Maternal Inheritance of a Promoter Variant in the Imprinted *PHLDA2* Gene Significantly Increases Birth Weight

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Birth weight is an important indicator of both perinatal and adult health, but little is known about the genetic factors contributing to its variability. Intrauterine growth restriction is a leading cause of perinatal morbidity and mortality and is also associated with adult disease. A significant correlation has been reported between lower birth weight and increased expression of the maternal *PHLDA2* allele in term placenta (the normal imprinting pattern was maintained). However, a mechanism that explains the transcriptional regulation of *PHLDA2* on in utero growth has yet to be described. In this study, we sequenced the *PHLDA2* promoter region in 263 fetal DNA samples to identify polymorphic variants. We used a luciferase reporter assay to identify in the *PHLDA2* promoter a 15 bp repeat sequence (RS1) variant that significantly reduces *PHLDA2*-promoter efficiency. RS1 genotyping was then performed in three independent white European normal birth cohorts. Meta-analysis of all three (total n = 9,433) showed that maternal inheritance of RS1 resulted in a significant 93 g increase in birth weight ($p = 0.01$; 95% confidence interval [CI] = 22–163). Moreover, when the mother was homozygous for RS1, the influence on birth weight was 155 g ($p = 0.04$; 95% CI = 9–300), which is a similar magnitude to the reduction in birth weight caused by maternal smoking.

Very low birth weight shows a strong association with perinatal mortality and morbidity and is linked to an increased risk of developing adulthood diseases, such as obesity and type 2 diabetes (MIM 125853).[1,2] Fetal growth relies on an effective nutrient supply from the mother to the fetus via the placenta; this nutrient supply is influenced by a complex interrelationship between the environment and genetics. Of particular interest are imprinted genes, which show expression from only one allele in a parent-of-origin dependent manner. Genomic imprinting is found almost exclusively in placental mammals. Its evolution is probably best explained by the "conflict hypothesis," which suggests that paternally expressed imprinted genes promote fetal growth and ensure inheritance of the paternal genome to successive generations, whereas maternally expressed imprinted genes limit growth in order for the mother to survive and reproduce again.[3]

PHLDA2 (MIM 602131) encodes the pleckstrin homology-like domain, family A, member 2 protein and is a maternally expressed imprinted gene found in one of the most extensively studied imprinting clusters in human chromosomal region 11p15.5. Consistent with the "conflict hypothesis," *Phlda2*-null mice exhibit placenta overgrowth, whereas doubling the *Phlda2* expression in transgenic mice results in placental stunting accompanied by a 13% reduction in fetal weight; both of these findings suggest that *Phlda2* has a growth-suppressing role.[4,5] In humans, *PHLDA2* is expressed in a variety of tissues but is predominantly expressed in the villous cytotrophoblast of the placenta throughout gestation,[6,7] and upregulation has been observed in intrauterine growth restriction (IUGR) placentas.[8–10] This complements our previous finding that *PHLDA2* expression is significantly higher in the term placenta of lower-birth-weight babies.[11] However, sequence analysis of all informative samples in the "Moore cohort" of white European normal births confirmed that only maternal, monoallelic *PHLDA2* expression was present.[11] This indicates that loss of imprinting (LOI) was not responsible for the increased *PHLDA2* expression and suggests that additional regulatory mechanisms, including the *PHLDA2* promoter, other than imprinting must be involved.

In this study, we examined the *PHLDA2* promoter region for genetic polymorphisms that might affect *PHLDA2* transcriptional activity and therefore could affect birth weight. From the Moore cohort (n = 263), recruited from Queen Charlotte and Chelsea Hospital,[11] we sequenced a ~2 kb upstream region beginning at the transcription start site and overlapping the promoter CpG island. The UCSC Genome Browser (build GRCh37/hg19) listed 20 SNPs, encompassing rs12798267 to rs412300, in this region.

