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Maternally-inherited *Grb10* reduces placental size and efficiency

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ABSTRACT

The control of foetal growth is poorly understood and yet it is critically important that at birth the body has attained appropriate size and proportions. Growth and survival of the mammalian foetus is dependent upon a functional placenta throughout most of gestation. A few genes are known that influence both foetal and placental growth and might therefore coordinate growth of the conceptus, including the imprinted *Igf2* and *Grb10* genes. *Grb10* encodes a signalling adapter protein, is expressed predominantly from the maternally-inherited allele and acts to restrict foetal and placental growth. Here, we show that following disruption of the maternal allele in mice, the labyrinthine volume was increased in a manner consistent with a cell-autonomous function of *Grb10* and the enlarged placenta was more efficient in supporting foetal growth. Thus, *Grb10* is the first example of a gene that acts to limit placental size and efficiency. In addition, we found that females inheriting a mutant *Grb10* allele from their mother had larger litters and smaller offspring than those inheriting a mutant allele from their father. This grandparental effect suggests Grb10 can influence reproductive strategy through the allocation of maternal resources such that offspring number is offset against size.

Introduction

Foetal growth in late gestation is critically dependent upon the correct growth and development of the placenta. Intrauterine growth restriction (IUGR) is often the result of placental insufficiency (Chaddha et al., 2004), and low birth weight is associated with poor perinatal survival as well as significantly increased risk for the metabolic syndrome in later life (Hales and Barker, 2001). Many human and animal studies of IUGR have focused on the insulin-like growth factor (IGF) signalling pathway, specifically on the action of IGF2 (reviewed by Klammt et al., 2008; Efstratiadis, 1998). Deficiency of IGF2 signalling in mice (DeChiara et al., 1990) and humans (Guo et al., 2008; McMinn et al., 2006) causes growth restriction of the placenta and the foetus. Moreover, in the mouse, deletion of a placenta-specific transcript of *Igf2* (*Igf2P0*) leads to placental insufficiency in late gestation and growth restriction of the foetus (Constancia et al., 2002). *Igf2P0* is expressed in the labyrinthine zone of the placenta, the site of nutrient exchange. Depletion of *Igf2P0* reduces placental exchange volume thus reducing the transfer of maternal resources to the foetus (Sibley et al., 2004). In contrast, mechanisms also exist to limit growth *in utero*, as evidenced by overgrowth of mice following inactivation of genes such as *Gpc3* (Chiao et al., 2002) and *Grb10* (Charalambous et al., 2003). These genes normally function to prevent the foetus from growing to its full potential and perhaps by opposing growth-promoting factors provide greater control over body size and proportions. They might also guard against excessive growth of the foetus that could obstruct delivery and have catastrophic consequences for the mother (Henriksen, 2008).

Whilst experiments that modulate IGF2 signalling provide a clear example of the relationship between placental physiology and foetal growth, not all IUGR can be explained by disruption to this pathway. However, few non-IGF2 pathway genes have been found that are able to modulate placental and embryonic weight concordantly (Efstratiadis, 1998). One such gene encodes the growth factor receptor-bound substrate 10 (Grb10), an adaptor molecule that can negatively regulate embryonic and placental growth (Charalambous et al., 2003). *In vitro* studies have placed GRB10 downstream of several receptor tyrosine kinases such as the epidermal-like growth factor receptor (EGFR), the insulin-like growth factor 1 receptor (IGF1R) and the insulin receptor (IR) (Liu and Roth, 1995; Morrione et al., 1996; Ooi et al., 1995). *In vivo* we and others have shown that Grb10 negatively regulates the insulin signalling pathway in postnatal life (Smith et al., 2007; Wang et al., 2007). In addition, GRB10 ablates NEDD4-mediated degradation of the IGF1R, causing increased flux through this pathway

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(Monami et al., 2008). Surprisingly, despite its known interaction with the IGF1R, our genetic experiments demonstrated that *Grb10* is not epistatic to *Igf2*, such that GRB10 does not significantly modulate IGF2-mediated growth of the embryo or of the placenta (Charalambous et al., 2003).

Grb10, like *Igf2*, is epigenetically regulated by genomic imprinting (Miyoshi et al., 1998). Imprinting regulates small, overlapping sets of genes in mouse and man that are important for foetal and perinatal resource acquisition and placentation (Reik et al., 2003). *Grb10* transcriptional regulation is complex and results in unique transcripts arising from the paternally-inherited chromosome and from the maternally-inherited chromosome by a mechanism involving differential germline methylation of a 5′ control element (Arnaud et al., 2003; Hikichi et al., 2003). During embryogenesis the maternally-inherited copy of *Grb10* is expressed widely in the embryo, but is largely absent from the developing nervous system. In contrast, the paternally-inherited copy is expressed at low levels in peripheral tissues but at high levels in the CNS (Arnaud et al., 2003). In the adult, maternal transcription of *Grb10* is retained in insulin-responsive tissues such as skeletal muscle and adipose tissue, whereas paternally-inherited *Grb10* transcription is restricted to parts of the CNS (Smith et al., 2007). Both parental transcripts are predicted to encode the same protein which initiates from a shared translational start in exon 3 (Arnaud et al., 2003).

Deletion of the maternally-inherited copy of *Grb10* results in embryonic and placental growth enhancement, whereas deletion of the paternal copy has no associated growth phenotype (Charalambous et al., 2003). In this paper we show that Grb10 expression in the mature mouse placenta is predominantly from the maternally-inherited chromosome. In the late gestation placenta Grb10 expression is confined to the labyrinthine compartment, including both the foetal endothelium and in at least one trophoblast cell layer. Loss of maternally-derived Grb10, predominantly from the vascular endothelium of the foetal circulation, results in expansion of the labyrinth by about 50% and increased placental efficiency. Furthermore, mothers who are deficient in Grb10 in their peripheral tissues have an increase in brood size with a concomitant decrease in embryonic and placental weight, suggesting that Grb10 acts on more than one pathway of maternal resource allocation.

