Placenta 34 (2013) 174–181

Contents lists available at SciVerse ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Dual specificity phosphatase 9 (DUSP9) expression is down-regulated in the severe pre-eclamptic placenta

M.J. Czikk [a,b,*], S. Drewlo [b,c], D. Baczyk [c], S.L. Adamson [b,c], J. Kingdom [a,b,c]

[a] Department of Obstetrics & Gynaecology, Mount Sinai Hospital, 600 University Ave, Toronto, ON, Canada M5G 1X5
[b] Department of Obstetrics & Gynaecology, University of Toronto, Canada
[c] Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 25 Orde Street, Toronto, ON, Canada M5T 3H7

ARTICLE INFO

Article history:
Accepted 22 November 2012

Keywords:
DUSP9
Pre-eclampsia
Intrauterine growth restriction
Placenta
Trophoblast

ABSTRACT

Objectives: Intrauterine growth restriction (IUGR) and pre-eclampsia are severe and clinically important manifestations of placental insufficiency. In the mouse, dual specificity phosphatase 9 (DUSP9) is critical to the normal development of the placenta, where knock-outs are growth restricted and have a placental phenotype similar to that seen in syndromes of human placental insufficiency. Our purpose was to characterize DUSP9 expression in normal human pregnancy and in cases of placental insufficiency.
Study design: We used RT-PCR, immuno-histochemistry and Western blotting to characterize DUSP9 gene expression and protein levels across human gestation and in pregnancies complicated by severe IUGR and/or severe pre-eclampsia. *DUSP9* promoter methylation was studied in pathologic and pre-term control placentas to investigate potential epigenetic regulation. First trimester villous explants and BeWo cells were treated with *DUSP9* silencing RNA to determine the effect on downstream pathways. Placental hypoxia is a hallmark of pre-eclampsia; therefore explants were subjected to hypoxic culture conditions to determine the effect of oxygen on *DUSP9* expression *in vitro*.
Results: DUSP9 expression was evident in villous trophoblast and declined during development. DUSP9 protein was significantly lower in severe pre-eclamptic placentas compared to severe growth restriction. This was not epigenetically mediated by promoter hyper-methylation, and the downstream pathway ERK1/2 was not significantly affected. *DUSP9* expression in first trimester explants was significantly decreased by 74 ± 20% in hypoxic (3% oxygen) culture conditions. In BeWo cells and explanted placental villi treated with *DUSP9* silencing RNA, expression of *DUSP9* was down-regulated by 61% and 62% respectively. There was a trend to increased phosphorylation of the downstream target ERK1/2 in *DUSP9* down-regulated BeWo cells and explanted placental villi.
Conclusion: DUSP9 protein levels were markedly suppressed in severe pre-eclampsia, but not in severe IUGR. This suppression might be attributable to the prolonged hypoxic conditions found in pre-eclampsia.

1. Introduction

Placental insufficiency is a term used to describe a number of pathological conditions that include inadequate uteroplacental blood flow, maternal inflammation, reduced placental size and inadequate development of the placental villi [1]. Intrauterine growth restriction (IUGR) is a severe manifestation of placental insufficiency, leading to increased fetal and neonatal morbidity and mortality. The maternal syndrome of pre-eclampsia, which can result in hypertensive emergencies with neurological manifestations, renal failure and liver failure, can also occur in conjunction with or in the absence of IUGR. Although prenatal diagnosis of placental insufficiency has improved significantly based on the combination of placental morphology, biochemical markers and uterine artery Doppler waveforms [2], the underlying molecular basis leading to these clinical manifestations is still unclear and the focus of much investigation.

Mouse transgenic studies aimed at identifying genes involved in the development of placental insufficiency have revealed an

Abbreviations: IUGR, intrauterine growth resitriction; DUSP9, dual specificity phosphatase 9; MAPK, mitogen activated protein kinase; sIUGR, severe intrauterine growth restriction; sPE, severe pre-eclampsia; ERK1/2, extracellular signal related kinase 1/2; REB, research ethics board; AEDFV, absent end diastolic flow velocity; REDFV, reversed end diastolic flow velocity; PTC, pre-term control; TC, term control; qRT-PCR, quantitative RT-PCR; PVDF, Polyvinylidene Fluoride; VEGF, vascular endothelial growth factor; BMP, bone morphogenic protein.

* Corresponding author. Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 25 Orde Street, Room 6-1020, Toronto, ON, Canada M5T 3H7. Tel.: +1 416 586 8322; fax: +1 416 586 8565.
E-mail address: mczikk@mtsinai.on.ca (M.J. Czikk).

0143-4004/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.placenta.2012.11.029

important candidate. Dual specificity phosphatase 9 (DUSP9) is a mitogen activated protein kinase (MAPK) phosphatase which acts to negatively regulate the MAPK pathways responsible for a range of cellular responses including cell differentiation, proliferation, cell cycle regulation and apoptosis [3,4]. DUSP9 has a substrate preference for the MAPK pathways ERK1/2 (extracellular signal related kinases 1/2) and p38 [5,6], both of which play a role in trophoblast differentiation [7] and placental angiogenesis [8,9]. The *DUSP9* gene is located on the X-chromosome and has been localized to the placenta, kidney and fetal liver [10]. DUSP9 is critically important in mouse placental development, as shown by Christie et al. in 2005 [11]. Mice lacking *DUSP9* expression show evidence of IUGR that precedes intrauterine fetal demise. Tetraploid rescue of the embryos leads to normal fetal growth and development, confirming that the effect of deficient *DUSP9* is at the level of the trophoblast. Placental pathology was consistent with the tetraploid rescue data as the knock-out mouse placentas exhibited an underdeveloped vasculature and a thin and more compact placental exchange region. Although this mouse model demonstrated a dramatic placental phenotype, to date no studies of DUSP9 expression or function have been reported in either normal human pregnancy or in pregnancies complicated by severe placental insufficiency disorders. We therefore characterized placental DUSP9 expression across normal human gestation and in syndromes of placental insufficiency including severe IUGR (sIUGR) and severe early onset pre-eclampsia (sPE). We used BeWo cells and first trimester villous explants as *in vitro* models to study the effect on the downstream MAPK pathways ERK1/2 and p38. *DUSP9* promoter methylation and hypoxic culture conditions were also studied as possible regulators of *DUSP9* gene expression.