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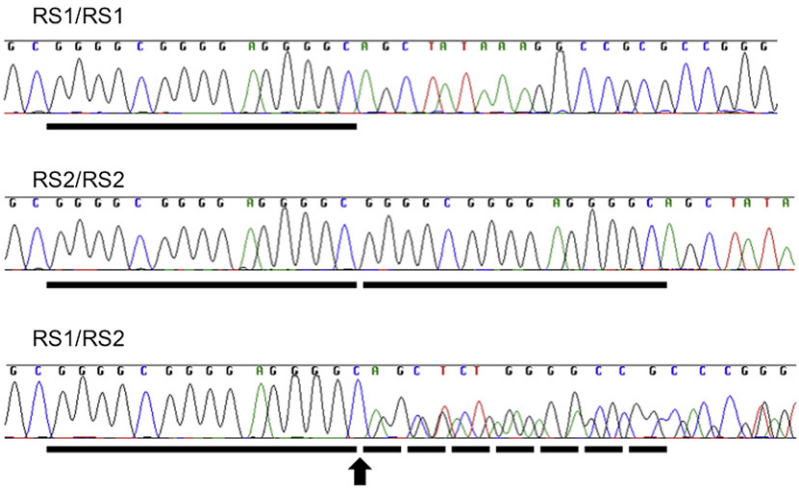

Figure 1. Sequence Electropherograms Showing the 15 bp RS in the *PHLDA2* Promoter Region

RS1/RS1 homozygous, RS2/RS2 homozygous, and RS1/RS2 heterozygous sequences are shown. Each black bar represents the location of a single 15 bp copy. The start of the overlapping sequence in the heterozygous sample is indicated by the black arrow and dotted bars.

However, none of these SNPs were identified in this cohort, suggesting that they either are rare in the white European population or have not been accurately validated. We only detected one variable sequence: a tandem 15 bp (5′-GGGG CGGGGAGGGGC- 3′; bp 4,934–4,967 of NG_009266.1) repeat sequence (RS) variant present 48 bp upstream of the *PHLDA2* transcription start site (Figure S1, available online). The tandem repeat (RS2) is most common (it is present in 87% of chromosomes), and the minor allele is a single copy (RS1) that is found in the remaining 13% of chromosomes (Figure 1). In addition, RS1 was not found to be in linkage disequilibrium (LD) with nearby SNPs rs3847646 (located ~3 kb upstream) or rs13390 or rs1056819 (present within *PHLDA2* exons 1 and 2, respectively).

We investigated the effect of the *PHLDA2* RS on the gene's promoter activity by transiently transfecting luciferase reporter constructs into the transformed human embryonic kidney (HEK) 293T cell line and the human trophoblast cell line 1 (TCL-1). We made the promoter constructs by cloning 300 bp (with the use of HindIII and Xho1) and 600 bp (with the use of HindIII and Sac1) DNA fragments upstream of the *PHLDA2* start site and inserting them into the pGL3.1-Basic vector (Promega, UK). These sequences contained either RS1 or RS2. RS1 showed significantly lower *PHLDA2* promoter activity for both the 300 bp (74% decrease; t test, $p = 0.004$) and 600 bp (42% decrease; t test, $p = 0.001$) constructs (Figure 2). The experiments were performed in duplicate for HEK293T cells and in triplicate for TCL-1 cells (Figure S2), and each assay included six replicates. TFSEARCH (Transcriptional Factor Search) shows that the RS2 allele potentially harbors four SP1 and two MZF1 binding sites. However, losing a 15 bp copy (RS1) removes three of these sites, suggesting the possibility that the number of available transcription-factor binding sites might be important for promoter efficiency.

Given that high expression of *PHLDA2* is associated with lower birth weight[11] and that RS1 reduces the *PHLDA2* promoter efficiency in vitro, we investigated whether RS1 could be associated with increased birth weight. Because *PHLDA2* is maternally expressed and paternally silenced, RS1 homozygotes and heterozygotes with a maternally inherited RS1 were grouped and deemed the "RS1 effect group." RS2 homozygotes and heterozygotes with a paternally inherited RS1 were named the "unaffected group" (Table S1). We assessed the parental origin of the RS1 allele in the heterozygous babies by genotyping their corresponding parental DNA samples. Uninformative cases were those in which both parents were heterozygotes.