Materials and methods

Mice

Grb10Δ2-4 animals were generated and maintained according to Charalambous et al. (2003), on a mixed C57BL/6:CBA genetic background.

Gene expression analysis

RNA was obtained from whole placentae 17 days after the morning of the observation of a vaginal plug (e17.5) using TRI Reagent (Invitrogen). cDNA was generated from 2 μg total RNA which had been treated with DNase I (Ambion), using Superscript III (Invitrogen) with random primers according to the manufacturer's instructions. Real-time quantitative PCR with SYBR Green was performed with SensiMix (Quantace) according to the manufacturer's instructions using the primers in Supplementary Table S1. Quantification was performed using the relative standard curve method (Pfaffl, 2001), and target gene expression was normalised to the expression of *Hprt*, the expression of which did not differ between the groups (not shown).

Stereology

We performed cell composition analysis essentially according to Kurz et al. (1999). Briefly, we chose one midline and one lateral section from each placenta (+/Igf2Δ and +/+ littermates $n = 6/19$ placentae from 5/10 litters, Grb10Δ2-4/+ $n = 8$ placentae from 5 litters, +/+ $n = 9$ placentae from 7 litters, +/Grb10Δ2-4 $n = 6$ placentae from 4 litters). A counting grid was overlaid onto six randomly chosen fields/section, and cell type was scored. Final volume fraction was approximated by multiplying the cell type proportion by the placental weight.

In situ hybridisation

Immunohistochemistry, beta-galactosidase staining and RNA in situ hybridisation were carried out as described previously (Charalambous et al., 2003). The probe for the gene encoding trophoblast-specific protein (*Tpbp*, also known as trophoblast-specific cDNA clone 4311) was derived from a cDNA described by Carney et al. (1993). The *Igf2* probe was derived from the cDNA described by Stempien et al. (1986), sub-cloned as an approximately 1.5 kb EcoRI fragment into the pBluescript II KS+ vector (Stratagene) such that RNA probes can be synthesised from either a bacteriophage T3 promoter (sense strand) or T7 promoter (antisense) following digestion with XbaI or HindIII, respectively.

Results

Grb10 is imprinted in the placenta and expressed predominantly from the maternally-inherited chromosome

We previously generated a mouse model of *Grb10* loss of function by introducing a β-galactosidase gene trap cassette into the locus. Insertion of the gene trap cassette resulted in deletion of 36 kb genomic sequence including exons 2–4. The translational start of all described isoforms of *Grb10* is found in exon 3, predicting that the gene trap insertion interrupts all full length Grb10 function *in cis* (Charalambous et al., 2003).

In order to assess whether *Grb10* is imprinted in the mature placenta we compared levels of *Grb10* mRNA between wild-type, maternal deletion (*Grb10Δ2-4/+*) and paternal deletion (*Grb10+/Δ2-4*) placentae at e17.5 by quantitative real-time PCR. We used primers within the deleted region to assess expression from the wild-type allele as well as primers within exons 11–16 to evaluate total

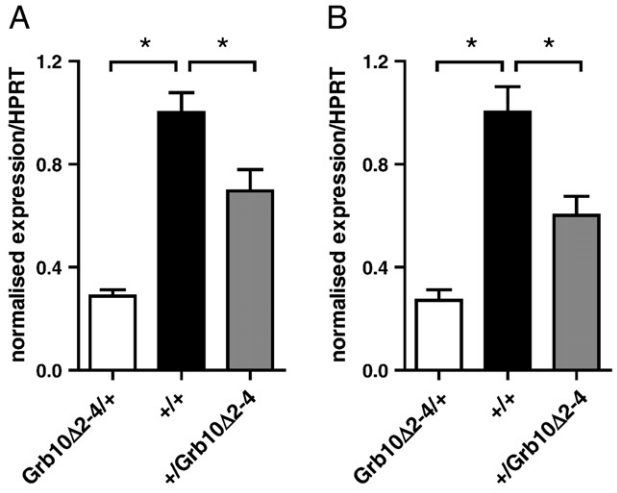

Fig. 1. Assessment of allele-specific expression of *Grb10* in the placenta. Quantitative real-time PCR was performed on eight placentae of each genotype using two sets of primers, one within the *Grb10Δ2-4* deleted region (A) and another in exons 11–16 (B), which are common to all transcripts. Values are expressed as a proportion of wild-type expression levels. The mean of each genotype was compared using the Kruskal–Wallis test, with Dunn's multiple comparison test to compare each mutant group to the wild-type $*p < 0.05$.

expression of *Grb10*. *Grb10* mRNA expression was normalised to wild-type levels and the effect of the deletion is apparent with both primer assays (Fig. 1). Maternal transmission of the *Grb10* deletion resulted in a 70% reduction in expression, whereas paternal transmission of the deleted allele caused a complementary 30–40% reduction of *Grb10* mRNA (deletion primers *Grb10Δ2-4/+*, 0.29 ± 0.07; *Grb10+/Δ2-4*, 0.70 ± 0.08 relative to wild-type; exon 11–16 primers *Grb10Δ2-4/+*, 0.27 ± 0.04; *Grb10+/Δ2-4*, 0.60 ± 0.07 relative to wild-type; eight placentae from at least three litters were analysed per genotype).

Therefore the majority of *Grb10* mRNA is derived from the maternally-inherited chromosome, though a significant minority is expressed from the paternally-inherited copy. Moreover, since the sum of expression from the maternal and paternal chromosomes assayed separately approaches that of the wild-type, our data suggest that in the presence of a deleted *Grb10* allele, the wild-type chromosome is unable to compensate for loss of *Grb10* by upregulating gene expression.

