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Disproportional effects of *Igf2* knockout on placental morphology and diffusional exchange characteristics in the mouse

P. M. Coan[1], A. L. Fowden[1], M. Constancia[2], A. C. Ferguson-Smith[1], G. J. Burton[1] and C. P. Sibley[3]

[1]*Department of Physiology, Development & Neuroscience and the Centre for Trophoblast Research, University of Cambridge, Downing Street, Cambridge CB2 3EG, UK*

[2]*Department of Obstetrics & Gynaecology Metabolic Research Laboratories, University of Cambridge, Box 223, The Rosie Hospital, Robinson Way, Cambridge CB2 2SW, UK*

[3]*Maternal and Fetal Health Research Group, Research School of Clinical and Laboratory Sciences, University of Manchester, St. Mary's Hospital, Manchester M13 OJH, UK*

Both complete knockout of the *Igf2* gene (*Igf2*null$^{+/-}$) and knockout of its placental specific transcript alone (*Igf2*P0$^{+/-}$) lead to fetal growth restriction in mice. However, in the *Igf2*null$^{+/-}$ this growth restriction occurs concurrently in gestation with placental growth restriction, whereas, placental growth restriction precedes fetal growth restriction in the *Igf2*P0$^{+/-}$ mouse. Previous studies have shown that the *Igf2*P0$^{+/-}$ placenta has proportionate reductions in its cellular compartments and its diffusional exchange characteristics. Yet, nothing is known about the structural development or diffusional exchange characteristics of the *Igf2*null$^{+/-}$ mouse. Hence, this study compares the structural properties (using stereology) and diffusional exchange characteristics (using measurement of permeability–surface area product, P.S, of three inert hydrophilic tracers) of the *Igf2*null$^{+/-}$ and the *Igf2*P0$^{+/-}$ placenta to identify the role of *Igf2* in the development of the labyrinthine exchange membrane and its functional consequences. Our data show disproportionate effects of complete *Igf2* ablation on the compartments of the placenta, not seen when the placental-specific transcript alone is deleted. Furthermore, although the theoretical diffusing capacity (calculated from the stereological data) of the *Igf2*null$^{+/-}$ placenta was reduced relative to control, there was no effect of the complete knockout on permeability surface area available for small hydrophilic tracers. This is in contrast to the *Igf2*P0$^{+/-}$ placenta, where theoretical diffusion capacity and P.S values were reduced similarly. Total ablation of the *Igf2* gene from the fetoplacental unit in the mouse therefore results in a disproportionate growth of placental compartments whereas, deleting the placental specific transcript of *Igf2* alone results in proportional placental growth restriction. Thus, placental phenotype depends on the degree of *Igf2* gene ablation and the interplay between placental and fetal *Igf2* in the mouse.

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Corresponding author P. M. Coan: Department of Physiology, Development & Neuroscience and the Centre for Trophoblast Research, University of Cambridge, Downing Street, Cambridge CB2 3EG, UK. Email: p.m.coan.02@cantab.net

Intrauterine growth restriction (IUGR) is a major complication of pregnancy. The perinatal mortality rate of the IUGR fetus is 4–10 times higher than that of normally grown babies (Chiswick, 1985) and approximately 5–10% of all pregnancies complicated by IUGR will result in stillbirth or neonatal death (McIntire *et al.* 1999; Thornton *et al.* 2004). Furthermore, there is a strong inverse correlation between size at birth and risk of adult-onset disease including hypertension and type 2 diabetes (Barker, 1994; Fowden *et al.* 2005; Hanson & Gluckman, 2008). In the Western world, where mothers generally have an adequate diet, the principal cause of IUGR is placental insufficiency, i.e. abnormal development of the placenta leading to inadequate structural and functional capacity to supply nutrients to the fetus (Fox, 1976; Fowden *et al.* 2006*b*). The causes of placental insufficiency are not known but there is evidence for both environmental and genetic effects

Genetically, the imprinted genes have been shown to have an important role in regulating placental development (Reik *et al.* 2003*a*; Coan *et al.* 2005*a*; Fowden *et al.* 2006*a*). These genes are expressed selectively from