We first genotyped the parental DNA samples corresponding to the heterozygous babies in the Moore cohort, and 28 babies were revealed to have maternally inherited RS1 (Table 1). To investigate the effect of maternally inherited RS1 on birth weight, we applied a linear-regression model and corrected for the following covariates: the gender of the baby, maternal weight, gestational age, parity and maternal diabetes, hypertension, and smoking habits (Table S2). A two-tailed test was used throughout; p values were based on Wald tests, and standard residual plots were examined and showed no evidence of departure from model assumptions. This analysis showed that babies in the RS1 effect group tended to have an average birth weight 122 g higher than that of the babies in the unaffected group ($p = 0.15$; 95% confidence interval [CI] = −43–286) (Table 1). We then carried out the same analysis on the UCL-FGS cohort (baby-parent trios, $n = 385$) from the University College London Fetal Growth Study.[12] This produced a similar trend to the Moore cohort: babies in the RS1 effect group ($n = 16$) were on average 68 g heavier ($p = 0.61$; 95% CI = −196–332) (Table 1). The reproducibility of this trend suggests a potentially valid finding despite the fact that statistical confidence could not be achieved because too few individuals (approximately 13%) had maternal RS1 (Table 1).

To address this, we then introduced a third and larger collection, the ALSPAC cohort ($n = 8,785$) from the Avon Longitudinal Study of Parents and Children study.[13] Because this cohort only includes samples from the mother and child and because *PHLDA2* is a maternally expressed transcript, the RS1 effect group ($n = 179$) consisted of homozygous RS1/RS1 babies and heterozygous babies with homozygous RS1/RS1 mothers (Table S3). Using the

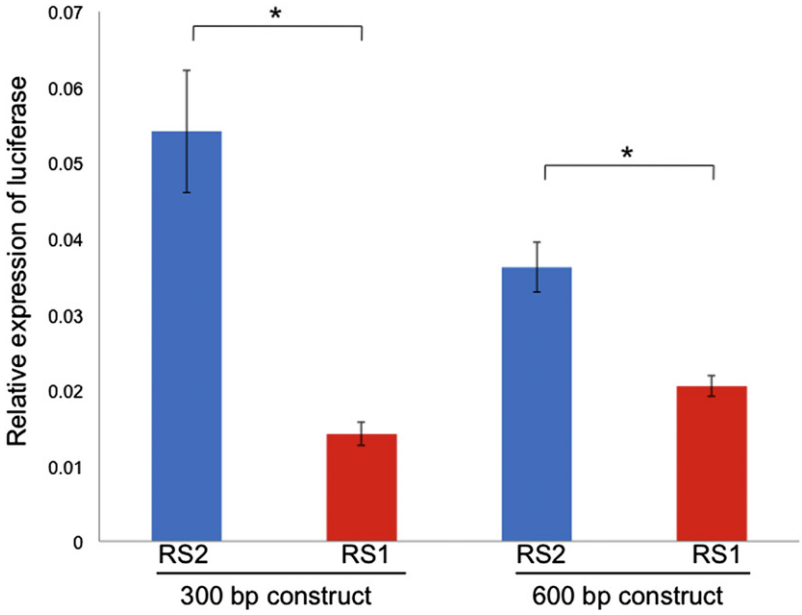

Figure 2. The Effects of RS1 and RS2 on *PHLDA2* Promoter Efficiency in HEK 293T Cells
The bars indicate the firefly luciferase expression relative to *Renilla* luciferase activity in HEK 293T cells for the 300 bp and 600 bp constructs with either RS2 or RS1. Luciferase activity was measured 30 hr after transfection. This data shows the mean of six replicate samples ± SEM (standard error of the mean). Asterisks represent $p < 0.05$.