2. Materials & methods

2.1. Patient selection and placental collection

Ethics approval was granted and written informed consent was obtained from all participants for donation of samples of their placenta to the hospital Research Centre for Women and Infant's Health Biobank (REB approval 04-0018-U). Women were recruited from outpatient clinics, the high risk antenatal and labor and delivery units. Placental collection was performed as per protocol (Standard Operating Procedures for the Collection of Perinatal Specimens for Research 2010, last viewed July 25, 2012, PDF available at http://biobank.lunenfeld.ca/?page=SpecimenArchive). Once collection was complete, the placenta was sent to the Department of Pathology for analysis as per hospital placental examination protocol [12].

A search was performed of the Biobank database to select placentas from singleton pathologic pregnancies and controls of live born infants delivered preterm between 24^{+0} and 34^{+6} weeks of gestation. They were classified into three groups, with characteristics summarized in Table 1:

1) Severe intrauterine growth restriction (sIUGR): Birth weight $\leq$ 10th centile, abnormal umbilical artery Doppler (absent end diastolic flow velocity (AEDFV) or reversed end diastolic flow velocity (REDFV)), including normotensive women and women with any form of coexisting hypertension [13].
2) Severe pre-eclampsia (sPE): 1) Systolic BP >140 systolic or >90 diastolic AND proteinuria of >1+ on dipstick or >300 mg/24 h AND delivery under 34^{+0} weeks, with a birth weight >10th percentile OR 2) Systolic BP >160 mmHg or a diastolic BP >110 mmHg AND proteinuria of >1+ on dipstick or >300 mg/24 h AND delivery at less than 34^{+6} weeks, with a birth weight >10th percentile. These criteria are based on the ACOG guideline *The Diagnosis and Management of Pre-eclampsia and Eclampsia* [14].
3) Pre-term control (PTC): Birth weight >20th centile, no abnormal umbilical artery Doppler measurements, BP < 140/90, no history of gestational diabetes, no histologic evidence of chorio-amnionitis.

First and early second trimester villous tissues from elective social terminations of pregnancy as well as healthy term control (TC) placentas ($>37^{+0}$ weeks gestation) were also collected for gestational age profile studies.

2.2. BeWo cell culture

The human choriocarcinoma cell line BeWo (passages 10–20) was maintained in F12K media (ATCC, Manassas, Va, USA) supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 units/mL streptomycin (Invitrogen, Burlington, ON, Canada), in atmospheric O_2/5% CO_2 at 37 °C. Cells were transfected at 50% confluency with siRNA to *DUSP9* in parallel to control experiments. 48 h post-transfection, RNA was extracted using the RNeasy kit (Qiagen, Mississauga, ON, Canada) while total protein was extracted using RIPA lysis buffer which included both protease and phosphatase inhibitors. Knock-down of *DUSP9* transcription was determined using quantitative RT-PCR (qRT-PCR).

2.3. Placental villous explant cultures

First trimester placental villous tissues were collected from ultrasound-dated viable singleton pregnancies after elective social termination of pregnancy at 8–13 weeks gestation. Explants were cultured using the floating explant method [15] in serum-free media (Dulbecco's modified Eagle's media (DMEM)/F12K) with 1% liquid media supplement ITS + 1 (Sigma, St Louis, MO, USA), 100 units/mL penicillin, 100 units/mL streptomycin, 2 mM L-glutamine, 100 μg/mL gentamicin and 2.5 μg/mL fungizone. Explants were cultured and maintained in physiologic conditions (8% ambient oxygen) or mildly-hypoxic conditions (3% ambient oxygen). Culture was maintained for 48 h and explants were then collected for RNA and protein extraction as above, or were fixed with 4% paraformaldehyde and wax embedded for histology and immuno-histochemistry. DUSP9 repression was assessed with qRT-PCR.

2.4. DUSP9 siRNA treatment

Double-stranded experimentally-verified siRNA directed against the human *DUSP9* sequence was purchased from Qiagen (SI02665572, Cat No. NM_001395) and used as previously described [15]. BeWo cells and villous explants were incubated in the presence of either 100 nM siRNA or non-silencing control ($N = 4$) (as previously described) [15].

2.5. Reverse transcription and real-time PCR

Total RNA was extracted from BeWo cells or placental villous explants using the TRIsol®Reagent extraction method (as per manufacturer's instructions) (Ambion Life Technologies, Burlington ON, Canada) and purified using the RNeasy kit (Qiagen). Reverse transcription and real-time PCR were performed on DNAse treated RNA samples as previously described [16]. Comparative CT Method (ABI technical manual) was used to analyze the real-time PCR. The expression of the *DUSP9* gene was normalized to the geometric mean of the housekeeping genes SDHA, and one of YWHAZ or TBP (sequences in Table 2) [17]. All data was expressed relative to non-silenced (NS) control.

2.6. Protein isolation

Tissues were extracted in RIPA buffer (50 mM TrisHCl, 150 mM NaCl, 1% Triton ×100, 1%Na Deoxycholate and 0.1% SDS) containing protease inhibitor and sodium vanadate. Protein concentration was determined by a Bradford protein assay (Bio-Rad, Mississauga ON, Canada) using BSA as a standard or using a Pierce®BCA Protein Assay Kit (Thermo Scientific, Rockford IL, USA). All measurements were performed in triplicate. Aliquots were stored at −80 °C.

2.7. DUSP9 Western blotting

Western blotting was performed as recently described [16]. Pathologic and control lysates were diluted in Nu-Page LDS Sample Buffer (Invitrogen) and β-mercaptoethanol and then boiled for 5 min at 95 °C. Samples extracts containing 60 μg of protein were loaded into a Novex® 10% Tris-Glycine 1.5 mm pre-cast gel (Invitrogen), separated at 100–125 V, then transferred at 4 °C to a Polyvinylidene Fluoride (PVDF) membrane (Millipore, Billerica MA, USA) at 80–90 V. Blots were blocked for 1 h with 5% milk in TBS at room temperature. Primary antibody to DUSP9 (mouse monoclonal anti-DUSP9, Abcam, Cambridge MA, USA) was diluted 1:1000 with 5% milk in TBS and incubated overnight at 4 °C. Blots were then washed in TBS, and incubated at room temperature for 1 h in secondary antibody (anti-mouse HRP) diluted at 1:2000 with 5% milk + TBS. An enhanced chemi-luminescence system (Western Lightening™ Plus-ECL, PerkinElmer, Woodbridge ON) was used to detect the signal using autoradiography. Blots were then stripped using a mild stripping buffer (glycine, SDS and Tween 20) followed by a series of PBS and TBST (0.05%) washes and then re-probed using an antibody against actin (Santa Cruz Biotechnology, Santa Cruz CA) diluted at 1:3000. Internal control samples were run on each gel in order to standardize measurements between gels. Data are presented as the ratio of relative optical density of DUSP9 to actin as compared to the standard control sample. Western blots of all samples were done in three separate technical replicates on different days, with the results averaged for the three runs.