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either the paternal or the maternal allele and are believed to have an important role in the allocation of maternal resources to fetal growth (Constancia *et al.* 2002; Reik *et al.* 2003*a*; Constancia *et al.* 2005). Consistent with this hypothesis, most of the known imprinted genes are expressed in the placenta, the main site of maternal–fetal nutrient transfer (Reik *et al.* 2003*b*; Fowden *et al.* 2006*a*). One of the first imprinted genes to be discovered, the insulin-like growth factor 2 gene, *Igf2*, is known to have a key role in regulating feto-placental development (Salmon & Daughaday, 1957; DeChiara *et al.* 1990; Ferguson-Smith *et al.* 1991; Baker *et al.* 1993; Ludwig *et al.* 1996; Louvi *et al.* 1997; Morrione *et al.* 1997; Murrell *et al.* 2001; Constancia *et al.* 2002). Deletion of this gene leads to placental and fetal growth restriction while, conversely, deletion of the type II insulin-like growth factor clearance receptor is associated with placentomegaly, putatively due to excess circulating IGF-II (Ludwig *et al.* 1996). Similarly, over expression of the *Igf2* gene by imprint relaxation leads to placental and fetal overgrowth (Eggenschwiler *et al.* 1997). IGF-II enhances growth via paracrine and/or autocrine actions, which stimulate cell proliferation and survival (Morrione *et al.* 1997; Burns & Hassan, 2001; Carter *et al.* 2006).

The *Igf2* gene has four fetal promoters that are expressed in a tissue specific manner in the fetus and placenta. In the labyrinthine zone of the mouse placenta, *Igf2* is driven from two different promoters; 10% of *Igf2* transcripts in the placenta derive from an upstream extra-embryonic specific promoter (P0), expressed exclusively in the labyrinthine trophoblast, whilst fetal promoters control further expression of *Igf2* in the trophoblast and fetal endothelial cells (Redline *et al.* 1993; Constancia *et al.* 2000). In the junctional zone of the mouse placenta, *Igf2* is expressed from fetal promoters; firstly from the spongiotrophoblasts and from E13 onwards from the glycogen cells (Redline *et al.* 1993).

Two mouse models have been generated using deletions of cell-type specific promoters to investigate the roles of *Igf2* in the placenta and fetus. Mice lacking the labyrinthine trophoblast-specific *Igf2*P0 transcript (*Igf2*P0$^{+/-}$) exhibit placental and fetal IUGR, with the former preceding the latter so that there is an increase in the fetal to placental weight ratio shortly after mid-gestation onwards (Constancia *et al.* 2002). These mutant placentas were also found to have perturbed diffusional exchange characteristics (as measured using inert hydrophilic tracers which can only cross the placenta by passive diffusion), which were related to alterations in placental morphology (Sibley *et al.* 2004). Furthermore, the placenta increases expression of *Slc2a3* and *Slc38a4* genes, enhancing glucose and amino acid transfer, respectively, and manages to maintain a normal growth trajectory until E16 (Sibley *et al.* 2004; Constancia *et al.* 2005). Despite these alterations in placental transport capacity, the *Igf2*P0$^{+/-}$ placenta is still limiting to fetal growth in late gestation as by term fetuses are smaller than their wild-type littermates (Constancia *et al.* 2005).

Complete ablation of *Igf2* in the *Igf2*null$^{+/-}$ mouse results in both fetal and placental growth restriction, which occur concurrently and are more severe than seen in the *Igf2*P0$^{+/-}$ mutant that continues to express *Igf2* in its fetal tissues (Constancia *et al.* 2005). The complete *Igf2*null$^{+/-}$ placenta supported less fetus per gram weight and, by the end of gestation, transferred less methyl-aminoisobutyric acid (MeAIB) per gram of placenta than its wild-type littermate in contrast to the *Igf2*P0$^{+/-}$ placenta (Constancia *et al.* 2005). Moreover, there is down-regulation of the gene expression of *Slc38a2* and of certain other cationic and anionic amino acid transporters in the *Igf2*null$^{+/-}$ mouse, whereas in the *Igf2*P0$^{+/-}$ placenta there is an up-regulation of *Slc38a4* in the presence of fetal *Igf2* (Matthews *et al.* 1999; Constancia *et al.* 2005). Thus, fetal and placental *Igf2* appear to play an important role in regulating the relationship between fetal and placental growth and placental capacity for transport of nutrients by facilitated and active transport. However, no published information on the morphology or diffusional exchange characteristics of the *Igf2*null$^{+/-}$ placenta is currently available. This is despite the fact that deficiency in placental-specific expression of *Igf2* has been reported to proportionately affect both placental exchange barrier morphology and passive diffusion and this is likely to contribute to the growth restriction found close to term (Sibley *et al.* 2004). This study therefore investigates the interplay between fetal and placental *Igf2* in controlling placental development by comparing the structure and diffusional exchange properties of the placenta in the *Igf2*null$^{+/-}$ and *Igf2*P0$^{+/-}$ mouse during late gestation when the effects of *Igf2* are maximal.

