RESEARCH ARTICLE

Isolating the role of elevated Phlda2 in asymmetric late fetal growth restriction in mice

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ABSTRACT

Pleckstrin homology-like domain family A member 2 (PHLDA2) is a maternally expressed imprinted gene whose elevated expression has been linked to fetal growth restriction in a number of human studies. In mice, Phlda2 negatively regulates placental growth and limits the accumulation of placental glycogen. We previously reported that a three-copy transgene spanning the Phlda2 locus drove a fetal growth restriction phenotype late in gestation, suggesting a causative role for PHLDA2 in human growth restriction. However, in this mouse model, Phlda2 was overexpressed by fourfold, alongside overexpression of a second imprinted gene, Scl22a18. Here, we genetically isolate the role of Phlda2 in driving late fetal growth restriction in mice. We furthermore show that this Phlda2-driven growth restriction is asymmetrical, with a relative sparing of the brain, followed by rapid catch-up growth after birth, classic features of placental insufficiency. Strikingly, fetal growth restriction showed strain-specific differences, being apparent on the 129S2/SvHsd (129) genetic background and absent on the C57BL6 (BL6) background. A key difference between these two strains is the placenta. Specifically, BL6 placentaee possess a more extensive endocrine compartment and substantially greater stores of placental glycogen. Taken together, these data support a direct role for elevated Phlda2 in limiting fetal growth but also suggest that growth restriction only manifests when there is limited placental reserve. These findings should be taken into account in interpreting the results from human studies.

KEY WORDS: Phlda2, Fetal growth restriction, Asymmetric

INTRODUCTION

Low birth weight (LBW) is one of the most problematic conditions affecting human populations. It is a very common complication of pregnancy, affecting up to 19% of all births in the developing world and between 5 and 7% of births in developed countries (Valero de Bernabe et al., 2004). Either as a consequence of poor growth in utero, preterm birth or a genetic abnormality, some babies are born too small and are consequently more vulnerable to complications at the time of birth, with increased risk of mortality (Witt et al., 2012). Importantly, growth restriction was negatively regulated by using transgenic mice carrying a single-copy transgene. We report that as little as twofold increased expression of Phlda2 was sufficient to reduce birth weight by 10%. Importantly, growth restriction was asymmetric, with relative sparing of the brain, followed by rapid catch-up growth after birth, classic features of placental insufficiency.

RESULTS

In our previous paper, gene expression analysis of a newly generated BAC transgenic line on mixed genetic background suggested the presence of at least two copies of the BAC transgene (Tunster et al., 2010). However, when we bred this line fully into the 129S2/SvHsd (129) genetic background (>eight generations) and re-examined...
gene expression, the data was more consistent with the presence of a single copy (Fig. 1A). When we examined fetal weight in the context of twofold gene expression, growth restriction of Phlda2 single-copy BAC transgene fetuses was apparent at embryonic day 18.5 (E18.5; 1184.4±8.7 mg versus 1073.8±18.5 mg, P=5.33×10−7) but not at E14.5 or E16.5 (Fig. 1B), similar to the growth profile we reported in response to fourfold expression (Tunster et al., 2010). Transgenic placentae were 10-20% lighter than normal-birth-weight babies. Although the causes of low birth weight are numerous and include both genetic and environmental factors, elevated placental expression of the imprinted gene PHLDA2 has been reported in a number of studies on low birth weight. However, because correlation does not always equal causation, determination of the relevance of elevated PHLDA2 expression using an animal model is of crucial importance.

Results

In this study, the authors generated a mouse model in which Phlda2 expression was elevated twofold, a similar increase in expression to that reported in human studies. The authors observed late fetal growth restriction in the transgenic mice, with the pups being born with a low birth weight. Crucially, growth restriction was asymmetric, with relative sparing of the brain, followed by rapid catch-up growth after birth, which are classic features of placental insufficiency. Moreover, growth restriction showed strain-specific differences and the effects of Phlda2 overexpression were only apparent in mouse strains in which the placenta was working at maximum capacity.