2.8. ELISA for ERK1/2 and p38

Invitrogen ERK1/2 (total and phosphorylated) and p38 (total and phosphorylated) ELISA kits were used to determine levels of these proteins in human pathologies and controls, BeWo cells and first trimester explants according to

Table 1
Clinical characteristics of pregnancies for placentas studied.

Parameter	PTC	sIUGR	sPE	*vs PTC †sIUGR vs sPE
N	14	27	18	
Gravidity (median, range)	2 (1–4)	2 (1–7)	2 (1–5)	ns
Parity (median, range)	0 (0–2)	1 (0–3)	0 (0–4)	ns
Gestational age (mean ± SD)	32.3 ± 2.2	29.9 ± 2.5*	30.1 ± 2.2**	$^{*}p < 0.05$, $^{**}p < 0.01$
Maternal age (mean ± SD)	31.1 ± 5.4	32.4 ± 6.8	31.7 ± 6.7	ns
Race				
Black	2	6	8	nd
White	10	10	5	nd
Other/unknown	2	10	5	nd
Drugs				
Cigarettes	1	1	0	nd
Alcohol	0	0	0	nd
Other	0	1 (marijuana)	0	nd
Unknown	2	2	1	nd
Max systolic BP (mean ± SEM)	125 ± 3	156 ± 4***	174 ± 5***†	$^{***}p < 0.001$, $\dagger p < 0.05$
Max diastolic BP (mean ± SEM)	75 ± 3	97 ± 3***	107 ± 2***	$^{***}p < 0.01$
Chronic hypertension	0	5	6	nd
Maternal diabetes				
GDM	0	1	1	nd
Pre-gestational	0	0	2	nd
Antenatal steriods				
Yes	10	23	16	nd
GA (wks) if yes	28 ± 4	28 ± 3	29 ± 2	nd
No	3	3	1	nd
Unknown	1	1	1	nd
Magnesium sulfate				
Yes	0	7	11	nd
No	2	0	0	nd
Unknown	12	20	7	nd
Delivery mode				
C-section no labour	2	27	15	nd
C-section with labour	9	0	2	nd
Vaginal delivery	3	0	1	nd
Birth weight (g) (mean ± SD)	2163 ± 525	875 ± 255***	1279 ± 358*††	$^{*}p < 0.05$, $^{***}p < 0.001$, $\dagger\dagger p < 0.01$
Birthweight percentile, *N* (%)				
<5%ile	0	16 (59)	0	nd
5–10%ile	0	11 (41)	0	nd
10–50%ile	3 (21)	0	18 (100)	nd
>50%ile	11 (79)	0	0	nd
Placental weight (g) (mean ± SD)	370 ± 123	146 ± 33***	219 ± 60***††	$^{***}p < 0.001$, $\dagger\dagger p < 0.01$
Baby's sex, *N* (%)				
Male	8 (57)	13 (48)	7 (39)	ns
Female	6 (43)	14 (52)	11 (61)	ns

Clinical characteristics of pregnancies of all placentas studied. PTC – pre-term control, sIUGR – severe intrauterine growth restriction, sPE – severe pre-eclampsia, GA –gestational age, SD – standard deviation, SEM – standard error of the mean, ns – not statistically significant, nd – statistics not done (data presented for descriptive purposes only).

manufacturer instructions. Proteins were extracted as noted above. All samples were run in duplicate to derive mean values.

2.9. Immuno-histochemistry

Paraformaldehyde-fixed samples were wax embedded and sectioned onto charged slides for immuno-histochemical analysis of DUSP9 as previously described [16]. Sections were incubated overnight with primary anti-serum (rabbit anti-DUSP9 1:100 dilution, Sigma, Oakville ON, Canada) at 4 °C. The following day, signal amplification with biotinylated anti-rabbit immunoglobulin (1:300) was performed as previously described [16]. All slides were stained on the same day using the same solutions in order to minimize variation in intensity of stain. Negative controls included substitution of the primary anti-serum with non-immune serum to rule out non-specific binding.

2.10. Methylation studies

In-silico analysis revealed an island of 1228 base pairs with 115 CpG sites in the *DUSP9* promoter region; thirteen of these CpG sites were analyzed. DNA was extracted from tissue samples using the AllPrep DNA/RNA Mini Kit (Qiagen) and DNA methylation was determined using EpiTYPER analysis (Sequenome, San Diego, CA, USA) at the UHN Gene Profiling Facility (Toronto, ON).

2.11. Statistical analysis

Data are presented as mean ± SEM unless otherwise noted. Means or medians were derived from duplicate or triplicate values. Groups were analyzed for normality by the D'Agostino & Pearson omnibus normality test. To analyze characteristics of pregnancies for placentas studied, either a one-way ANOVA with a Bonferroni post-hoc test or a Kruskal–Wallis test with a post-hoc Dunn's comparison test were used for continuous data and a Chi-squared test was used if data were categorical. A one-way ANOVA was used to determine differences in DUSP9 gene expression across gestation. A two-way ANOVA was used to determine the effect of fetal sex and pathological group on DUSP9 protein expression and whether there was an interaction between these two factors. A paired *t*-test was used to determine if the silencing of BeWo cells and first trimester villous explants was significantly different from the non-silenced control (referenced at 1) and if there were differences in the explants cultured in hypoxic conditions compared to those in normoxic conditions (referenced at 1). Data for BeWo cells and first trimester placental villous explants were also analyzed separately using a one-way ANOVA to determine if there were differences in ERK1/2 phosphorylation in silenced vs non-silenced controls. CpG islands were analyzed individually by a two-way ANOVA to determine if there was an effect of group, sex or an interaction

Table 2
Primer sequences used for RT-PCR.