Grb10 is expressed in the foetal endothelium and the labyrinthine trophoblast layers of the mature mouse placenta

Immunohistochemistry for Grb10 on mature (e14.5 and e17.5) wild-type placentae revealed that the protein is largely confined to the labyrinthine compartment and the chorionic plate (Fig. 2A and B), though we occasionally detected weak immunostaining in the maternal deciduum. Closer examination allowed us to conclude that Grb10 is expressed in both the foetal endothelium and in at least one of the three trophoblast layers of the labyrinth (note large nuclei of stained cells, Fig. 2D). The spongiotrophoblast, glycogen cells and

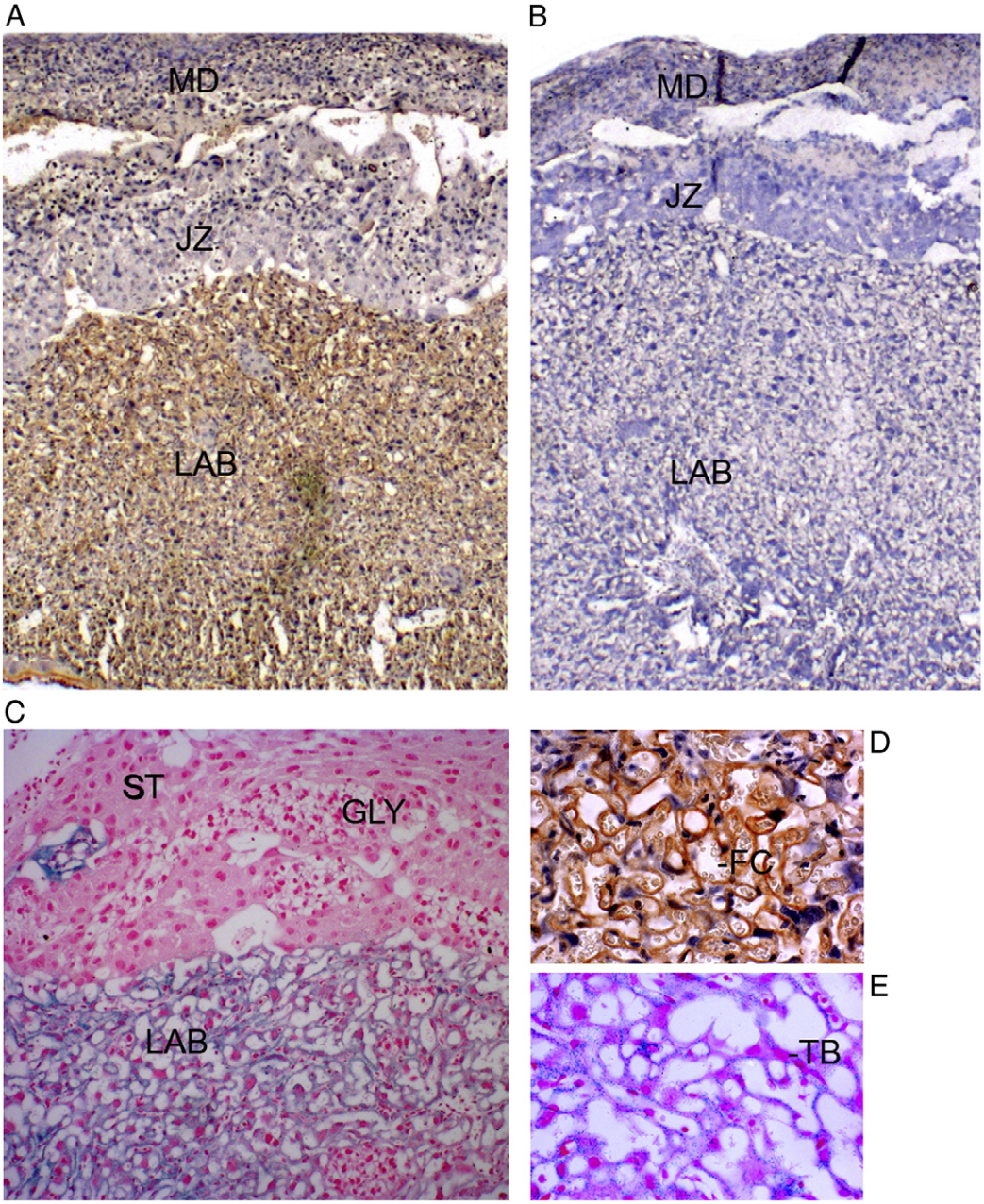

Fig. 2. Sites of Grb10 expression within the placenta. Immunohistochemistry of wild-type e17.5 placenta with an anti-Grb10 antibody shown at magnifications of 2.5× (A) and 40× (D). For comparison, a no primary antibody control is shown at 2.5× (B). Brown staining represents Grb10 immunopositivity. Beta-galactosidase staining of e14.5 placenta following maternal transmission of the gene trap allele at magnifications of 2.5× (C) and 40× (E). A blue precipitate marks the sites of reporter gene expression. MD, maternal deciduum; JZ, junctional zone; LAB, labyrinthine zone; ST, spongiotrophoblast; GLY, glycogen cells; FC, foetal capillary; TB trophoblast.

giant cells were not Grb10-positive. Following maternal transmission of the *Grb10Δ2-4* allele, the β-galactosidase reporter was detected most abundantly in the labyrinth, chorionic plate and umbilicus (Fig. 2C). Reporter expression was also clearly detectable in the foetal endothelium and labyrinthine trophoblast (Fig. 2E). Therefore by two independent methods we are able to show that Grb10 is expressed in more than one developmental lineage of the placenta, the trophectoderm and the extraembryonic mesoderm.