Implications and future directions

These findings support a causal role for elevated PHLDA2 in the aetiology of human low birth weight. Moreover, the observation of both asymmetrical growth restriction and rapid catch-up growth in this new mouse model of Phlda2 overexpression suggests that human infants born with a low birth weight owing to this specific alteration might be at higher risk of later-life health complications than infants born with a low birth weight for other reasons. This possibility can be investigated by re-examining data from human cohorts and through further work on the animal model. Finally, these findings suggest that elevated PHLDA2 expression might be most harmful in scenarios in which the placenta is working at maximum capacity, such as in pregnancies subject to prenatal adversity.

Fig. 1. Characterisation of the single-copy line BACx1 on a 129 strain background. (A) QPCR analysis of Phlda2 and Slc22a18 expression in E14.5 Phlda2+/+BACx1(129) placentae. (B) Fetal wet weights on the 129 genetic background at E14.5, E16.5 and E18.5. Phlda2+/+BACx1(129) (2×) fetuses (n=36) were significantly lighter at E18.5, weighing 90.7% of the weight of their non-transgenic counterparts (P=5.33×10−7). (C) Placental wet weights on the 129 genetic background. Phlda2+/+BACx1(129) (2×) placentae were significantly lighter that non-transgenic placentae at each time point. Numerical data is given in supplementary material Table S1. NS, not significant (P>0.05), *P<0.05, ***P<0.005.

To formally test whether the line carried a single copy of the transgene, we crossed males carrying the BAC with females carrying a paternally inherited targeted allele of Phlda2, also bred for ≥8 generations into the 129 genetic background. This cross generated four genotypes: Phlda2+/−(129) (one active copy of Phlda2 and Slc22a18), Phlda2+/−BACx1(129) (two active copies Phlda2 and Slc22a18), Phlda2−/−(129) (maternal-knockout Phlda2, equivalent to loss of function) and Phlda2+/−BACx1(129) (double transgenic; one active copy of Phlda2 and two active copies of Slc22a18). At E14.5, Phlda2 was expressed at wild-type levels in double-transgenic fetuses, whereas Slc22a18 remained elevated, confirming that the line carried a single extra copy of Phlda2 (Fig. 2A). Importantly, these double-transgenic fetuses exposed to a single dose of Phlda2 and a double dose of Slc22a18 were not significantly different in weight to controls at E18.5 (1123.3±13.8 mg versus 1144.3±23.4 mg, P=0.415; Fig. 2B), whereas fetuses carrying the single-copy BAC transgene, exposed to a double dose of both genes (Phlda2+/−BACx1(129)), weighed less than controls (1123.3±13.8 mg versus 1038.7±23.4 mg, P=0.00180; Fig. 2B). This finding genetically assigned growth restriction to the twofold-elevated Phlda2 expression. As observed with a different single-copy BAC transgene (Tunster et al., 2010), normalising the dose of Phlda2 also returned placental weights to normal (Fig. 2C).

Loss of function of Phlda2 has previously been characterised on the C57BL/6 genetic background (Frank et al., 2002; Salas et al., 2004). We similarly observed substantial placental overgrowth on the 129 background. Phlda2+/−(129) placentae weighed 33% more...
Fig. 2. Isolating a causative role for elevated Phlda2 in inducing late fetal growth restriction (129 strain background). (A) QPCR analysis of Phlda2 and Slc22a18 expression in E14.5 Phlda2<sup>+/−</sup> (wild type; non-transgenic) versus Phlda2<sup>+/−</sup>BACx1<sup>(129)</sup> (1×) (double transgenic) from litters containing all four genotypes. Phlda2 expression was normal in double-transgenic placenta. (B) E18.5 fetal wet weights for Phlda2<sup>+/−</sup>BACx1<sup>(129)</sup> (WT), Phlda2<sup>+/−</sup>BACx1<sup>(129)</sup> (BACx1; 2×), Phlda2<sup>+/−</sup>KO<sup>(129)</sup> (KO; 0×) and Phlda2<sup>+/−</sup>BACx1<sup>(129)</sup> (KO & BACx1; 1×) from litters containing all four genotypes on the 129 genetic background. Fetal growth restriction was attenuated in double-transgenic fetuses. Phlda2<sup>+/−</sup> fetuses were similar in weight to controls. (C) E18.5 placental wet weights for the litters used to generate data in Fig. 2B. Placental growth restriction was apparent only when Phlda2 expression was elevated (Phlda2<sup>+/−</sup>BACx1<sup>(129)</sup>). Phlda2<sup>+/−</sup> placenta were 33% heavier than controls. Numerical data is given in supplementary material Table S2. NS, not significant (P>0.05), **P<0.01, ***P<0.005.

than control placenta at E18.5 (94.9±4.1 mg versus 71.5±0.9 mg; P=8.41×10<sup>−8</sup>; Fig. 2C). As in the study of Phlda2 loss of function on the BL6 background, Phlda2<sup>+/−</sup>BACx1 fetuses, which lacked the maternal Phlda2 allele, were not advantaged by the possession of a larger placenta and weighed the same as their non-transgenic counterparts at E18.5 (1116.1±19.1 mg versus 1123.3±13.8; P=0.753; Fig. 2B). These data formally assigned the late fetal growth restriction phenotype to twofold-elevated expression of Phlda2 and confirmed that loss of function of Phlda2 does not induce fetal overgrowth.

Fetal growth restriction due to placental insufficiency is often asymmetric, with a relative reduction in fetal kidney, liver, pancreas and lung size but a sparing of the brain. When organ weights of the four genotypes were compared at E18.5, the brain weights of the Phlda2<sup>+/−</sup>BACx1 fetuses were 10% heavier relative to body weight as compared to the non-transgenic fetuses, suggestive of brain sparing (Fig. 3A). The lungs and heart were proportionately growth restricted, whereas the liver and kidney, sites of embryonic Phlda2 expression (Qian et al., 1997), were growth restricted as a proportion of body weight (Fig. 3A). Brain sparing was similarly apparent in newborn mice carrying three copies of the transgene (Phlda2<sup>+/−</sup>BACx3<sup>(2×)</sup>) examined on the 129 genetic background (Fig. 3B). Normalising the dose of Phlda2 restored symmetry to the fetus (Fig. 3A). These data genetically demonstrated that twofold elevated Phlda2 restricts fetal growth asymmetrically. Moreover, loss of function of Phlda2 had no significant consequence for these organ weights, excluding a reciprocal function for Phlda2 in regulating organ weights.

Catch-up growth is a key feature of extrinsically driven fetal growth restriction (Saenger et al., 2007). When postnatal weights for line Phlda2<sup>+/−</sup>BACx3<sup>(2×)</sup> were examined from birth (P0) until 4 weeks of age (P28), pups were born weighing 11% less than their non-transgenic counterparts (1.25±0.014 g versus 1.41±0.009 g; P=3.29×10<sup>−7</sup>). Within 7 days, there was no significant difference in the weights between transgenic and non-transgenic pups (P=0.147; Fig. 3C).

Placental weight and fetal:placental (F:P) ratios are widely used as a parameter of placental functional capacity (Fowden et al., 2009). The F:P ratio for mice of the 129 genetic background at E18.5 is relatively high, at 16.0±0.23, whereas that of C57BL/6 (BL6) mice is lower, at 12.3±0.18 (Tunster et al., 2012). This suggests that BL6 placenta might have a greater reserve capacity than 129 placenta. To investigate whether Phlda2 similarly restrained fetal growth in the context of a more favourable F:P ratio, we bred Phlda2<sup>+/−</sup>BACx1<sup>(129)</sup> and Phlda2<sup>+/−</sup>BACx3<sup>(3 copy line)</sup> mice onto the BL6 genetic background for >eight generations. We observed no difference in fetal weight at E14.5, E16.5 or E18.5 (Fig. 4A) despite transgenic placenta weighting 10-15% less than non-transgenic placenta at each time point (Fig. 4B). When we examined F:P ratios, there was a significant difference from normal at every time point due to the reduction in weight of the placenta but not the fetus on the BL6 background (Fig. 4C). F:P ratios were also significantly different from normal at E14.5 and E16.5 on the 129 background. At E18.5, there was no significant difference in F:P ratio because, at this later time point, both placental and fetal weights were reduced. This suggests that 129 placenta function at maximum capacity late in gestation and consequently cannot compensate for the reduction in capacity induced by elevated Phlda2, resulting in fetal growth restriction.

A reduced junctional zone and loss of placental glycogen have been linked to fetal growth restriction in several studies (Hitz et al., 2005; Oh-McGinnis et al., 2011; Withington et al., 2006; Zheng-Fischhöfer et al., 2007). Furthermore, placental glycogen has been suggested as an important source of easily utilisable energy to support late embryonic growth (Coan et al., 2006). In addition to the difference in F:P ratios, BL6 placenta have a disproportionately larger junctional zone and significantly greater stores of placental glycogen than 129 placenta (Tunster et al., 2012). In situ hybridisation with trophoblast specific protein alpha (Tphpa), a marker for the junctional zone (Leschin et al., 1988), revealed a reduction of the junctional zone at E14.5 on both the 129 and the BL6 genetic backgrounds for line Phlda2<sup>+/−</sup>BACx1 (Fig. 5A). To establish to what extent elevated Phlda2 compromised glycogen accumulation on the two genetic backgrounds, a biochemical determination of glycogen was performed at E14.5, E16.5 and
E18.5. Twofold expression of Phlda2 induced a 50-60% decrease in total glycogen on the 129 background and 40-45% decrease on the BL6 background, relative to the respective non-transgenic counterparts, at each time point (Fig. 5B,C). A similar reduction was apparent for the three-copy line at E18.5 on both backgrounds (Fig. 5B,C). Thus, elevated Phlda2 similarly limited the accumulation of glycogen on both genetic backgrounds. However, although the amplitude of the effect was similar, a comparison of the amount of glycogen stored, expressed either relative to the weight of the placenta (mg/g) or as a total amount (mg), highlighted a striking finding. Whereas glycogen stores were compromised on both genetic backgrounds by elevated Phlda2, the compromised BL6 placentae (Phlda2^{+/+}BACx1(BL6)) still contained three times more glycogen than the genetically uncompromised 129 placentae (Phlda2^{+/+}(129)) (Fig. 5D-F).

Taken together, these data genetically define a role for elevated Phlda2 in asymmetrically restricting late fetal growth but suggests that the growth-restricting properties of Phlda2 manifest only in unfavourable circumstances in which the placenta is already functioning at its upper limit to support the growth demands of the rapidly growing fetus.

**DISCUSSION**

Elevated expression of the imprinted gene PHLDA2 has been reported in a number of studies on LBW and fetal growth restriction, but correlation does not always equal causation. Here, we have demonstrated, using a mouse model, that twofold elevated expression of Phlda2 can restrain fetal growth late in gestation. Moreover, we have also shown that Phlda2-induced growth restriction is asymmetric, with a relative sparing of the brain alongside rapid postnatal catch-up growth. However, we did not observe a similar growth restriction on a different genetic background.

Mouse and human placentae differ substantially not only with respect to histologically classified lineages but also with respect to...
Diplas et al. reported a threefold increase in placental PHLDA2 from growth-restricted babies showed increased expression of PHLDA2 (McMinn et al., 2006). Kumar et al. similarly reported elevated placental PHLDA2 in relation to fetal growth restriction, and Diplas et al. reported a threefold increase in placental PHLDA2 in very LBW babies (Kumar et al., 2012; Diplas et al., 2009). These data suggest that elevated PHLDA2 might be a relatively common cause of LBW in human populations. Our findings in the mouse model that Phlda2-induced growth restriction was asymmetric, with relative sparing of the brain, followed by rapid catch-up growth, is consequently of significant importance. Human babies with these characteristics, particularly those with accelerated postnatal weight gain after in utero growth restriction, have a greatly enhanced risk of developing type 2 diabetes, obesity and cardiovascular disease (Barker, 1990; Barker, 1994; Barker, 2001; Barker and Medical Research Council, Environmental Epidemiology Unit (UK), 1992; Lackland et al., 2000; Lackland et al., 2001). These diseases are thought to reflect the programming of metabolic abnormalities in utero in response to limited nutrient supply acerbated by optimal or even excessive nutrition after birth. This suggests that babies with elevated placental PHLDA2 might be at risk of these later-life health complications. Further work is required to follow up the later-life outcomes for babies with elevated PHLDA2 and also our transgenic mice to identify the predictive value of PHLDA2 as a biomarker.

Several human studies suggest that PHLDA2 expression inversely correlates with birth weight even within normal pregnancies (Apostolidou et al., 2007; Guo et al., 2008; Lim et al., 2012). Moreover, in a study of >7000 samples over three cohorts, a PHLDA2 promoter polymorphism was positively correlated with birth weight (Ishida et al., 2012). An inverse correlation with LBW was not reproduced in two other studies (Lambertini et al., 2012; 2013).
Lewis et al., 2012, although these studies only looked at ~100 samples. In our previous investigation of PHLDA2 expression in placenta from the Southampton Women’s Study, we did not find a significant inverse association between placental PHLDA2 and birth weight, but we did observe a slowdown in fetal femur growth late in gestation and a significant loss of bone density at age 4 years (Lewis et al., 2012). In this current study, we found that the growth-restricting properties of Phlda2 were apparent on the 129 and not the BL6 genetic background, despite similar proportional reductions in placental weight and glycogen stores. Both the human data and our mouse data can be reconciled if elevated PHLDA2 only restricts fetal growth under certain circumstances, i.e. when the placenta is working at maximum capacity or when other factors, such as environmental exposures, limit placental capacity extrinsically. LBW is particularly prevalent in populations under severe socioeconomic stress; this could be due to a multitude of factors including poor diet, maternal stress and smoking. Human placental PHLDA2 has been reported to be upregulated in response to maternal smoking; additionally, in an experimental rat model, both maternal calorie restriction and alcohol were found to result in significantly upregulated placental Phlda2 (Bruchova et al., 2010; Shukla et al., 2011). We did not identify a correlation between placental PHLDA2 expression and maternal lifestyle in our relatively small study (Lewis et al., 2012), but it might be pertinent to further explore the data on PHLDA2 both with respect to asymmetry and catch-up growth while taking into account the degree of prenatal adversity experienced by the mother.

In summary, our work has identified a causative role for elevated Phlda2 in inducing asymmetrical late fetal growth restriction. Importantly, our work also suggests that elevated Phlda2 might be more harmful in scenarios where the placenta is working at maximum capacity, such as in pregnancies subject to prenatal adversity.

**MATERIALS AND METHODS**

**Mouse strains and genotyping**

Animal studies and breeding were approved by the Universities of Cardiff ethical committee and performed under a UK Home Office Project licence (RJH). All mice were housed together in one room throughout the study on a 12-hour light-dark cycle with lights coming on at 06.00 hour and a temperature range of 21±2°C with free access to water (tap water) and standard chow. The Phlda2 transgenic lines Phlda2+/BACx1(129) (previously Tg10-12) and Phlda2+/BACx2(129) (previously Tg10-13) were bred for >eight generations onto either the C57BL6 (Harlan, BL6) or the 129S2/SvHsd (Harlan, 129) strain backgrounds for phenotypic assessment. The Phlda2 targeted allele was either maintained by paternal transmission on the BL6 background or crossed into the 129 background for >eight generations.

**Weighing studies and biochemical determination of placental glycogen concentration**

Fetal and placental wet weights were taken at the stated time points after a discorable plug. Genotyping data was obtained from yolk sac DNA as previously described (Frank et al., 2002; John et al., 2001). Glycogen was extracted from whole placenta, and resuspended in 1 ml of H2O and assayed according to the method of Lo et al. (Lo et al., 1970) at a dilution of 1 in 2 (129) or 1 in 10 (BL6).

**Quantitative RNA analysis**

Quantitative PCR of reverse-transcribed RNA (QRT-PCR) was performed and analysed as described (Schmittgen and Livak, 2008; Tunster et al., 2010).

**Statistical analyses**

Statistical significance (probability values) was determined using the Student’s t-test (two-tailed distribution and two-sample unequal variance) or the Mann-Whitney U-test.