Gene	Primer sequences
DUSP9	Forward 5′-GCCATTGAGTTCATTGATGA-3′ Reverse 5′-ACGGTGACAGAACGGCTGAC-3′
SDHA	Forward 5′-TGGGAACAAGAGGGCATCTG-3′ Reverse 5′-CCACCACTGCATCAAATTCATG-3′
YWHAZ	Forward 5′-ACTTTTGGTACATTGTGGCTTCAA-3′ Reverse 5′-CCGCCAGGACAAACCAGTAT-3′
TBP	Sequences are described in Bieche et al. (1999) [17]

between group and sex on the methylation status. All statistical calculations were performed using GraphPadPrism®4.02 software and p-values of <0.05 were considered significant.

3. Results

Table 1 summarizes the patient characteristics. The gestational age of the control group was slightly but significantly greater than the sIUGR and sPE groups (32.3 ± 0.6 weeks vs 29.9 ± 0.5 and 30.1 ± 0.5 weeks for sIUGR and sPE respectively, $p < 0.05$) but this small difference is unlikely to be biologically significant. Maximum systolic blood pressure was significantly higher in the sPE group (174 ± 5 mmHg) compared with the sIUGR group (159 ± 4 mmHg, $p < 0.001$) and the PTC group (125 ± 3 mmHg, $p < 0.001$). Maximum diastolic blood pressures in the sIUGR and sPE groups (99 ± 3 and 107 ± 2 mmHg respectively) were both significantly greater than in the control group (75 ± 3 mmHg, $p < 0.01$). In the sIUGR group, 18/27 patients (67%) met criteria for pre-eclampsia and 16/27 (59%) had birth weights < 5th percentile. All infants in the sPE group were normally grown with a birth weight between the 10th–50th percentile. In the control group 21% were 10th–50th percentile and 79% were >50th percentile. There were no significant differences in gravidity, parity, maternal age or fetal sex across groups.

3.1. DUSP9 expression and immuno-staining declined during development

Representative photomicrographs of DUSP9 immuno-staining across gestation are shown in Fig. 1A. Visual intensity of staining was highest in the first trimester and diminished towards term. In the early 1st trimester, DUSP9 protein was localized in the cytotrophoblast layer of the placental villi, with little to no staining in the villous stroma. In the late 1st trimester, DUSP9 staining was also evident in the syncytiotrophoblast. DUSP9 staining thereafter declined with gestation. These findings were confirmed by measuring *DUSP9* mRNA levels by RT-PCR in placentas from the first, second and third trimester as shown in Fig. 1B. *DUSP9* mRNA expression declined over the course of gestation, reaching low levels at term.

3.2. DUSP9 protein levels are decreased in severely pre-eclamptic placentas

Representative photomicrographs of DUSP9 immuno-staining in PTC, sIUGR and sPE placentas are shown in Fig. 2A. DUSP9 was again localized to the villous trophoblast layer. sPE placental villi displayed abnormal morphology with characteristic syncytial knots as expected [16]. Parallel Western blot analysis of DUSP9 protein expression are shown in Fig. 2B and C. DUSP9 protein expression was significantly lower, in the sPE group compared to the sIUGR group, with most samples in the sPE group having undetectable levels of DUSP9 protein as measured by Western blotting. Fetal sex did not influence DUSP9 expression as shown in Fig. 2C. DUSP9 has been shown to dephosphorylate downstream MAPK proteins, specifically ERK1/2 and p38 [3], however phosphorylated levels of ERK1/2 were low and showed no significant differences in the sPE group compared to the sIUGR group or PTC group (0.7 ± 0.2% vs 0.6 ± 0.2% vs 0.5 ± 0.1%, ns). Phosphorylated and total levels of p38 were mostly undetectable and thus ratios of phosphorylated to total p38 levels were not calculated (data not shown).

3.3. DUSP9 supression in severe pre-eclampsia is not epigenetically mediated

Methylation status of the *DUSP9* promoter was assessed independently in male and female sPE and PTC placentas and is summarized in Supplemental Table 1. Methylation status was low across all loci, ranging from 0 to 19.3% in males and 0.2 to 28.4% in females. Statistical analysis demonstrated an overall significant effect of sex on *DUSP9* methylation, with increased *DUSP9* methylation in females consistent with the location of the gene on the X-chromosome. Male and female sPE placentas demonstrated reduced or similar CpG-rich island methylation status in comparison with PTC placentas indicating that hyper-methylation is not responsible for suppression of *DUSP9* expression in this disease.

3.4. Hypoxia down-regulates DUSP9 gene expression in first trimester floating villous explants

To assess the role of local tissue hypoxia as a regulator of *DUSP9* gene expression, first trimester floating villous explants were cultured in untreated media under either 3% O_2 (mild hypoxia) or 8% O_2 (normoxic) conditions. Explants cultured in this mildly-hypoxic condition showed significant down-regulation of *DUSP9* mRNA expression by ~72% (0.28 ± 0.08 compared to 8% O_2 control expression set to 1.00, $N = 6$, $p < 0.01$).

3.5. ERK1/2 phosphorylation is marginally increased in DUSP9 knock-down BeWo cells and first trimester villous explants

DUSP9 gene knock-down experiments were performed to determine the downstream impact on MAPK signaling pathways in both the BeWo cell culture model and the floating villous explant model. SiRNA-mediated *DUSP9* gene suppression significantly reduced *DUSP9* mRNA expression in both BeWo cells (0.39 ± 0.12 compared to NS control referenced at 1.00, $N = 4$, $p < 0.01$) and in first trimester villous explants (0.38 ± 0.13 compared to NS control referenced at 1.00, $N = 4$, $p < 0.01$). Phosphorylation of ERK1/2 (as a percentage of total ERK1/2 levels as measured by ELISA) was very low in both BeWo and floating villous explants. However, increases of ~2 fold in phosphorylation were observed in response to DUSP9 knock-down in both BeWo cells and explants (1.3 ± 0.4% ($N = 4$) vs 0.7 ± 0.1% ($N = 3$), 1.6 ± 0.5 ($N = 4$) vs 0.8 ± 0.1% ($N = 3$) respectively), though neither trend reached statistical significance. Phosphorylated and total levels of p38 were again mostly undetectable in BeWo cells and explants (data not shown).

