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Placental PHLDA2 expression is increased in cases of fetal growth restriction following reduced fetal movements

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Abstract

Background: Maternal perception of reduced fetal movements (RFM) is associated with increased risk of fetal growth restriction (FGR) and stillbirth, mediated by placental insufficiency. The maternally expressed imprinted gene PHLDA2 controls fetal growth, placental development and placental lactogen production in a mouse model. A number of studies have also demonstrated abnormally elevated placental PHLDA2 expression in human growth restricted pregnancies. This study examined whether PHLDA2 was aberrantly expressed in placentas of RFM pregnancies resulting in delivery of an FGR infant and explored a possible relationship between PHLDA2 expression and placental lactogen release from the human placenta.

Methods: Villous trophoblast samples were obtained from a cohort of women reporting RFM (N = 109) and PHLDA2 gene expression analysed. hPL levels were assayed in the maternal serum (N = 74).

Results: Placental PHLDA2 expression was significantly 2.3 fold higher in RFM pregnancies resulting in delivery of an infant with FGR (p < 0.01), with highest levels of PHLDA2 expression in the most severe cases. Placental PHLDA2 expression was associated with maternal serum hPL levels (r = −0.30, p = 0.008, n = 74) although this failed to reach statistical significance in multiple linear regression analysis controlling for birth weight (p = 0.07).

Conclusions: These results further highlight a role for placental PHLDA2 in poor perinatal outcomes, specifically FGR associated with RFM. Furthermore, this study suggests a potential relationship between placental PHLDA2 expression and hPL production by the placenta, an association that requires further investigation in a larger cohort.

Keywords: Reduced fetal movements, Stillbirth, Fetal growth restriction, Placenta, PHLDA2

Background

Maternal perception of reduced fetal movements (RFM) is associated with an increased risk of poor perinatal outcomes such as fetal growth restriction (FGR), preterm birth, fetal distress, and stillbirth [1–7]. Placental insufficiency, the reduced ability of the placenta to supply nutrients and oxygen to the fetus, could explain the co-occurrence of RFM and FGR [8]. Placental insufficiency is thought to primarily result in asymmetric FGR with prolonged exposure resulting in RFM (as a mechanism for conserving energy for vital functions) and, if this remains undetected, stillbirth [8]. Indeed, altered placent al structure and function has been reported in RFM pregnancies [9–12]. For example, RFM placentas are smaller, lighter and exhibit a specific reduction in the syncytiotrophoblast layer of the placenta [9], which synthesises hormones such as human placental lactogen (hPL) for release into the maternal circulation [13].

Imprinted genes are monoallelically expressed with expression depending on the parent of origin [14]. These genes have well-established roles in controlling fetal growth and placental development, and are regulated by epigenetic marks that may respond to environmental stimuli [15–17]. A subset of imprinted genes also converge on the endocrine lineages of the mouse placenta to regulate placental hormone production [18]. The maternally expressed imprinted gene PLECKSTRIN HOMOLOGY-LIKE DOMAIN FAMILY A MEMBER 2 (PHLDA2) exemplifies all of these functions. Importantly, abnormally elevated placental PHLDA2

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expression has been associated with FGR and/or low birth weight in a number of studies (reviewed in [16]). Modelling similar overexpression of Phlda2 in a mouse model results in FGR [19, 20], inferring that the association observed in human pregnancies is causal. Moreover, the growth restriction which occurs in response to Phlda2 overexpression is late onset and asymmetric, followed by rapid postnatal catch up growth [20], which is typical of human pregnancies complicated by placental insufficiency. The Phlda2 overexpression mouse model also exhibits stunted placental growth with a specific reduction in a key endocrine lineage and impaired placental lactogen production [19, 21], consistent with the altered endocrine function observed in human RFM placentas [9, 12].