Foetal endothelial expression of Grb10 is largely derived from the maternally-inherited allele

The spatial contribution of gene expression from each parentally-inherited copy of *Grb10* was assessed by immunohistochemistry in the context of a *Grb10Δ2-4* deletion allele. In the wild-type labyrinth (Fig. 3A, +/+) Grb10 can be detected at high levels in the membranes of the endothelium apposed to the foetal circulation (FC). A lower level of immunostaining was detected in the labyrinthine trophoblast (note staining close to large trophoblast nuclei), and does not appear to be membrane-bound. A similar pattern was observed following paternal transmission of the deletion (representing expression from the maternally-inherited chromosome, Fig. 3A, *Grb10+/Δ2-4*), with Grb10 abundantly expressed in the endothelium, and weakly in the trophoblast. Immunostaining in the maternal transmission mutants reflects expression of Grb10 from the paternally-inherited chromosome (Fig. 3A, *Grb10Δ2-4/+*). In this case Grb10 was detected in both endothelial and trophoblastic cells, but the relative abundance was shifted, Grb10 was relatively less abundant in the endothelial compartment. Thus, the maternally-inherited copy of *Grb10* is expressed predominantly in the foetal endothelium of the labyrinth and both parental copies are expressed in the labyrinthine trophoblast.

Maternal Grb10 deletion (Grb10Δ2-4/+) placentae are large and correctly express markers of the labyrinthine and junctional zones

We previously showed that maternal deletion of *Grb10* results in placental growth enhancement from e12.5, whereas paternal deletion had no effect on placental size (Charalambous et al., 2003). At e17.5,

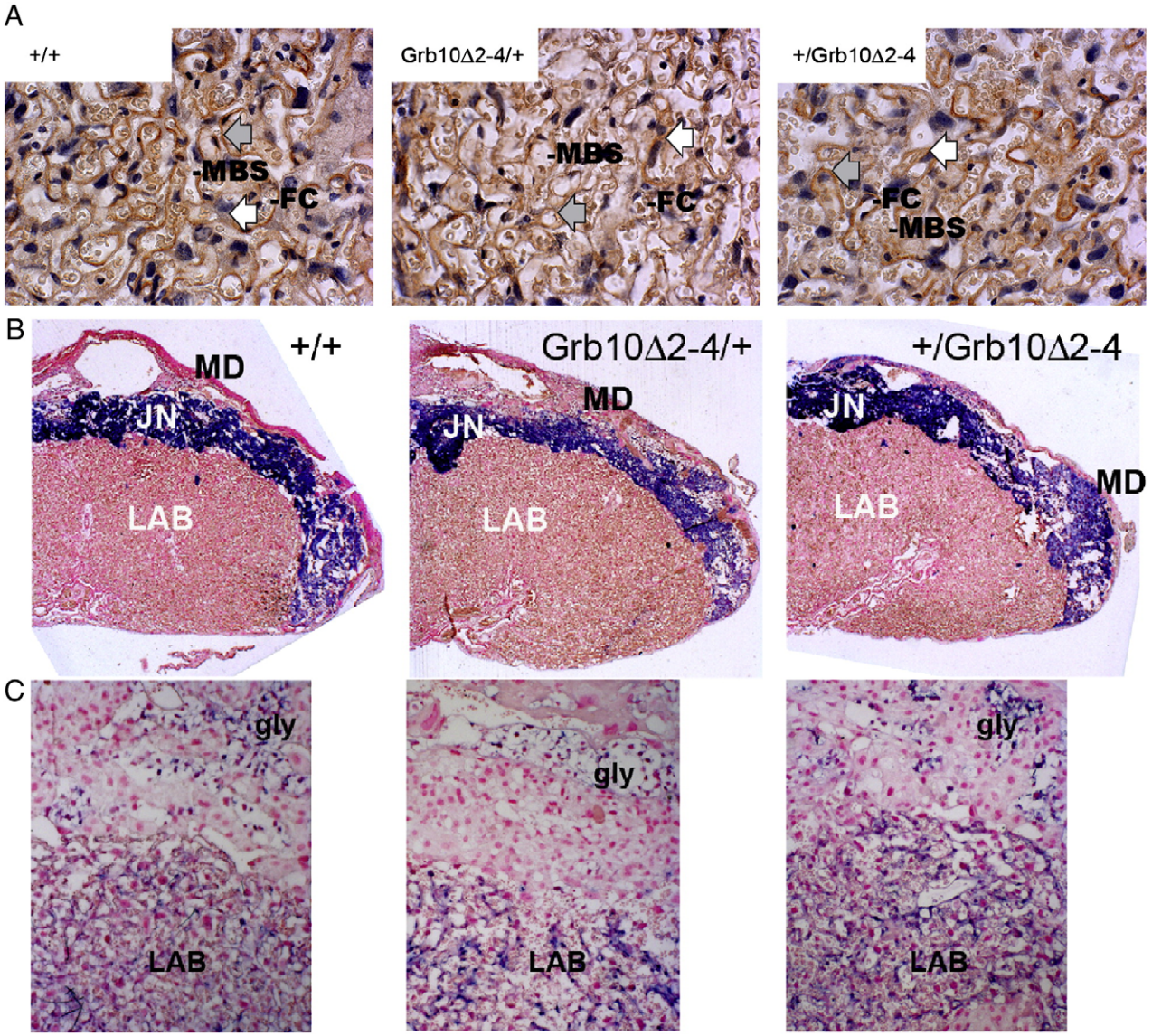

Fig. 3. Allele-specific expression of *Grb10* and assessment of placental organisation in *Grb10Δ2-4* mutant placentae. (A) Immunohistochemistry of wild-type (+/+), maternal transmission (*Grb10Δ2-4/+*) and paternal transmission (*Grb10+/Δ2-4*) placentae at e17.5 using an anti-Grb10 antibody at 40× magnification. Grey arrows indicate foetal endothelial cells, and white arrows indicate trophoblast (note large nuclei). (B) RNA *in situ* hybridisation of *Tpbp* as a marker of spongiotrophoblast and (C) *Igf2* which labels the labyrinth and glycogen cells of the junctional zone. MBS, maternal blood space; FC, foetal capillary.

maternal *Grb10Δ2-4* placentae are 30% larger in mass than wild-types (*Grb10Δ2-4*/+ 130 mg, $n=$ 85, +/+ 100 mg, $n=$ 90, from 20 litters, see Table 1). The overgrown placentae appeared grossly morphologically normal, with three clearly defined zones, the maternal deciduum, junctional zone and labyrinthine zone. In addition, the spongiotrophoblast in the junctional zone expressed the lineage marker *Tpbp* (Fig. 3B) (Carney et al., 1993), and expression of *Igf2* was correctly confined to the labyrinthine compartment and the glycogen cells of the junctional zone (Fig. 3C) (Redline et al., 1993).