4. Discussion

When pregnancies end with pre-term delivery due to severe forms of IUGR and/or sPE they are highly likely to be associated with multiple pathologic observations in the placenta [1]. These elements of placental insufficiency include gross disorders of placental size and shape, accompanied by placental infarction, tissue inflammation and abnormal formation of the peripheral villi. The outer trophoblast layer of the placental villi exhibits a variety of defects including focal necrosis, or apoptosis accompanying wave-like syncytial knot formation [18]. Taken with the observation that these structurally-abnormal areas of villous trophoblast hyper-secrete the VEGF decoy protein sFlt-1 [19] these observations suggest a central role for villous trophoblast in the pathogenesis of placental insufficiency disorders. In this context, DUSP9 has recently been shown to be critically important in the development and function of the exchange region of the mouse placenta. Lack of *DUSP9* expression in trophoblasts resulted in placental insufficiency, IUGR, and early intrauterine lethality [11]. We found that placental *DUSP9* expression is high in early gestation and then decreases as gestation advances, with little to no expression at term. Our most striking observation was an almost complete loss of DUSP9 protein expression in placentas of patients with severe early onset pre-eclampsia without intrauterine growth restriction, in

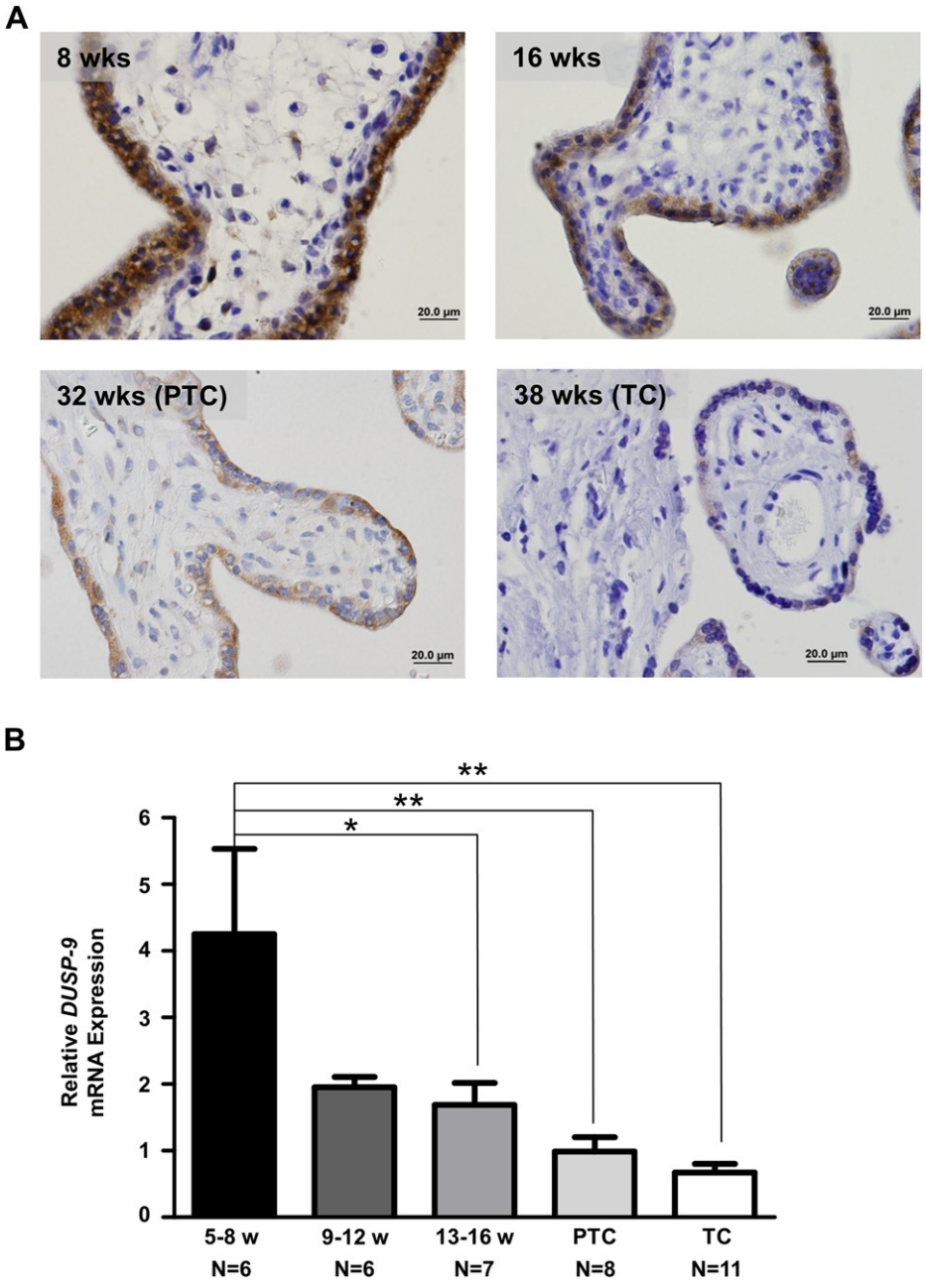

Fig. 1. (A) Representative photomicrographs of DUSP9 immuno-histochemistry in a gestational profile of control subjects at 8, 16, 32 and 38 weeks. Note increased intensity of DUSP9 staining in first trimester placentas vs those at term. (B) Relative *DUSP9* mRNA expression across gestation. PTC – pre-term control, TC – term control. $^{*}p < 0.05$, $^{**}p < 0.001$.

contrast to the *DUSP9* knock-out mouse which exhibited growth restriction as a primary finding. Whether placental DUSP9 deficiency in mice causes maternal pre-eclamptic signs is unknown.

DUSP9 has been shown to play a role in a number of biological processes in addition to its importance in placental development. Over-expression of *DUSP9* in mice resulted in improved glucose tolerance and a reduced risk of developing insulin resistance [20]; interestingly, in humans it has recently been identified as a type 2 diabetes susceptibility locus [21]. Decreased expression of *DUSP9* is a marker for poor prognosis in clear cell renal carcinoma [22] and *DUSP9* is a negative marker in epithelial carcinogenesis, with loss of *DUSP9* (also known as *MKP4*) leading to tumor growth and restored levels causing tumor suppression through the disruption of microtubules and G_2-M associated cell death [23]. Recent work has also demonstrated that *DUSP9* is a critical mediator of bone morphogenic protein (BMP) signaling involved in early neural differentiation [24]. DUSP9 clearly has an emerging role in a variety of biological systems. Despite this wide interest in DUSP9, its expression patterns in the human placenta have not been reported and we thus focused on this as a primary objective in our study.

