterations observed with *STOX1A* over expression to alterations seen in deciduas of pre-eclamptic women delivering FGR neonates. Conceivably, these findings might reflect a role for the *STOX* genes in the pathophysiology of pre-eclampsia with FGR.

Authors' roles

M.H.F. wrote the paper, contributed substantially to acquisition of gene expression and epidemiology data and to data analysis and interpretation of results. M.P.J., J.B., E.K.M. and R.A. contributed substantially to conception and design, analysis and interpretation of data, revising and final approval of manuscript. M.L., S.B.M., A.K.J. and T.D.D. contributed substantially to acquisition and analysis of expression data, revising and final approval of manuscript. L.T.R. acquisition and interpretation of genotype data, revising and final approval

of manuscript. I.P.E. contributed substantially to acquisition of data, revising and final approval of manuscript. S.F. contributed substantially to acquisition of epidemiology data, analysis and interpretation, revising and final approval of manuscript. L.B. and R.K.S. recruitment of patients, collection and cryopreservation of decidual tissues, establishment of clinical database, revising and final approval of manuscript.

Acknowledgements

The authors thank Dr Mette Langaas for valuable advice. The Nord-Trøndelag Health Study (the HUNT study) is a collaboration between the HUNT Research Centre, Faculty of Medicine, The Norwegian University of Science and Technology, the Norwegian Institute of Public Health and the Nord-Trøndelag County Council.

Funding

This work was supported by grants from the Research Council of Norway (to M.H.F.); the Fulbright foundation for educational exchange (to M.H.F.); the Central Norway Regional Health Authority (to M.H.F. and L.T.R.); the Norwegian University of Science and Technology (M.H.F., L.T.R., M.L., S.B.M. and R.A.); National Institutes of Health (HD049847 to E.K.M. and MH059490 to J.B.), the Southwest Foundation Forum (to M.P.J.). This investigation was conducted, in part, in facilities constructed with support for the Research Facilities Improvement Program from National Institutes of Health (RR013556 and RR017515).

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