Labyrinthine volume is increased in maternal Grb10 deletion (Grb10Δ2-4/+) placentae

We performed a stereological assessment of cell composition of *Grb10Δ2-4* mutant and wild-type placentae (Fig. 4A). The composition of maternal deletion *Grb10Δ2-4*/+ placentae was altered, with the labyrinthine zone expanded by about 50% in this region. Paternal inheritance of the *Grb10Δ2-4* deletion had no significant effect upon placental cell composition. In comparison, *Igf2*+/- placentae were reduced in size in both the junctional zone and in the labyrinth, as previously described (Coan et al., 2008b; Lopez et al., 1996).

Closer examination of the junctional zone confirmed that *Grb10* deletion affects placental cell composition in a manner consistent with a cell-autonomous function (Fig. 4B). There were no differences in volume fraction of the spongiotrophoblast, the glycogen cells or the giant cells following paternal or maternal transmission of the *Grb10Δ2-4* allele, consistent with lack of Grb10 expression at these sites. In contrast, and as previously described, loss of *Igf2* gene function resulted in reduction in glycogen cell fraction (Coan et al., 2008b; Lopez et al., 1996).

Maternal Grb10 deletion (Grb10Δ2-4/+) placentae are more efficient than wt placentae

At e17.5, placentae with a maternal *Grb10* deletion are about 10% more efficient than those of their wild-type littermates, as judged by the ratio of foetal:placental mass. This increase in efficiency is first apparent from e14.5 (Fig. 4C). Therefore, at least in mice maintained under standard laboratory conditions, the normal action of Grb10 is not only to reduce the overall size of the placenta, but to also limit foetal resource acquisition via the placenta.

Expression of imprinted genes associated with improved placental efficiency is unaffected in Grb10 deletion placentae

Elevated *Igf2P0* transcription correlates with improved placental efficiency in wild-type mice (Coan et al., 2008a). However, we were unable to detect any change in either placental-specific or total levels of *Igf2* in either *Grb10Δ2-4*/+ or *Grb10*+/*Δ2-4* placentae. Expression of the imprinted system A transporter *Slc38a4* also correlates with increased placental efficiency, in *Igf2P0*-deletion placentae (Constancia et al., 2005). We found no change in the abundance of *Slc38a4* transcripts between wild-type, *Grb10Δ2-4*/+ and *Grb10*+/*Δ2-4* placentae, suggesting that Grb10 does not mediate placental efficiency by this molecular pathway (Fig. 4D).

Wild-type littermates of Grb10Δ2-4/+ mice are small

We noted that wild-type littermates of *Grb10Δ2-4*/+ offspring were at e17.5 smaller than the wild-type littermates of *Grb10*+/*Δ2-4* offspring (wild-type sibs of *Grb10Δ2-4*/+, 0.906 ± 0.012g, $n=$ 90; wild-type sibs of *Grb10*+/*Δ2-4*, 1.046 ± 0.027g, $n=$ 20, $p<$ 0.001, Student's t-test, Table 1). We initially interpreted this as a suggestion that intrauterine competition was taking place, since *Grb10Δ2-4*/+ embryos are much larger than *Grb10*+/*Δ2-4* embryos. We assumed that if intrauterine competition were in effect, wild-type offspring in a litter with a high proportion of large *Grb10Δ2-4*/+ embryos would be smaller than those in a litter with fewer mutants. However, we could find no correlation between the proportion of *Grb10Δ2-4*/+ conceptuses in a litter and the size of the wild-type embryos or placentae in that litter (embryo: Spearman $r=$ 0.152, placenta: $r=-$0.150, $n=$ 20, $p=$ 0.528). In addition the effect was not due to local crowding, as a wild-type embryo with two large (*Grb10Δ2-4*/+) neighbours was not smaller than a wild-type conceptus flanked by two wild-type neighbours (data not shown). We also failed to find an alleviation of intrauterine competition in litters containing +/*Igf2Δ* conceptuses, despite the reduction in embryo and placental size of approximately half of the litter by over 40% (Table 1); wild-type littermates of +/*Igf2Δ* embryos did not differ in weight of either the embryo or the placenta when compared to wild-type littermates in purely wild-type litters (wild-type sibs of +/*Igf2Δ*, 0.959 ± 0.007 g, $n=$ 102; wild-type sibs of +/+, 0.951 ± 0.009 g, $n=$ 47, $p=$0.511, Student's t-test, Table 1). Consistent with this, we could not detect a neighbour effect in such crosses at e16.5 nor at e17.5 (data not shown).

Since imprints are reset in the germline, *Grb10Δ2-4*/+ conceptuses can arise from mothers who are themselves *Grb10Δ2-4*/+, or from mothers who are *Grb10*+/*Δ2-4* (Supplementary Fig. 1A). Wild-type offspring with a *Grb10Δ2-4*/+ mother were approximately 10% smaller than wild-type offspring with a *Grb10*+/*Δ2-4* mother. This size difference was seen both in the placenta and in the embryo (Table 1). Litter size is well known to negatively correlate with embryonic weight (McLaren, 1965), and we observed such an effect in our crosses (Supplementary Fig. 1B). We were able to apportion the

Table 1
Litter size and foetal and placental weight according to genotype.