We found that *DUSP9* mRNA expression was high in villous trophoblast in early gestation and then decreased significantly as gestation advanced. In mice, *DUSP9* (also known as *Pyst3*), is also highly expressed in early gestation in the ectoplacental cone and chorion regions (E7.5 and E8.5) and expression tended to decline by E10.5 [25]. Whether levels continued to decrease to term in mice, as we found in humans, is unknown. Nevertheless, in both humans and mice, *DUSP9* is expressed at high levels in early gestation. It is

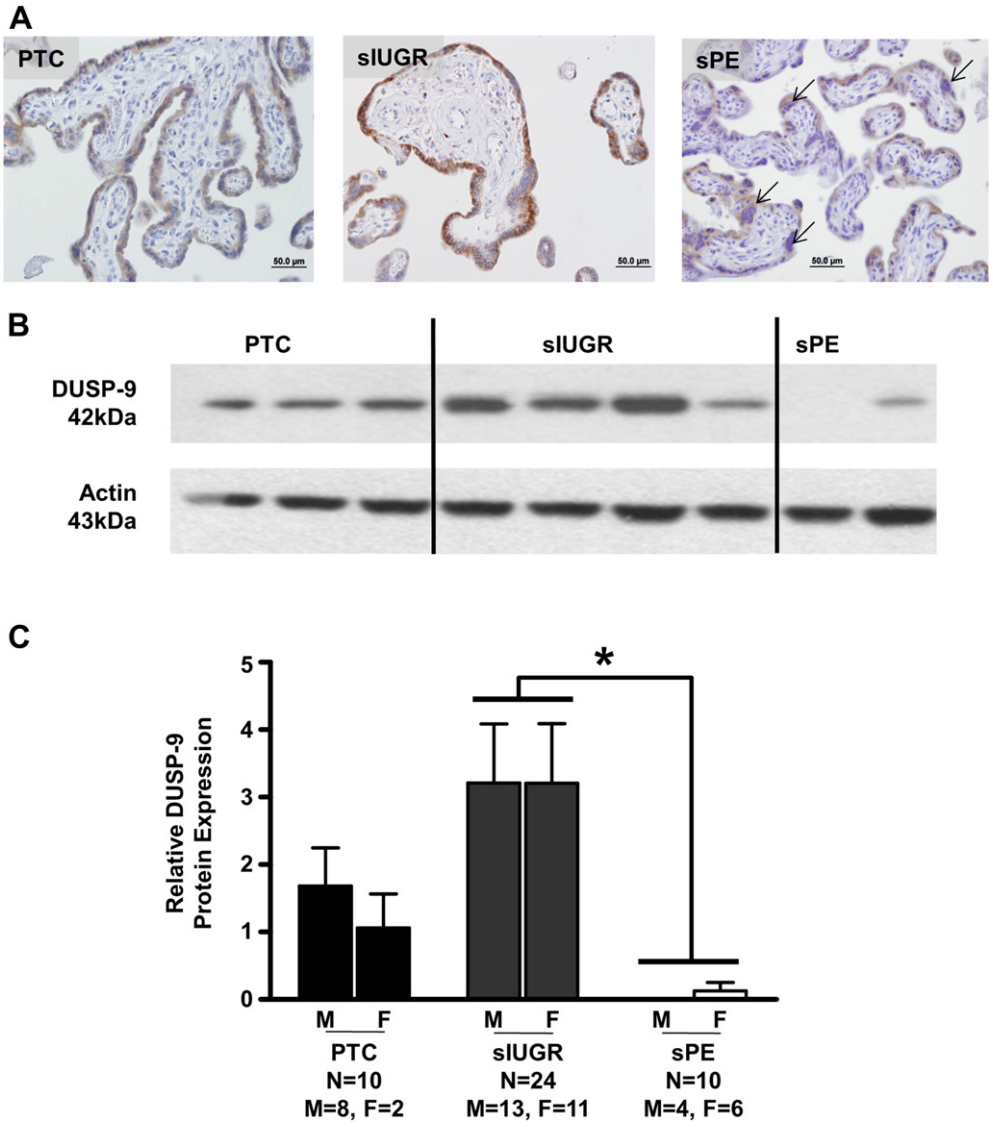

Fig. 2. (A) Representative photomicrographs of DUSP9 immuno-histochemistry in PTC, sIUGR, and sPE groups. Note abnormal morphology of sPE placenta with multiple syncytial knots (noted by arrows) and decreased DUSP9 intensity. (B) Representative Western blot analysis of DUSP9 protein expression in the placentas of PTC, sIUGR and sPE patients. (C) DUSP9 protein in males (M) and females (F) expressed as a relative measure compared to actin and normalized to a control sample run on each gel. Expression was significantly lower in the sPE group compared to the sIUGR group (Combined males and females, sPE 0.07 ± 0.07 vs sIUGR 3.21 ± 0.61, $p < 0.01$).

possible that the initial elevated levels of DUSP9 are important in regulating and controlling the intense trophoblast proliferation in the early first trimester, which then declines as gestation advances. Furthermore, this lower expression in late gestation would be an important mechanism of maintaining a baseline level of trophoblast proliferation to term as has been shown in the human placenta [26].

Contrary to what was found in the *DUSP9* knock-out mouse model, where absent expression was associated with growth restriction, we found sustained expression of DUSP9 in human pregnancies complicated by sIUGR. We did however find significant suppression of DUSP9 protein in the sPE group with normally grown babies compared to the sIUGR group, with no difference between males and females. Our results are consistent with previous reports of differential gene and protein expression in cases of pre-eclampsia with vs without fetal growth restriction [27–29]. The mechanism mediating the significant suppression of DUSP9 in pre-eclampsia is unclear, but proved not to be due to an epigenetic mechanism involving promoter hyper-methylation. It has been well established that hypoxia is a major contributor to the abnormalities seen in the pre-eclamptic placenta [30]. Previous studies culturing placental explants in hypoxic conditions suggest that they may switch to a pre-eclamptic phenotype, which is characterized by abnormal trophoblast development [31] and increased production of sFlt-1 [32]. Hypoxia caused significant down-regulation of *DUSP9* gene expression in placental explants *in vitro*. This suggests that hypoxia or oxidative stress could also be a potential *in vivo* mechanism responsible for the decrease in protein levels that we observed in severe pre-eclampsia. Given the lack of an identified epigenetic regulatory mechanism, an alternative mechanism could be intermittent ischemia–reperfusion injury that is arresting protein translation through endoplasmic reticulum (ER) stress [33,34]. To our knowledge, this is the first report identifying hypoxia as a possible regulator of *DUSP9* RNA expression.