In this study we analysed PHLDA2 expression in a cohort of women reporting RFM (N = 109). We hypothesised that placental PHLDA2 expression is specifically increased in RFM pregnancies complicated by FGR (N = 20). We also analysed maternal serum hPL levels (N = 74) in the RFM cohort to explore the potential link between PHLDA2 expression and placental lactogen in the human placenta.

Methods
Participant recruitment
Study participants included women delivering within one week of presentation with maternal perception of RFM (after 28 weeks gestation) [2, 9]. Written informed consent was obtained from the participants and the study was approved by Oldham and Greater Manchester North Research Ethics Committees (REC no. 08/1011/83 and 11/NW/0664). Fetal growth restriction was defined as customised birth weight centile <10th [2]. Participant demographics are shown in Table 1.

Sample collection
Villous trophoblast samples were obtained within 30 min of delivery [9]. As intraplacental variation in PHLDA2 expression has previously been demonstrated [22], samples were consistently taken midway between the cord insertion and distal edge. Maternal venous serum samples were obtained on admission (N = 74) and hPL levels measured by ELISA (Immunodiagnostic systems, Boldon, UK) as previously described [2].

Gene expression analysis
Total RNA was extracted from the placental tissue samples using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Dorset, UK). 5 μg of RNA was reverse transcribed using M-MuLV reverse transcriptase (Promega, Southampton, UK) with random hexamers, according to manufacturer’s instructions. Quantitative RT-PCR was performed using Chromo 4 Four Colour

Table 1 Participant demographics of RFM participants (N = 109)

| Birth outcomes                          | Mean (SD)/Range or Number (%) | Mean (SD)/Range or Number (%) |
|----------------------------------------|-------------------------------|-------------------------------|
| Fetal sex                              |                               |                               |
| Male                                   | 56 (51 %)                     | 73 (67 %)                     |
| Female                                 | 53 (49 %)                     | 10 (9 %)                      |
| Gestational age (days)                 | 278 (13)/211 – 295            | 21 (19 %)                     |
| Birth weight (g)                       | 3306 (599)/850 - 4680         | 5 (5 %)                       |
| Custom birth weight centile            | 40 (29)/0 - 100               | 1 (1.22)/0 – 7                |
| Placental Weight (g)                   | 587 (127)/353 – 854           |                               |
| Apgar Scores (1 min)                   | 9 (1.66)/0 - 10               | 17 (16 %)                     |
| Apgar Scores (5 min)                   | 10 (1.39)/0 – 10              | 92 (84 %)                     |
| Mode of Delivery                       |                               |                               |
| SVD                                    | 61 (56 %)                     | None                          |
| ELCS                                   | 10 (9 %)                      | 1-5 units/week                |
| EMCS                                   | 14 (13 %)                     | 2 (2 %)                       |
| Instrumental                           | 24 (22 %)                     | Obstetric complications       |
| Maternal characteristics               |                               | None                          |
| Age (years)                            | 29 (5.81)/17 – 46             | Preeclampsia/PIH/Proteinuria  |
| Maternal BMI                           | 25.78 (5.26)/17 – 46          | Oligohydramnios  |
|                                        |                               | Suspected growth restriction   |
|                                        |                               | Other                          |

Mean (SD)/Range or Number (%)

RFM reduced fetal movements, BMI body mass index, PIH pregnancy induced hypertension
Real Time Detector (MJ Research) in a 20 μl reaction containing 5 μl of cDNA (diluted 1 in 50), 1X Buffer 2 mM MgCl₂, 2 mM dNTPs, 0.65 Units Taq (Fermentas (Thermo), Loughborough, UK), 1 μM of each primer (Sigma-Aldrich, Dorset, UK) and 0.12X Sybr Green (Invitrogen, Paisley, UK). All samples were run in triplicate and duplicate plates were performed. Conditions for amplification were: 1) 15 min at 94 °C, 2) 30 s at 94 °C, 3) 30 s at 60 °C, 4) 30 s at 72 °C and 5) 30 s 75 °C, with steps 2–5 repeated for a total of 40 cycles. Melt Curve was performed from 70 to 94 °C, reading every 0.5 °C and holding for 2 s. Primer sequences were as follows: YWHAZ forward: TTCTTGATCCCCAATGCTTC & reverse: AGTTAAGGGCCAGACCCAGT, PHLDA2 forward: GAGCCGACGGGCAAGTA & reverse: CAGCG GAAGTGCATCTCCCT [23] and L19 forward: CCAACT TCCGTGCAGCAGATC & reverse: CAAGGTGGTTTT CCGGCATC [24].

Statistical analysis
Gene expression data is presented as the ΔCT (target gene expression relative to the housekeeping gene YWHAZ) and as the fold change in expression, calculated using the 2⁻ΔΔCT method [25]. The geometric mean of two housekeeping genes, YWHAZ and L19, was used to confirm results obtained using the single housekeeping gene, YWHAZ, for a subset of samples. Parametric statistical tests were used to analyse normally distributed data. The effect of potential confounders (infant birth weight, gender and gestational age) was examined using multiple linear regression analysis. To ease interpretation, ΔCT values have been inverted [x(−1)] such that lower values represent decreasing gene expression.

Results
Placental PHLDA2 expression was significantly 2.3 fold higher in RFM pregnancies resulting in delivery of a growth restricted compared with a normal birth weight infant (Fig. 1a). Results remained statistically significant when comparing normalisation to a single housekeeping gene YWHAZ with the geometric mean of YWHAZ and L19 expression in a subset of samples (Additional file 1: Figure S1).

When participants were further classified according to severity of growth restriction, PHLDA2 expression was significantly 1.7 fold higher in cases with custom birth weight centile <10th (p = 0.01, n = 12. v. 82) and 3.2 fold higher in cases with custom birth weight centile <3rd (p < 0.001, n = 8 v. 82). Placental PHLDA2 expression was also significantly inversely associated with birth weight (r = −0.24, p = 0.01, n = 109), custom
birth weight centiles ($r = -0.25$, $p = 0.01$, $n = 109$) and, in this study, also placental weight ($r = -0.39$, $p = 0.03$, $n = 31$).

We subsequently analysed placental PHLDA2 expression in relation to preterm delivery and NICU admission at birth, which are other common poor perinatal outcomes associated with RFM. There was no significant correlation between placental PHLDA2 expression and gestational age ($r = -1.41$, $p = 0.14$, $n = 109$) and was no significant difference in expression between preterm and term deliveries (Fig. 1b). Placental PHLDA2 expression was not significantly altered in infants admitted to NICU at delivery (for perinatal asphyxia) (Fig. 1c) and there was no significant correlation between placental PHLDA2 and other measures of infant wellbeing at delivery including Apgar scores at 1 min ($r = 0.01$, $p = 0.93$, $n = 106$) and 5 min ($r = -0.11$, $p = 0.27$, $n = 105$) or with arterial cord blood pH ($r = -0.7$, $p = 0.52$, $n = 76$).

Imprinted genes have been demonstrated to regulate the endocrine lineage of the mouse placenta, in particular expression of placental lactogens [18]. In this study there was a significant inverse association between placental PHLDA2 expression and maternal serum hPL levels (Fig. 1d) suggesting that PHLDa2 may regulate the production of placental hormones in human pregnancies. In further multiple linear regression analysis controlling for infant birth weight, offspring gender and gestational age ($F(4,69) = 5.34$, $p = 0.001$, $R^2 = 0.24$), the association between placental PHLDA2 expression and serum hPL levels failed to reach statistical significance ($p = 0.07$), with only infant birth weight significantly associated with maternal serum hPL levels ($p = 0.01$). This is perhaps not surprising since, in the mouse model, elevated Phlda2 drives both growth restriction and reduced placental lactogen expression. hPL plays an important role in maternal glucose management during pregnancy and in the control of fetal growth [28]. This relationship between placental PHLDA2 expression and placental hPL production would therefore be of considerable interest in understanding the mechanisms driving poor growth in utero.

**Discussion**

A number of studies have demonstrated abnormally elevated placental PHLDA2 in pregnancies complicated by FGR (reviewed in [16]). The Phlda2 overexpression mouse model exhibits FGR and impaired placental development supporting a causal role for elevated PHLDA2 in human FGR [19, 20]. Uniquely, this study examined a group of women reporting RFM, a group who are at high risk of poor perinatal outcomes. Within this group, there was a significant association between increased placental PHLDA2 expression and FGR. Moreover, the highest levels of PHLDA2 expression were present in the most severely growth restricted cases which also had the most severe placental phenotype [12]. Placental weight was also significantly inversely associated with PHLDA2 expression, which has not previously been reported. This is of clinical interest as RFM is thought to represent a fetal adaptation to prolonged placental insufficiency which, if undetected, may result in stillbirth [8]. While all participants in the current study delivered live born infants, increased expression of PHLDA2 has previously been reported in cases of spontaneous miscarriage or fetal death [26] and more specifically, in cases of fetal death attributed to FGR [27]. These results further highlight a role for placental PHLDA2 in poor perinatal outcomes.

Our second key finding in this study was the significant association between elevated placental PHLDA2 expression and decreased maternal hPL serum levels. After controlling for birth weight, this association failed to reach statistical significance ($p = 0.07$) but this can be explained if, as in the mouse [19, 21], elevated PHLDA2 drives both growth restriction and reduced placental lactogen expression. hPL plays an important role in maternal glucose management during pregnancy and in the control of fetal growth [28]. This relationship between placental PHLDA2 expression and placental hPL production would therefore be of considerable interest in understanding the mechanisms driving poor growth in utero.

**Conclusion**

This study demonstrated significantly elevated PHLDA2 expression in placentas of RFM pregnancies resulting in delivery of a growth restricted infant and identified an association between placental PHLDA2 expression and serum hPL levels. It has previously been suggested that PHLDA2 expression in the placenta could be used to post-natally identify types of fetal growth restriction and thus inform subsequent infant care [16]. Identifying hormonal biomarkers of abnormal placental PHLDA2 expression during pregnancy could be further used as a prenatal diagnostic tool to identify RFM pregnancies at risk of poor perinatal outcomes. This is of particular importance in the context of a recent UK report highlighting that more appropriate, better informed management of RFM could have prevented one third of the stillbirth cases examined [29].

**Availability of data and materials**

Cohort data and details on availability of material [2, 9].

**Additional file**

Additional file 1: Figure 1. Placental PHLDA2 expression in RFM pregnancies, normalised to the geometric mean of YWHAZ and L19 expression. PHLDA2 expression was significantly increased in placentas from pregnancies complicated by fetal growth restriction when normalized to YWHAZ expression (A) or to the geometric mean of YWHAZ and L19 expression (B) in a subset of samples from the full cohort (N = 37). Expression of the housekeeping genes YWHAZ and L19 was significantly correlated (r = 0.94, p < 0.001, n = 37). There was no significant difference in expression between RFM placentas from normal birth weight pregnancies and pregnancies resulting in fetal growth restriction ($p = 0.37$ and $p = 0.45$ respectively, n = 3 (TIF 2931 kb))
Abbreviations

FGR: fetal growth restriction; hPL: human placental lactogen; PHLDA2: Pleckstrin homology-like domain family A member 2; RFM: reduced fetal movements.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

ABJ, AEPH and RMJ conceived the project. AEPH was involved in the collection of samples and clinical data for the cohort. ABJ and SJT carried out the laboratory and statistical analysis. ABJ, SJT, AEPH and RMJ wrote the manuscript and all authors have read and approved the final version of the manuscript.

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