Cross	Paternal genotype	Maternal genotype	Foetal genotype	Mean embryo weight	Mean placental weight	*n*	Number of litters	Mean litter size
1	Grb10Δ2-4/+ and Grb10+/Δ2-4	+/+	+/+	1.046 ± 0.027	0.096 ± 0.003	20	5	7.6 ± 0.9
			Grb10+/Δ2-4	1.010 ± 0.027	0.099 ± 0.003	18		
2	+/+	Grb10Δ2-4/+ and Grb10+/Δ2-4	+/+	0.906 ± 0.012	0.100 ± 0.002	90	20	8.9 ± 0.5
			Grb10Δ2-4/+	1.326 ± 0.015	0.130 ± 0.002	85		
3	+/+	Grb10+/Δ2-4	+/+	0.953 ± 0.011	0.106 ± 0.002	45	12	8.1 ± 0.5
			Grb10Δ2-4/+	1.350 ± 0.019	0.134 ± 0.003	52		
4	+/+	Grb10Δ2-4/+	+/+	0.851 ± 0.015	0.094 ± 0.002	40	8	10.0 ± 0.8
			Grb10Δ2-4/+	1.293 ± 0.024	0.125 ± 0.003	38		
5	+/+	+/+	+/+	0.951 ± 0.009	0.088 ± 0.002	47	6	7.8 ± 0.3
6	Igf2Δ/+	+/+	+/+	0.959 ± 0.007	0.083 ± 0.001	102	22	7.9 ± 0.3
			Igf2+/Δ	0.592 ± 0.006	0.056 ± 0.001	72		

Summary of crosses performed between animals inheriting the *Grb10Δ2-4* allele and the *Igf2Δ*/+ allele with their respective foetal and placental weights at e17.5 and mean litter sizes. *n* refers to the total number of animals examined by genotype in each cross. Data shown for cross 2 is the combined data from cross 3 and cross 4.

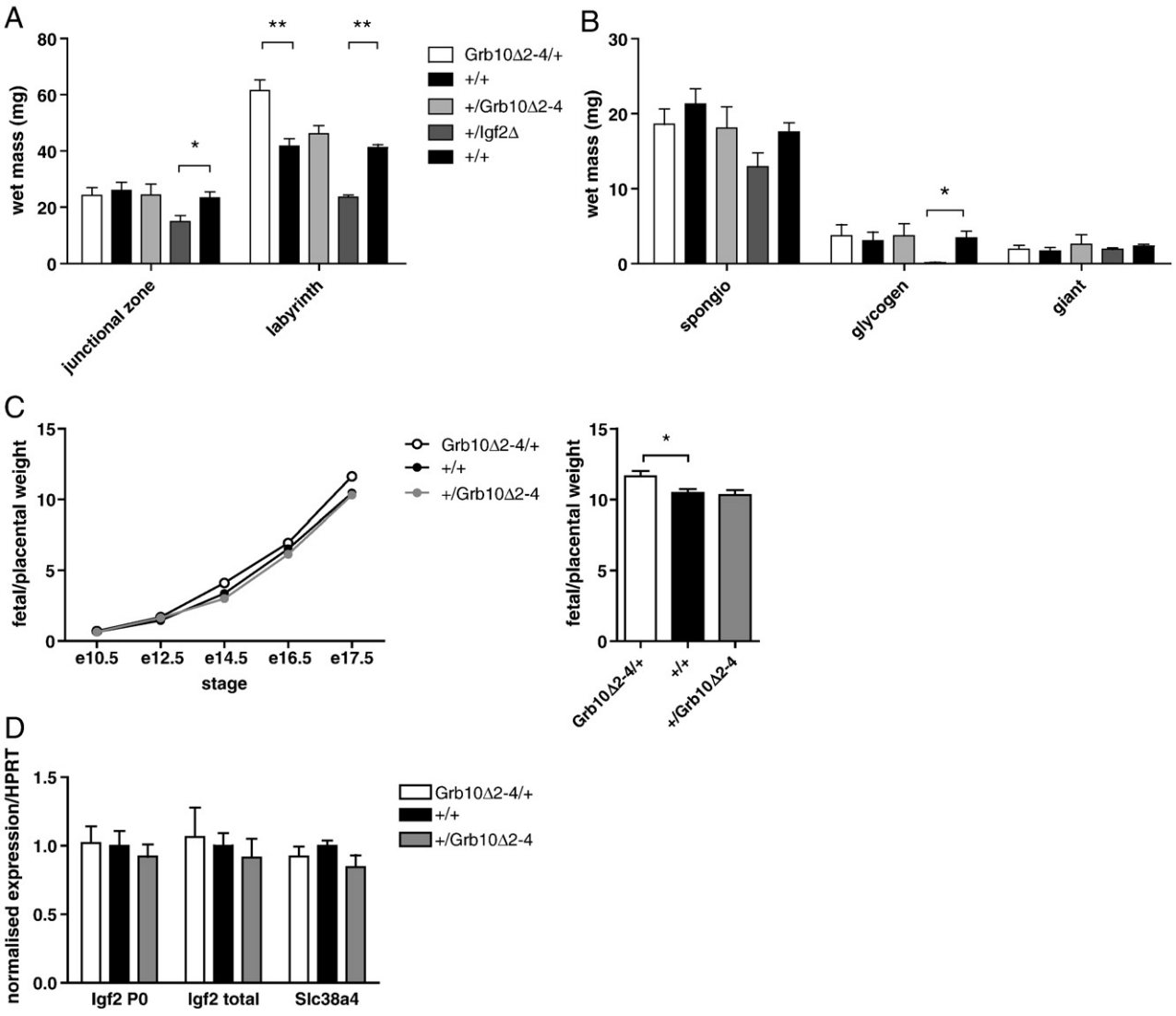

Fig. 4. Placental stereology, placental efficiency and expression of *Igf2* and *Slc38a4*, imprinted genes previously associated with improved placental efficiency. Whole placental (A) and junctional zone (B) stereology was performed on e17.5 placentae from wild-type (+/+), maternal transmission (*Grb10Δ2-4/+*) and paternal transmission (*Grb10+/Δ2-4*) mutant mice, as well as from wild-type (+/+) and paternal transmission *Igf2Δ* mice. Paternal and maternal transmission *Grb10Δ2-4* placentae were compared to wild-type placentae from littermates using a Kruskal–Wallis test with post hoc comparison of each mutant genotype to wild-type using Dunn's test $*p<0.05$, $**p<0.01$. *Igf2+/Δ* placentae were compared to wild-type placentae from littermates using a Mann–Whitney U test, $p<0.05$. (C) Foetal/placental weight was calculated from weights collected from at least five litters per developmental stage: e10.5 ($n=23$ *Grb10+/Δ2-4*, $n=29$ *Grb10Δ2-4/+* and $n=45$ +/+, $p=0.877$), e12.5 ($n=30$ *Grb10+/Δ2-4*, $n=36$ *Grb10Δ2-4/+* and $n=60$ +/+, $p=0.260$), e14.5 ($n=22$ *Grb10+/Δ2-4*, $n=25$ *Grb10Δ2-4/+* and $n=52$ +/+, $p<0.01$), e16.5 ($n=13$ *Grb10+/Δ2-4*, $n=30$ *Grb10Δ2-4/+* and $n=24$ +/+, $p<0.001$). The p value is shown for the comparison of *Grb10Δ2-4/+* with wild-types, compared by one-way ANOVA with Bonferroni's multiple comparison test; *Grb10+/Δ2-4* embryos never exhibited altered placental efficiency. Bar graph shows foetal/placental weight at e17.5 ($n=20$ *Grb10+/Δ2-4*, $n=85$ *Grb10Δ2-4/+* and $n=108$ +/+), compared by one-way ANOVA with Bonferroni's multiple comparison test to compare each mutant genotype with wild-type, $*p<0.05$. (D) Quantitative real-time PCR was performed on eight placentae of each genotype using primers to the placental-specific transcript of *Igf2* (*Igf2P0*), to exons 4–5 of *Igf2* shared by all transcripts (*Igf2* total), and to *Slc38a4* exons 14–15. No significant differences were detected between the genotypes when compared using the Kruskal–Wallis test.

reduction in wild-type weight to an increased litter size resulting from the maternal transmission cross (Table 1 and Fig. 5). *Grb10Δ2-4/+* mothers (10.0 ± 0.8, $n=8$) had larger litters (and thus smaller embryos) than any of the controls, including crosses involving *Grb10+/Δ2-4* mothers (8.1 ± 0.5, $n=12$, $p<0.05$), two wild-type parents (7.8 ± 0.3, $n=6$, $p<0.05$) or a wild-type mother and *Igf2Δ* father (7.8 ± 0.3, $n=22$, $p<0.01$) (overall $p=0.016$, by a Kruskal–Wallace test with Dunn's multiple comparison testing). Therefore a component of maternal nutrient allocation, as well as placental size and efficiency, is modulated by *Grb10* function.

Discussion

By utilising a model of loss of function of *Grb10* we were able to conduct a quantitative analysis of *Grb10* imprinting in the mature mouse placenta. We found that transcripts from the maternally-inherited chromosome comprise the majority of gene expression, but that a significant minority of transcripts arise from the paternally-inherited chromosome.

Grb10 is expressed in the labyrinthine compartment of the placenta, the site of maternal foetal exchange of nutrients. The labyrinthine zone arises at midgestation in the mouse by the fusion of the chorionic trophoblast with the allantoic mesoderm (Watson and Cross, 2005). The mesoderm differentiates into a highly branched foetal endothelium which comprises the foetal circulation. This is interdigitated with maternal blood spaces that are bounded by three layers of trophoblastic cells. Nutrients from the maternal blood must therefore cross the trophoblast and epithelial cell layers to reach the foetal circulation, and this exchange occurs by both passive diffusion and active transport (Sibley et al., 1997). In the labyrinth Grb10 is

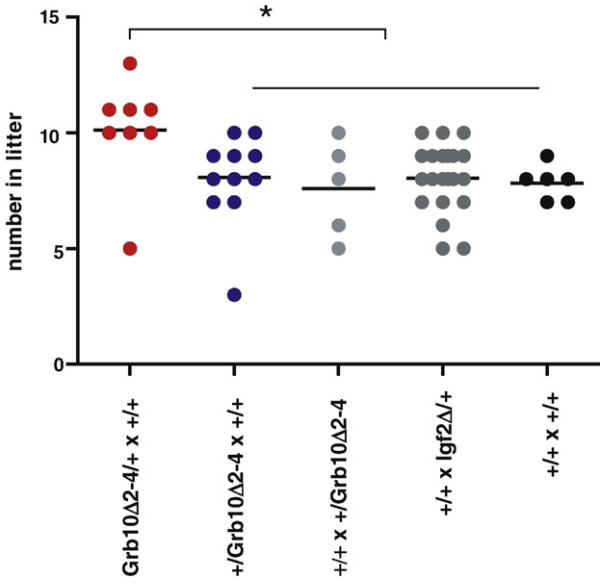

Fig. 5. Effect of maternal genotype on litter size. Number of animals in litters of crosses between *Grb10Δ2-4* mice, wild-type mice, and *Igf2*+/Δ mice. The only significant difference detected in litter size was in litters from mothers that had themselves inherited a maternal *Grb10Δ2-4* mutant allele (*Grb10Δ2-4*/+ mothers), which have significantly larger litters than any other cross (data from Table 1 compared by a Kruskal–Wallis test with Dunn's multiple comparison test applied to compare *Grb10Δ2-4*/+×+/+ with each other cross, *$p < 0.05$).

abundantly expressed in both the trophoblast and in the foetal endothelium. While both parental alleles contribute Grb10 to each of these cell layers, the majority of Grb10 in the foetal endothelium arises from the maternally-inherited chromosome.

Loss of the maternally-inherited copy of *Grb10* results in foetal and placental growth enhancement (Charalambous et al., 2003). Placentae with a maternally-inherited *Grb10* deletion are ~30% larger than wild-type placentae at e17.5, whereas fetal mass is increased by over 40%. Fetal growth may be influenced directly by Grb10 expression that is widespread in fetal tissues (Charalambous et al., 2003), and/or indirectly as a consequence of the changes we have observed in the *Grb10Δ2-4*/+ placentae. The approximate 10% increase in the efficiency of *Grb10Δ2-4*/+ placentae suggests that at least some of the enhanced fetal growth may be attributed to the increase in placental size, which is due to a 50% expansion of the labyrinthine zone.

To our knowledge this is the first example of a genetic manipulation that increases placental efficiency in an overgrown placenta, though examples of either increased efficiency (Constancia et al., 2002) or size (Frank et al., 2002; Takahashi et al., 2000; Chiao et al., 2002; Wang et al., 1994) exist. Loss of the paternally-inherited copy of *Grb10* has no effect on placental or embryonic weight.

Transcripts arising from the maternally- and paternally-inherited copies of the *Grb10* gene differ in their 5′ untranslated exons, but encode the same protein (Arnaud et al., 2003). We have demonstrated that imprinting directs *Grb10* expression to distinct but overlapping compartments of the placenta, and that it is the maternally-inherited copy that mediates placental regulation of embryonic growth. Recently, Monk et al (2009) showed by RT-PCR analysis that in RNA samples prepared from whole human placenta *GRB10* expression was biallelic or biased towards the maternal allele (Monk et al., 2009). These data are consistent with our quantitative PCR data but should be interpreted cautiously in light of our immunohistochemical staining experiments, showing that Grb10 expression occurs from either the maternal or paternal alleles in closely apposed tissues within the mouse placenta. Interestingly, in isolated villous trophoblast human *GRB10* was robustly imprinted, with expression exclusively from the maternal allele (Monk et al., 2009). This suggests some conservation of *Grb10* imprinted regulation, and perhaps function, between the human and mouse placenta. A role for paternally-inherited *Grb10* has yet to be elucidated.

Our immunohistochemistry experiments localised Grb10 to the foetal side of the labyrinthine endothelium, suggesting that it is signalling molecules in the foetal circulation that are affecting placental growth via Grb10. Such molecules may arise from the placenta itself, or from the foetus. It has been postulated that imprinted genes mediate a network of supply and demand factors that mediate growth in utero (Reik et al., 2003). In such a context, Grb10 expressed from the maternally-inherited chromosome might modulate the placental growth response to a foetal demand signal. IGF2 is the most likely candidate for such a signal, and its expression in the embryo can affect placental size (Coan et al., 2008b). However, we showed previously that maternally-inherited *Grb10* deletion promotes growth of the embryo and placenta even in the absence of *Igf2* (Charalambous et al., 2003). Consistent with this, we found that expression of *Igf2* and the imprinted system A transporter protein gene *Slc38a4* were unaltered in *Grb10Δ2-4* mutant placentae. This means that the signalling pathway through which Grb10 regulates foetal and placental growth is still to be elucidated.

Finally, we observed that wild-type offspring of a *Grb10Δ2-4*/+ mother were small. We were able to associate this with increased litter sizes from females who lack maternal *Grb10*. The control of brood size is not well understood, but is likely to be under genetic control since, for example, inbred mouse strains differ in their average litter size (Hager and Johnstone, 2003). Such a trait may be dependent upon the number of ovulated oocytes as well as the frequency of implantation. The insulin/IGF1 signalling pathway is well known to modulate fertility, and acts both centrally and in the reproductive organs to control oocyte maturation and to mediate the release and sensitivity to gonadotrophins (Bruning et al., 2000; Burks et al., 2000). Female mice lacking the insulin-receptor substrate 2 (IRS2), known to mediate signalling from both the insulin and the IGF1 receptor, have severely compromised fertility (Burks et al., 2000; Neganova et al., 2007). The maternally-inherited copy of *Grb10* modulates postnatal insulin signalling and is expressed in adult tissues, including the female reproductive tract, in the uterus and ovaries (Smith et al., 2007). Our future work will test the hypothesis that loss of *Grb10* increases litter size by altering the number of ovulations, or by an improved rate of implantation.

In summary, despite expression of *Grb10* from both parentally-inherited copies, only the predominant maternally-inherited *Grb10* transcripts modulate placental size and efficiency. Maternally-inherited *Grb10* is abundantly expressed in the foetal endothelium of the labyrinthine zone of the placenta, and loss of this expression results in an increase in the size of the nutrient exchange compartment. The role of *Grb10* as a growth inhibitor is consistent with the parent–offspring conflict hypothesis that predicts an inhibitory effect of maternally-expressed imprinted genes on foetal growth (Moore and Haig, 1991). Crosses between two species of deer mice (*Peromyscus*) produce hybrid offspring that display parent-of-origin specific growth phenotypes affecting both fetal and placental size. Growth retarded offspring result from *P. maniculatus* females mated with *P. polionotus* males, whereas the reciprocal cross results in overgrowth (Rogers and Dawson, 1970). These phenotypes have been correlated with the disruption of genomic imprinting (Vrana et al., 1998) and with altered levels of expression from a number of imprinted genes, including *Grb10* (Duselis and Vrana, 2007). These data from natural populations suggest that the effects of selection on imprinted genes can be extended beyond those observed in laboratory mice. Thus, it would be interesting to investigate the effects on placental size and efficiency of placental-specific manipulations of *Grb10* expression, and also the effects of more subtle changes in *Grb10* levels. Nevertheless, the germline disruption of *Grb10*

described in this paper indicates that the normal role for maternally-inherited *Grb10* is to limit labyrinthine exchange of nutrients and thereby limit foetal growth. Such a mechanism may bring the benefit of extra control over proportionate growth by opposing growth-promoting forces. In addition, placental Grb10 might protect the mother from foetal overgrowth and the related complications of pregnancy. Consistent with this function, Grb10 dosage in the mother may modulate maternal resource allocation between different reproductive strategies such as generating a small brood with large pups or a large brood with small pups.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.10.011.

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