same analysis as previously described, we found that maternal inheritance of RS1 in this cohort results in an average 88 g increase in the baby's birth weight ($p = 0.03$; 95% CI = 6–170) (Table 1). We then performed a meta-analysis to combine the data from all three cohorts by using both fixed- and random-effects models. The results from both models showed that babies inheriting maternal RS1 have a 93 g heavier birth weight ($p = 0.01$; 95% CI = 22–163) (Figure 3). No evidence of heterogeneity was found across the studies ($p = 0.92$, $I^2 = 0\%$). In addition, no evidence of association was found between birth weight and paternally inherited RS1, consistent with the imprinting of *PHLDA2*. Medical records and clinical data for all three cohorts were obtained with informed consent, and the study was approved by the ALSPAC Law and Ethics Committee and the local research ethics committees of Hammersmith and Queen Charlotte's and Chelsea Hospital Trust and University College London.

The meta-analysis indicated that the fetal genotypes had a direct influence on the babies' birth weight; therefore, we further investigated the ALSPAC cohort to see whether the maternal genotypes would have an effect on the babies' birth weight. Because we cannot determine the parental origin of the RS1 allele of the heterozygous mothers without the grandparents' samples, we instead compared the effect of three maternal genotype groups on the babies' birth weight by using the homozygous RS2/RS2 group (n = 465) as the baseline. To test this, we used a linear-regression model corrected for the same covariates described in the previous analysis. Our analysis showed that the heterozygous group (n = 529) had a low impact on the babies' birth weight (+0.3 g; $p = 0.99$; 95% CI = −69–70); this result was expected because half of the babies should inherit a paternal RS1. However, when the mothers were homozygous for RS1 (n = 61), the babies were found to be 155 g heavier ($p = 0.04$; 95% CI = 9–300), indicating that maternal genotypes have an additional influence on fetal growth potentially through the intrauterine environment. This change is of similar magnitude to the reduction caused by maternal smoking (Table S2).

Notably, the effect of heterozygous mothers on birth weight was not midway between each homozygote, even though half would be expected to carry the maternal RS1. Instead, the homozygous RS1/RS1 group had considerably more than twice the effect on birth weight than did the heterozygous group. This suggests a three-generation cumulative effect, given that a homozygous RS1/RS1 mother also inherits maternal RS1 from her mother.

Table 1. Baby Genotypes and Influence on Birth Weight: Individual Studies and Meta-Analysis

Study	RS2/RS2	RS1/RS1	RS2/RS1	P[a]	M[b]	RS1 Effect Group	Effect Estimate (g)	95% CI (g)	p value
Moore	193	4	66	22	24	28	122	−43–286	0.15
UCL-FGS	292	5	88	20	11	16	68	−196–332	0.61
ALSPAC	6,649	128	2,008	465	51	179	88	6–170	0.03*
Combined[c]	7,134	137	2,162	507	86	223	93	22–163	0.01*

The RS1 effect group consists of babies with maternally inherited RS1. RS1/RS2 heterozygous babies with heterozygous parents are uninformative for the parental origin of RS1 and were therefore removed from the analysis. All effect estimates (g) have been adjusted for the following covariates: gender, parity, maternal weight, gestational age, maternal smoking, diabetes, and hypertension. The observed genotype frequency had no evidence of deviation from the Hardy-Weinberg equilibrium. Asterisks represent $p < 0.05$. Three further alleles with different numbers of repeats were identified at the *PHLDA2* RS locus in an extremely small number of individuals (n = 25) from the ALSPAC cohort and were thus excluded from the statistical analysis.
[a]The number of heterozygous babies with paternally inherited RS1.
[b]The number of heterozygous babies with maternally inherited RS1.
[c]The meta-analysis of all three cohorts.