Methods

Animals

All experiments were carried out in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986. Fetuses and placentas on a C57BL/6J background were collected at E19 (embryonic day 1 (E1) = day of plug). Wet weights of placentas and whole fetuses were recorded. *Igf2*P0$^{+/-}$ mutant animals were distinguished from wild-types with a Southern blotting-based assay (Constancia *et al.* 2000). Paternal transmission of the LacZDMR2– genotype (Murrell *et al.* 2001) was used to produce *Igf2*null$^{+/-}$ placentas and fetuses. Animals were identified by the previously published method (Murrell *et al.* 2001). Details on the generation of the *Igf2*P0$^{+/-}$ model and *Igf2*null$^{+/-}$ (LacZDMR2–) mice have been previously described (Constancia *et al.* 2000; Murrell *et al.* 2001).

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Measurement of permeability–surface area product (P.S) of inert, hydrophilic solutes

As described in detail in Sibley *et al.* (2004) the diffusional permeability of the placenta is usually described, based on Fick's law of diffusion, as the permeability–surface area product (P.S) for a series of inert hydrophilic tracers of increasing molecular size. The methodology for measuring P.S is also described in detail elsewhere (Sibley *et al.* 2004). Briefly, unidirectional materno-fetal transfer of non-metabolisable radioactive tracers was measured in pregnant mice at E19 (*Igf2*null$^{+/-}$, 30 litters; *Igf2*P0$^{+/-}$, 37 litters). The mice were anaesthetized with an intraperitoneal injection of 400 μl of fentanyl fluanisone and midazolam solutions in water (1 : 1 : 2 water, Janssen Animal Health). A neck incision was made, and the jugular vein was exposed. A 100 μl bolus of PBS either containing 1.295×10^4 Bq [^{14}C]mannitol (NEN NEC314; specific activity 1.98 MBq mmol^{-1}) or 2.59×10^5 Bq ^{51}Cr-labelled EDTA (NEN NEZ147; specific activity 1201.4 MBq mg^{-1}) or 2.59×10^5 Bq [^{14}C]inulin-carboxyl (ICN; specific activity 8.88 MBq g^{-1}) was then injected into the jugular vein via a short length of tubing attached to a 27-gauge needle. At times up to 6.5 min after injection of tracer, a maternal blood sample was taken and the animals were killed. Conceptuses were dissected out and killed by decapitation. Fetuses were minced and lysed overnight at 55°C in 4 ml of Biosol (National Diagnostics, Atlanta, GA, USA). Aliquots of the fetal lysates were then added to appropriate tubes containing scintillation liquid (Bioscint, National Diagnostics) for β counting (Packard Tri-Carb, 1900, GMI Inc. USA). Placental tissue was used for genotyping.

P.S for each tracer in μl min^{-1} (g of placenta)$^{-1}$ was calculated (Sibley *et al.* 2004) as:

$$\mathrm{P.S} = N_x/(\mathrm{AUC}_{0-x})W,$$

where N_x is counts in fetus taken at time x, when mother was killed; (AUC_{0-x}) is area under curve, from time 0 to time of killing mother, derived from a graph of maternal arterial counts *versus* time, where each time point is given by the single sample from an individual mouse; W is the wet weight of the placenta. For assessing membrane porosity, P.S is normalized to the diffusion constant for each tracer in water at 37°C (D_w).

Stereological analysis of placental structure

Four litters of each genotype were collected at E19. Mice were killed and fetuses and placentas dissected out. Fetuses were decapitated and weighed. Placentas were weighed and cut in half; one half was fixed in 4% glutaraldehyde, the other was fixed in 4% paraformaldehyde and processed as previously described (Coan *et al.* 2004). Two wild-types and two mutant placentas from each litter were randomly selected. Briefly, the paraformadehyde fixed half was dehydrated, embedded in paraffin wax and completely sectioned at 10 μm. The corresponding glutaraldehyde fixed half was dehydrated and embedded in Spurr's epoxy resin and a 1 μm thick section cut near to the placental midline. The Computer Assisted Stereological Toolbox (CAST v2.0, Olympus) was employed to superimpose count grids on random fields of view within systematic random paraffin sections or resin sections. Volume and surface densities were determined using superimposed point grids, surface densities with cycloid arc grids, total capillary length using a counting frame, mean diameter from measured capillary cross sectional areas, and harmonic mean thickness with orthogonal intercept lengths. Volume and surface densities were converted to absolute values by multiplying with the absolute volume of the placenta and adjusted for shrinkage (Coan *et al.* 2004). Theoretical diffusion capacity was calculated from mean surface area and thickness and using in this instance Krogh's constant for oxygen diffusion (Coan *et al.* 2004).