It has been previously described that DUSP9 exerts its actions through the MAPK pathways ERK1/2 and p38 [3]. Total ERK1/2 has been immunolocalized to the villous cytotrophoblasts throughout pregnancy, whereas phosphorylated ERK1/2 was identified only in

the first trimester, suggesting activation of this pathway early in pregnancy [35]. Daoud et al. (2005) investigated the expression of ERK1/2 and p38 in human term trophoblasts. They demonstrated that protein levels declined over the period of culture as trophoblast differentiation occurred, and that suppression of ERK1/2 and p38 lead to impaired trophoblast differentiation [7]. Targeted knock-out studies of *ERK2* and of *p38α* in the mouse lead to embryonic lethality due to abnormal placental development, with both pathways impacting placental angiogenesis [8,9]. These studies provided further rationale for studying the ERK1/2 and p38 pathways in relation to altered DUSP9 expression. In our cell and tissue based *in vitro* models, which are well established for use in manipulating gene expression and determining downstream effects [15,16], *DUSP9* down-regulation by 60% tended to increase the percentage of phosphorylated ERK1/2 by ~2 fold. Possibly this increase, which was expected based on the role that DUSP9 has in regulating ERK1/2 expression [24], would have been significant had greater *DUSP9* down-regulation been achieved. However, it is interesting that near elimination of DUSP9 in the sPE placentas lead to no significant changes in ERK1/2 phosphorylation. This finding is consistent with Christie et al. (2005) who did not show differences in downstream MAPK pathways in knock-out compared to wild-type placentas [11]. The lack of effect *in vivo* may be due to the chronic nature of the underlying placental pathology and likely also chronic DUSP9 deficiency. Additional compensatory mechanisms therefore may act on the MAPK pathways to compensate for loss of placental DUSP9 expression. Alternatively, DUSP9 may act via other as yet unknown downstream signaling pathways.

In conclusion, our research has demonstrated a marked reduction in DUSP9 protein expression in the placentas of women with severe early onset pre-eclampsia without fetal growth restriction. Consistent with the established role for hypoxia in the development of pre-eclampsia, *DUSP9* gene expression was also down-regulated in response to mild hypoxic culture conditions *in vitro*. Thus, hypoxia, or hypoxia-reoxygenation injury, could be an important mediator of DUSP9 repression *in vivo*, leading to the decreased protein levels we observed. Alternatively, decreased DUSP9 protein could reflect local tissue necrosis in the villous trophoblast, from substantial reductions in villous cytotrophoblast numbers, as we [18] and others [36] have shown. Differential placental expression of DUSP9 in pre-eclampsia with vs without growth restriction emphasizes the importance of separately studying these types of placental insufficiency syndromes because of differences in their placental molecular pathologies.

Author's roles

MC designed study, executed experiments, analyzed data, drafted manuscript and critical discussion.

SD executed experiments, analyzed data, and critical discussion.

DB immuno-histochemistry and critical discussion.

SA designed study and critical discussion.

JK designed study, analyzed data, drafted manuscript and critical discussion.

Acknowledgments

The authors would like to thank Dr. Sarah Keating for her assistance in the pathological assessment of the samples and Ms. Khrystyna Levytska for her critical reading of this manuscript. Furthermore, the authors thank the donors and the Research Centre for Women and Infant's Health Biobank Program of the CIHR Group in Development and Fetal Health (CIHR no MGC-13299), the Samuel Lunenfeld Research Institute and the MSH/UHN Department of Obstetrics and Gynecology for the human specimens used in this study. This study was supported by the Canadian Institutes of Health and Research (Grant no. 64302) to JK, Mount Sinai Hospital Department of Obstetrics and Gynecology Research Grant to MC and by the CIHR Interface Training Program, Applying Genomics to Human Health and Molly Towell Perinatal Research Foundation Fellowship to SD.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2012.11.029.

References

[1] Walker MG, Fitzgerald B, Keating S, Ray JG, Windrim R, Kingdom JC. Sex-specific basis of severe placental dysfunction leading to extreme preterm delivery. Placenta 2012;33(7):568–71.
[2] Costa SL, Proctor L, Dodd JM, Toal M, Okun N, Johnson JA, et al. Screening for placental insufficiency in high-risk pregnancies: is earlier better? Placenta 2008;29(12):1034–40.
[3] Patterson KI, Brummer T, O'Brien PM, Daly RJ. Dual-specificity phosphatases: critical regulators with diverse cellular targets. Biochem J 2009;418(3):475–89.
[4] Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 2001;22(2):153–83.
[5] Farooq A, Zhou MM. Structure and regulation of MAPK phosphatases. Cell Signal 2004;16(7):769–79.
[6] Camps M, Nichols A, Arkinstall S. Dual specificity phosphatases: a gene family for control of MAP kinase function. FASEB J 2000;14(1):6–16.
[7] Daoud G, Amyot M, Rassart E, Masse A, Simoneau L, Lafond J. ERK1/2 and p38 regulate trophoblasts differentiation in human term placenta. J Physiol 2005; 566(Pt 2):409–23.
[8] Hatano N, Mori Y, Oh-hora M, Kosugi A, Fujikawa T, Nakai N, et al. Essential role for ERK2 mitogen-activated protein kinase in placental development. Genes Cells 2003;8(11):847–56.
[9] Mudgett JS, Ding J, Guh-Siesel L, Chartrain NA, Yang L, Gopal S, et al. Essential role for p38alpha mitogen-activated protein kinase in placental angiogenesis. Proc Natl Acad Sci U S A 2000;97(19):10454–9.
[10] Muda M, Boschert U, Smith A, Antonsson B, Gillieron C, Chabert C, et al. Molecular cloning and functional characterization of a novel mitogen-activated protein kinase phosphatase, MKP-4. J Biol Chem 1997;272(8):5141–51.
[11] Christie GR, Williams DJ, Macisaac F, Dickinson RJ, Rosewell I, Keyse SM. The dual-specificity protein phosphatase DUSP9/MKP-4 is essential for placental function but is not required for normal embryonic development. Mol Cell Biol 2005;25(18):8323–33.
[12] Langston C, Kaplan C, Macpherson T, Manci E, Peevy K, Clark B, et al. Practice guideline for examination of the placenta: developed by the Placental Pathology Practice Guideline Development Task Force of the College of American Pathologists. Arch Pathol Lab Med 1997;121(5):449–76.
[13] Proctor LK, Toal M, Keating S, Chitayat D, Okun N, Windrim RC, et al. Placental size and the prediction of severe early-onset intrauterine growth restriction in women with low pregnancy-associated plasma protein-A. Ultrasound Obstet Gynecol 2009;34(3):274–82.
[14] ACOG. Diagnosis and management of preeclampsia and eclampsia. Am Coll Obstet Gynecol Practice Bulletin No 33 2002.
[15] Baczyk D, Drewlo S, Proctor L, Dunk C, Lye S, Kingdom J. Glial cell missing-1 transcription factor is required for the differentiation of the human trophoblast. Cell Death Differ 2009;16(5):719–27.
[16] Drewlo S, Czikk M, Baczyk D, Lye S, Kingdom J. Glial cell missing-1 mediates over-expression of tissue inhibitor of metalloproteinase-4 in severe pre-eclamptic placental villi. Hum Reprod 2011;26(5):1025–34.
[17] Bieche I, Laurendeau I, Tozlu S, Olivi M, Vidaud D, Lidereau R, et al. Quantitation of MYC gene expression in sporadic breast tumors with a real-time reverse transcription-PCR assay. Cancer Res 1999;59(12):2759–65.
[18] Fitzgerald B, Levytska K, Kingdom J, Walker M, Baczyk D, Keating S. Villous trophoblast abnormalities in extremely preterm deliveries with elevated second trimester maternal serum hCG or inhibin-A. Placenta 2011;32(4):339–45.
[19] Rajakumar A, Cerdeira AS, Rana S, Zsengeller Z, Edmunds L, Jeyabalan A, et al. Transcriptionally active syncytial aggregates in the maternal circulation may contribute to circulating soluble fms-like tyrosine kinase 1 in preeclampsia. Hypertension 2012;59(2):256–64.
[20] Emanuelli B, Eberle D, Suzuki R, Kahn CR. Overexpression of the dual-specificity phosphatase MKP-4/DUSP-9 protects against stress-induced insulin resistance. Proc Natl Acad Sci U S A 2008;105(9):3545–50.
[21] Voight BF, Scott LJ, Steinthorsdottir V, Morris AP, Dina C, Welch RP, et al. Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis. Nat Genet 2010;42(7):579–89.
[22] Wu S, Wang Y, Sun L, Zhang Z, Jiang Z, Qin Z, et al. Decreased expression of dual-specificity phosphatase 9 is associated with poor prognosis in clear cell renal cell carcinoma. BMC Cancer 2011;11:413.

[23] Liu Y, Lagowski J, Sundholm A, Sundberg A, Kulesz-Martin M. Microtubule disruption and tumor suppression by mitogen-activated protein kinase phosphatase 4. Cancer Res 2007;67(22):10711–9.
[24] Li Z, Fei T, Zhang J, Zhu G, Wang L, Lu D, et al. BMP4 signaling acts via dual-specificity phosphatase 9 to control ERK activity in mouse embryonic stem cells. Cell Stem Cell 2012;10(2):171–82.
[25] Dickinson RJ, Williams DJ, Slack DN, Williamson J, Seternes OM, Keyse SM. Characterization of a murine gene encoding a developmentally regulated cytoplasmic dual-specificity mitogen-activated protein kinase phosphatase. Biochem J 2002;364(Pt 1):145–55.
[26] Mayhew TM, Simpson RA. Quantitative evidence for the spatial dispersal of trophoblast nuclei in human placental villi during gestation. Placenta 1994; 15(8):837–44.
[27] Marzioni D, Todros T, Cardaropoli S, Rolfo A, Lorenzi T, Ciarmela P, et al. Activating protein-1 family of transcription factors in the human placenta complicated by preeclampsia with and without fetal growth restriction. Placenta 2010;31(10):919–27.
[28] Todros T, Bontempo S, Piccoli E, Ietta F, Romagnoli R, Biolcati M, et al. Increased levels of macrophage migration inhibitory factor (MIF) in preeclampsia. Eur J Obstet Gynecol Reprod Biol 2005;123(2):162–6.
[29] Cardaropoli S, Paulesu L, Romagnoli R, Ietta F, Marzioni D, Castellucci M, et al. Macrophage migration inhibitory factor in fetoplacental tissues from preeclamptic pregnancies with or without fetal growth restriction. Clin Dev Immunol 2012;2012:639342.
[30] Caniggia I, Winter J, Lye SJ, Post M. Oxygen and placental development during the first trimester: implications for the pathophysiology of pre-eclampsia. Placenta 2000;21(Suppl. A):S25–30.
[31] Huppertz B, Kingdom J, Caniggia I, Desoye G, Black S, Korr H, et al. Hypoxia favours necrotic versus apoptotic shedding of placental syncytiotrophoblast into the maternal circulation. Placenta 2003;24(2–3):181–90.
[32] Nevo O, Soleymanlou N, Wu Y, Xu J, Kingdom J, Many A, et al. Increased expression of sFlt-1 in in vivo and in vitro models of human placental hypoxia is mediated by HIF-1. Am J Physiol Regul Integr Comp Physiol 2006;291(4):R1085–93.
[33] Burton GJ, Yung HW, Cindrova-Davies T, Charnock-Jones DS. Placental endoplasmic reticulum stress and oxidative stress in the pathophysiology of unexplained intrauterine growth restriction and early onset preeclampsia. Placenta 2009;30(Suppl. A):S43–8.
[34] Yung HW, Calabrese S, Hynx D, Hemmings BA, Cetin I, Charnock-Jones DS, et al. Evidence of placental translation inhibition and endoplasmic reticulum stress in the etiology of human intrauterine growth restriction. Am J Pathol 2008;173(2):451–62.
[35] Kita N, Mitsushita J, Ohira S, Takagi Y, Ashida T, Kanai M, et al. Expression and activation of MAP kinases, ERK1/2, in the human villous trophoblasts. Placenta 2003;24(2–3):164–72.
[36] Longtine MS, Chen B, Odibo AO, Zhong Y, Nelson DM. Villous trophoblast apoptosis is elevated and restricted to cytotrophoblasts in pregnancies complicated by preeclampsia, IUGR, or preeclampsia with IUGR. Placenta 2012;33(5):352–9.