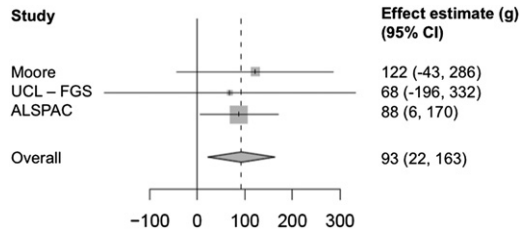

Figure 3. Meta-Analysis Showing the Relationship between Birth Weight and *PHLDA2* Promoter RS1 Effect
The data is depicted in a Forest plot; the 95% CI for each study is represented by a horizontal line, and the estimated effect sizes are shown as gray squares. The weight of the study in the meta-analysis is represented by the size of the squares. The scale used is in grams (g). The diamond shape indicates the mean and 95% CI for the total estimate of the effect. Both random- and fixed-effect models produced the same results, and the plot represents the results from the fixed-effect model.

Alternatively, homozygous RS1/RS1 mothers could also affect babies' birth weight by influencing the circulating *PHLDA2* protein/mRNA levels in the maternal blood, given that *PHLDA2* shows biallelic expression in adult blood.[14]

Maternal inheritance of RS1 did not affect the placental weight (+2.5 g; p = 0.93; 95% CI = −62–67) but did have a small and statistically-significant influence on head circumference (+0.23 cm; p = 0.04; 95% CI = 0.01–0.45). Interestingly, although the RS1 sequence is conserved in monkeys, the duplicated RS2 allele seems to be exclusive to humans (Figure S1). This implies an evolutionary role in human reproductive success. Consistent with the conflict hypothesis, maternal *PHLDA2* RS1 is associated with both increased growth of the baby and head circumference. Conversely, the net effect of the common (RS2/RS2) allele in humans is limited birth weight and head circumference, an effect which might provide an evolutionary advantage—protecting the mother and her birth canal.

Given the perinatal and life-long health complications associated with very low birth weight,[1,2,15] a number of studies have investigated the genetic contribution of putative growth-regulating genes, including the imprinted genes *IGF2* (MIM 147470) and *H19* (MIM 103280).[16–19] Genome-wide linkage or association studies have located several loci associated with birth weight,[20–23] although none have yet been directly associated with actual gene function. *PHLDA2* has not previously been detected in these screens, perhaps as a result of the complexity introduced by the parent-of-origin effect but also because *PHLDA2* RS1 is not in linkage disequilibrium with nearby SNPs and is therefore not well-represented on the genotyping platforms used. In addition, our study maximizes the information content for this allele because we specifically genotyped all informative individuals for what is essentially the functional and presumably causal variant. The biochemical function of PHLDA2 remains unknown. It is a small cytoplasmic protein that binds to phosphoinositide lipids via its PH domain.[24] A recent study showed a relationship between *PHLDA2* expression and lower growth velocity of the fetal femur; this relationship suggests that *PHLDA2* possibly plays a role in bone development.[25] Although increased *Phlda2* expression in transgenic mice resulted in a smaller placenta and a corresponding reduction in birth weight,[5] we could not replicate this finding on the human placenta either in a comparative study with *PHLDA2* expression[11] or indirectly via association with the promoter RS genotype. Nevertheless, a profound effect on the babies' birth weight was still detected, suggesting that placental weight was not the predominant regulatory factor. It also appears that this effect is controlled through the maternal expression of the gene, which is consistent with the conflict hypothesis and is mediated by the maternal genetic inheritance at the DNA level in the promoter. This provides the first example of a maternal genetic effect working together with a maternally driven epigenetic effect. We suspect it will be the first of many examples once further details of the interactions of the genome with the epigenome are unraveled. The *PHLDA2* promoter RS and its expression might serve as a useful genetic biomarker that can be used to predict birth size. Further insight into the function of *PHLDA2* along with other imprinted genes will help us understand the genetic basis of fetal growth as well as the common and serious complications—such as IUGR—of pregnancy.

Supplemental Data

Supplemental Data include three figures and three tables and can be found with this article online at http://www.cell.com/AJHG.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

ALSPAC, http://www.bristol.ac.uk/alspac
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org
TFSEARCH, http://www.cbrc.jp/research/db/TFSEARCH
UCSC Genome Browser, http://genome.ucsc.edu

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