Statistical analyses

Differences between fetal and placental weights and feto-placental weight ratios were assessed by ANOVA followed by Fisher's least significant difference *post hoc* test. Comparison of P.S and P.S/D_w between tracers within genotypes was assessed by ANOVA followed by Fisher's least significant difference *post hoc* test. Significant differences between mutants and wild-type littermates for individual tracer P.S and P.S/D_w values were assessed by paired *t* test. Stereological data were assessed for significant differences between mutants and wild-type siblings, as well as *Igf2*P0$^{+/-}$ *versus* *Igf2*null$^{+/-}$, using ANOVA followed by Fisher's least significant difference *post hoc* test. Values are presented as means ± S.E.M.

Results

Fetal and placental weights of the Igf2 mutants at E19

Lack of *Igf2* in the *Igf2*null$^{+/-}$ mice at E19 results in significant fetal and placental growth restriction compared to wild-type littermates (weights are 48% and 60% of wild-type, respectively) (Table 1). Compared to its wild-type counterpart, the *Igf2*null$^{+/-}$ placenta, at E19, supports significantly fewer grams of fetus per gram of placenta than its wild-type littermate (Table 1).

At E19, the *Igf2*P0$^{+/-}$ fetuses and placentas are growth restricted compared to their wild-type littermates (76% and 65% of wild-type, respectively). However, growth restriction in *Igf2*P0$^{+/-}$ fetuses and placentas is less severe than in *Igf2*null$^{+/-}$ mice (Table 1). Furthermore, significantly more grams of fetus are produced per gram of placenta in *Igf2*P0$^{+/-}$ mutants compared to their

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Table 1. Fetal and placental weights (mg) and feto-placental weight ratio

	Fetus	Placenta	Feto-placental weight ratio
WT (*Igf2*null)	1224 ± 17	85 ± 2	14.9 ± 0.3
*Igf2*null$^{+/-}$	595 ± 9*	51 ± 2*	12.2 ± 0.3*
WT (*Igf2*P0)	1240 ± 13	91 ± 3	14.1 ± 0.3
*Igf2*P0$^{+/-}$	949 ± 14*	60 ± 2*	15.9 ± 0.3*

WT, wild-type. Mean values ± s.e.m.; significant differences between mutant and wild-type littermate assessed by ANOVA with Fisher's protected least significant difference (PLSD) *post hoc* test; $^*P < 0.005$ to < 0.0001.

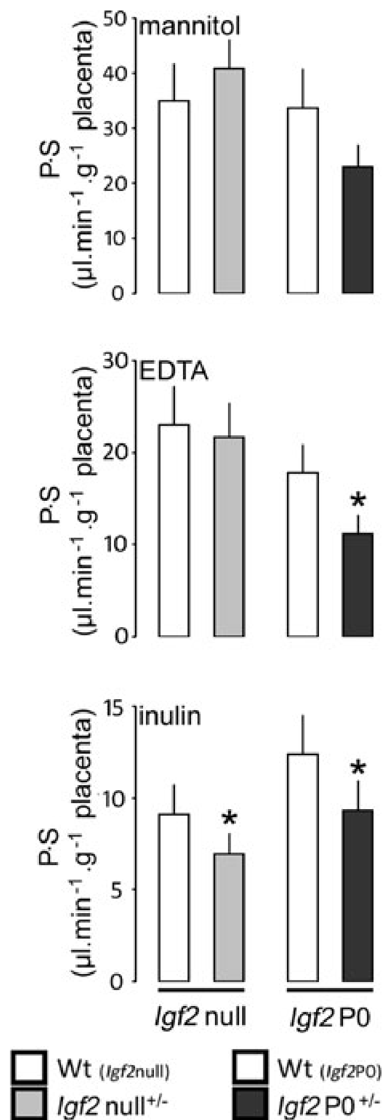

Figure 1. Permeability–surface area product (P.S) in relation to placental weight for three different sized radiolabelled solutes at E19 in *Igf2* mutants

Bars represent the mean + s.e.m. P.S *Igf2*null$^{+/-}$ litters: mannitol, 7; EDTA, 15, inulin, 8. *Igf2*P0$^{+/-}$ litters: mannitol, 6; EDTA, 23; inulin, 8. Significant differences between mutant and wild-type littermate were assessed by paired *t* test,$^*P < 0.05$ to < 0.0001.

wild-type littermates (Table 1). Fetal and placental weights of both mutants and their respective wild-type littermates are comparable to previously reported values (Constancia *et al.* 2005).

Diffusional exchange properties of the *Igf2* mutant placentas

In order to evaluate the passive diffusion properties of the *Igf2*P0$^{+/-}$ and *Igf2*null$^{+/-}$ placentas against their respective wild-type littermates, P.S per gram of placenta for inert tracers of increasing molecular size ([^{14}C]mannitol, ^{51}Cr-EDTA, and [^{14}C]inulin) was measured.

In the *Igf2*null$^{+/-}$ animals, there was no difference in P.S for [^{14}C]mannitol or ^{51}Cr-EDTA compared to wild-type littermates at E19 (Fig. 1). P.S for [^{14}C]inulin was significantly lower at E19 in *Igf2*null$^{+/-}$ compared to wild-type siblings.

In the *Igf2*P0$^{+/-}$ animals, P.S for [^{14}C]mannitol tended to be lower compared to their respective wild-type littermates at E19 but this did not reach significance (Fig. 1). P.S for both ^{51}Cr-EDTA and [^{14}C]inulin was significantly lower in *Igf2*P0$^{+/-}$ *versus* wild-type sibling animals at E19: these data for the P0 mice are similar to those we have reported previously (Sibley *et al.* 2004).

P.S was normalized to D_w for each tracer to assess potential differences in steric hindrance to diffusion of the tracers within each genotype through extracellular water filled channels (pores) (Stulc, 1989; Sibley *et al.* 2004). There were no significant differences in P.S/D_w between tracers for each genotype, for either mutants or wild-type littermates (Table 2).

Placenta structure

Labyrinthine and junctional zone volumes in the *Igf2*null$^{+/-}$ are reduced to 35% and 68% of wild-type, respectively, by E19 (Table 3). When expressed as a volume fraction, the labyrinthine zone is significantly reduced such that in the *Igf2*null$^{+/-}$ it occupies only 40% of the placenta compared to 52% in the wild-type littermate (Table 3). Furthermore, the junctional zone, which occupies 29% in the wild-type littermate, accounts for 38% in the *Igf2*null$^{+/-}$ placenta (Table 3).

In the *Igf2*P0$^{+/-}$, labyrinthine and junctional zone volumes are reduced to 53% and 56% of wild-type, respectively (Table 3). Comparison between the *Igf2*null$^{+/-}$ and *Igf2*P0$^{+/-}$ reveals that the *Igf2*null$^{+/-}$ has a significantly smaller labyrinthine zone, both in absolute terms and as a volume fraction (Table 3). Hence, the *Igf2*P0$^{+/-}$ placenta, unlike the *Igf2*null$^{+/-}$ placenta, is proportionately reduced with comparable volume fractions of labyrinthine and junctional zones to those of wild-type littermate placentas (Table 3).

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Table 2. P.S/D_w values ($cm^2\ s^{-1} \times 10^6$) for the trophoblast membrane in the *Igf2* mutant placentas

	D_w	WT (*Igf2*null)	*Igf2*null$^{+/-}$	WT (*Igf2*P0)	*Igf2*P0$^{+/-}$
[^{14}C]Mannitol	9.9	58.8 ± 11.4	68.7 ± 9.9	56.6 ± 11.9	38.7 ± 6.6
[^{14}C]EDTA	7.0	54.8 ± 6.7	50.9 ± 7.1	51.3 ± 7.5	33.8 ± 5.4*
[^{14}C]Inulin	2.6	58.5 ± 10.2	44.6 ± 7.1*	79.5 ± 13	59.6 ± 10*

Mean values ± S.E.M., significant differences between tracers for each genotype were assessed by ANOVA with Fisher's PLSD *post hoc* test $P > 0.05$. Significant differences between mutant and respective wild-type sibling for each tracer were assessed by paired *t* test; $^*P < 0.02$ to $P < 0.0001$.

Table 3. Stereological analysis of the *Igf2* mutant placentas

	WT (*Igf2*null)	*Igf2*null$^{+/-}$	% WT	WT (*Igf2*P0)	*Igf2*P0$^{+/-}$	% WT
Volume (mm^3)						
Placenta	94 ± 8	43 ± 2*†	46	116 ± 5	65 ± 4*	56
Labyrinthine zone	49 ± 6	17 ± 1*†	35	60 ± 2	32 ± 2*	53
Junctional zone	25 ± 1	17 ± 3*	68	34 ± 2	19 ± 3*	56
Decidua	16 ± 1	11 ± 1*	69	17 ± 2	13 ± 1	76
FC	11 ± 2	3 ± 0.3*†	27	15 ± 2	7 ± 1*	47
MBS	10 ± 1	3 ± 0.3*†	30	10 ± 1	6 ± 1*	60
LT	29 ± 3	10 ± 1*†	34	35 ± 1	19 ± 1*	54
Volume fraction (%)						
Labyrinthine zone	52 ± 2	40 ± 2*†	—	52 ± 3	49 ± 9	—
Junctional zone	29 ± 2	38 ± 6*	—	27 ± 3	29 ± 3	—
LT	52 ± 1	52 ± 1	—	50 ± 1	51 ± 1	—
MBS	26 ± 1	26 ± 1	—	24 ± 1	25 ± 1	—
FC	27 ± 1	26 ± 2	—	29 ± 2	27 ± 1	—
Surface area of the trophoblast membrane (cm^2)						
Fetal side	21 ± 3	6 ± 0.6*†	29	30 ± 2	15 ± 2*	50
Maternal side	25 ± 4	7 ± 0.8*†	28	30 ± 2	16 ± 2*	53
Total capillary length (m)	85 ± 5	22 ± 3*†	26	73 ± 10	37 ± 3*	51
Mean capillary diameter (μm)	14.6 ± 0.7	14.9 ± 0.7	—	13.6 ± 0.4	13.9 ± 0.8	—
Harmonic mean membrane thickness (μm)	3.1 ± 0.16	3.73 ± 0.08*†	120	3.31 ± 0.17	4.24 ± 0.13*	128
Theoretical diffusion capacity ($mm^2\ min^{-1}\ kPa^{-1}$)	13.1 ± 2.1	3.08 ± 0.19*†	24	15.9 ± 1.5	6.32 ± 0.51*	40

Abbreviations: FC, fetal capillaries; MBS, maternal blood spaces; LT, labyrinthine trophoblast; WT, wild-type littermate. $N = 4$ litters all groups, mean values ± S.E.M., significant differences assessed by paired *t* test between mutant and wild-type littermate $^*P < 0.05$ to < 0.0001. Significant differences assessed by unpaired *t* test between *Igf2*null$^{+/-}$ and *Igf2*P0$^{+/-}$ †$P < 0.05$ to < 0.001.

Labyrinthine zone architecture

The labyrinthine zone can be divided into several components: trophoblast (syncytial and cellular), fetal capillaries and maternal blood spaces. Alterations in each of these components can have implications for the function of the placenta and consequently fetal development.

The *Igf2*null$^{+/-}$ labyrinthine zone at E19 contains significantly less trophoblast, maternal blood spaces and fetal capillary volumes compared to the labyrinthine zone of its wild-type littermate (34%, 30% and 27% of wild-type, respectively) (Table 3). Surface areas on both the apical (maternal) side and basal (fetal) side are also reduced compared to the wild-type sibling placenta (28%, 29% of wild-type, respectively) (Table 3). Furthermore, total capillary length is significantly diminished to 30% compared with the length of capillaries in the wild-type (Table 3). However, mean capillary diameter in the *Igf2*null$^{+/-}$ placenta is similar to the wild-type littermate placenta (Table 3). Despite reduction in most physiologically relevant parameters, the harmonic mean thickness of the interhemal membrane is increased to 120% compared the wild-type littermate placenta

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(Table 3). Consequently, with a reduction in surface area and an expansion in membrane thickness, the theoretical diffusion capacity is considerably reduced in the *Igf2*null$^{+/-}$ placenta to 24% of placentas expressing all transcripts *Igf2* (Table 3).

Absence of the labyrinthine trophoblast-specific transcript *Igf2*P0 is also associated with a reduction in trophoblast, maternal blood spaces and fetal capillary volumes (54%, 60% and 47% of wild-type littermate placenta, respectively) (Table 3). