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Hypomethylation of the *LEP* gene in placenta and elevated maternal leptin concentration in early onset pre-eclampsia

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ABSTRACT

In pre-eclampsia, placental leptin is up-regulated and leptin is elevated in maternal plasma. To investigate potential epigenetic regulation of the leptin (*LEP*) gene in normal and complicated pregnancy, DNA methylation was assessed at multiple reported regulatory regions in placentae from control pregnancies ($n = 111$), and those complicated by early onset pre-eclampsia (EOPET; arising <34 weeks; $n = 19$), late onset pre-eclampsia (LOPET; arising $\geqslant 34$ weeks; $n = 18$) and normotensive intrauterine growth restriction (nIUGR; $n = 13$). The *LEP* promoter was hypomethylated in EOPET, but not LOPET or nIUGR placentae, particularly at CpG sites downstream of the transcription start site (−10.1%; $P < 0.0001$). Maternal plasma leptin was elevated in EOPET and LOPET ($P < 0.05$), but not nIUGR, compared with controls. EOPET cases showed a trend towards biallelic *LEP* expression rather than skewed allelic expression observed in control placentae, suggesting that loss of normal monoallelic expression at the *LEP* locus is associated with hypomethylation, leading to increased overall *LEP* expression.

1. Introduction

Pre-eclampsia is a multi-factorial disorder affecting 3–5% of pregnancies in the developed world and can progress to eclampsia, a common cause of pregnancy-related morbidity (Douglas and Redman, 1994; von Dadelszen et al., 2003). The syndrome is characterised by maternal hypertension and proteinuria arising after 20 weeks gestation (Magee et al., 2008) and can be sub-classified into early onset, arising before 34^{+0} weeks gestation, or late-onset, arising at or after 34^{+0} weeks gestation. Early onset pre-eclampsia (EOPET) carries a significantly greater risk of maternal and fetal mortality than late onset pre-eclampsia (LOPET) (MacKay et al., 2001), largely due to severe placental under-perfusion and prematurity (von Dadelszen et al., 2003; Ogge et al., 2011). Placental pathology in EOPET including perturbed growth, maturation, trophoblast shedding, oxygen sensing and vascularisation is reported, whereas LOPET placentae are less distinct from that of normal pregnancy (Moldenhauer et al., 2003; Egbor et al., 2006; Goswami et al., 2006; Rolfo et al., 2010; van der Merwe et al., 2010). The majority of newborns from early-onset pre-eclampsia affected pregnancies are born prematurely and many present with intrauterine growth restriction (IUGR). Such infants do not thrive as well and are at greater risk of developing disease in adult life. Low birth weight is associated with the development of metabolic and cardiovascular disease in adulthood and is an increasing burden on healthcare (Barker et al., 2002).

Maternal blood biomarkers that are associated with the development of pre-eclampsia include factors related to endothelial, hypoxic and inflammatory insults. Leptin, a multi-functional 16 kDa peptide hormone is encoded by the *LEP* gene, which is located on chromosome 7 (7q31), and is abundantly expressed by adipocytes and placenta as well as other tissues including muscle, liver, brain and ovary. Leptin has roles in numerous developmental and homeostatic processes (Zhang et al., 2005), regulation of body weight and metabolism (Halaas et al., 1995; Ioffe et al., 1998; Kershaw and Flier, 2004) and in maintaining reproductive function (Cervero et al., 2005; Henson and Castracane, 2006). Hyperleptinaemia is a physiological condition of pregnancy which occurs independently of body mass index (BMI) (Masuzaki et al., 1997). Leptin is abnormally elevated in maternal plasma of pre-eclampsia pregnancies even before the onset of symptoms (Anim-Nyame et al., 2000; Teppa et al., 2000; Chappell et al., 2002; Kocyigit et al., 2004; Laivuori et al., 2006; Mise et al., 2007; Molvarec et al., 2011).

In the human first trimester placenta, cytotrophoblast and syncytiotrophoblast express leptin and leptin receptor long, short and soluble isotypes (Ashworth et al., 2000). Term trophoblast also

Abbreviations: EOPET, early onset pre-eclampsia; LOPET, late onset pre-eclampsia; nIUGR, normotensive intrauterine growth restriction; TSS, transcription start site; SP1, specificity protein 1; C/EBP, CCAAT-enhancer binding protein; HRE, hypoxia response element; SD, standard deviation.

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express *LEP*, however less abundantly than in early gestation (Henson et al., 1998). Multiple placental roles of leptin have been reported *in vitro* including: the stimulation of matrix metalloproteinases and hCG release in first trimester cytotrophoblast (Castellucci et al., 2000; Islami et al., 2003a,b), induction of cell proliferation and inhibition of apoptosis in trophoblasts (Magarinos et al., 2007; Perez-Perez et al., 2008) and stimulation of pro-inflammatory cytokines and prostaglandin release in placental explants (Cameo et al., 2003; Lappas et al., 2005).

In pre-eclampsia, leptin mRNA and protein expression is increased in the placenta (Reimer et al., 2002; Haugen et al., 2006; Hoegh et al., 2010) whereas maternal adipose expression is unaffected (Haugen et al., 2006). Given that ~98% of placenta-produced leptin is released into the maternal circulation (Linnemann et al., 2000) this is likely the source of elevated serum leptin concentrations found in these women. Processes associated with pre-eclampsia, including placental hypoxia and decreased placental perfusion, increase *LEP* expression in the placenta and raise maternal serum leptin levels (Mise et al., 1998; Grosfeld et al., 2001; Moore et al., 2003). It is proposed that elevated leptin in pre-eclampsia occurs through a compensatory mechanism to increase nutrient delivery to the fetus, by stimulating placental angiogenesis, amino acid uptake and inhibiting apoptosis (Miehle et al., 2012).

Tissue-specific regulation of *LEP* is known to be epigenetically mediated through DNA methylation at a tissue-specific differentially methylated region in the *LEP* promoter (Stoger, 2006). An inverse relationship exists between DNA methylation in this region and gene expression in tissues such as adipocytes (low DNA methylation, high expression) and liver (high DNA methylation, low expression) (Marchi et al., 2011). Methylation of CpG dinucleotides proximal to transcription factor binding motifs upstream of the transcription start site (TSS), are consistent with such regulation. These include CpG sites proximal to the TATA box, specificity protein 1 (SP1) and CCAAT-enhancer binding protein (C/EBP) recognition sequences (Melzner et al., 2002; Marchi et al., 2011) of which binding of the respective transcription factors can regulate hormonal responsiveness, proliferation, differentiation and apoptosis (Diehl, 1998; Menard et al., 2002; Begay et al., 2004; Solomon et al., 2008).

We hypothesised that placental leptin levels are regulated by DNA methylation and predicted that DNA methylation of the *LEP* promoter would be decreased in pre-eclampsia and have an inverse relationship with increased circulating leptin concentrations. In addition, previous data from our laboratory suggested the presence of parent-of origin specific methylation at the *LEP* promoter region in placenta, with dominant expression from the maternal allele (Yuen et al., 2011); however, it is unknown whether allele-specific expression differs in pre-eclampsia. Therefore we aimed to (1) quantify DNA methylation at individual CpG sites proximal to the TSS and transcription factor binding sites including a reported hypoxia response element (HRE) located 120 bp upstream of the *LEP* TSS (Fig. 1) in control, EOPET, LOPET and nIUGR placentae, (2) correlate these findings with maternal plasma leptin concentration, and (3) determine the allelic bias in, and parent of origin of, placental *LEP* expression in control and EOPET placentae.

2. Materials and methods

2.1. Study samples

This study was undertaken with ethics approval from the University of British Columbia and the Children's and Women's Health Centre of British Columbia. Study participants were recruited at the BC Women and Children's Hospital, Vancouver, during their second trimester of pregnancy with full written consent. Diagnosis of pre-eclampsia was based on the Society of Obstetricians and Gynaecologists of Canada guidelines (Magee et al., 2008) which include the following criteria: hypertension (diastolic BP $\geqslant$ 90 mmHg measured twice, at least 4 h apart) after 20 weeks gestation, in combination with proteinuria ($\geqslant$0.3 g/d) or other indicated adverse condition (e.g. non-hypertensive and non-proteinuric hemolysis, elevated liver enzymes, low platelet count (HELLP) syndrome; fetal intrauterine growth restriction; or absent or reversed end-diastolic flow in the umbilical artery by Doppler velocimetry). Women were sub-classified into early or late onset by the manifestation of symptoms before or after 34 weeks gestation respectively (von Dadelszen et al., 2003). IUGR was defined as either: birth weight below the 3rd percentile for gender and gestational age using Canadian population parameters (Kramer et al., 2001), or birth weight below the 10th percentile in combination with (a) persistent uterine artery notching at 22^{+0} to 24^{+6} weeks, (b) absent or reversed end diastolic velocity on umbilical artery Doppler, and/or (c) oligohydramnios (amniotic fluid index <50 mm). Normotensive IUGR (nIUGR) was defined as IUGR occurring in the absence of maternal hypertension. Control women were selected based on the absence of the above criteria or any clear placental pathology. However, to enable collection of cases of earlier gestational ages, pregnancies of normal birth weight that were delivered early due to unrelated pregnancy complications such as premature labour, premature rupture of membranes or cervical incompetence were included. Exclusion criteria included maternal ages beyond 18–42 years, the presence of gestational diabetes, isolated pregnancy induced hypertension, cases of intrauterine death and fetal genetic anomalies. Some samples used in this study overlap those of previous placental DNA methylation studies published from our laboratory (Yuen et al., 2009, 2010; Bourque et al., 2010).

Placental DNA methylation was assessed in a cohort of control pregnancies (n = 111) with a gestational age at birth range of 28–41 weeks (mean 35.09 ± 4.21). *LEP* methylation was compared between controls and women fulfilling the diagnostic criteria for EOPET (n = 19), LOPET (n = 18) or nIUGR (n = 13; Table 1). IUGR was additionally present in 14/19 cases of EOPET and 5/18 cases of LOPET. Birth weight (measured in standard deviation (SD) points relative to normal birth weight appropriate for gestational age (Usher and McLean, 1969)) was significantly decreased in all case cohorts compared to controls. Gestational age was not matched between control and EOPET or LOPET cohorts, and was evaluated as a confounder in all analyses. Maternal age and infant gender were similarly distributed in all groups.

In a subset of women, plasma was collected during the third trimester and used to determine leptin hormone concentrations in control, EOPET, LOPET and nIUGR cases (Table 1). Clinical characteristics in the subset for which plasma was obtained over-lapped well with the larger cohort. There were no significant differences between the overall cohort and the plasma subset within control, EOPET, LOPET or nIUGR groups for birth weight (SD), maternal age or infant gender (data not shown). For gestational age at term, control women in the plasma subset gave birth later than those in the overall cohort (38.75 ± 1.06 vs. 35.09 ± 4.21; P < 0.01); however, there was no difference within the case-groups.

2.2. DNA methylation analyses

At placental collection, chorionic villous samples (~1 cm^3) were dissected from the fetal side from three representative anatomical sites within the placental disc: near umbilical cord, close to outer perimeter and mid-way between each site. DNA was extracted from each chorionic villous sample as previously described (Avila et al., 2010) and stored long term at −20 °C. Equal amounts of DNA from each of these three samples were subsequently pooled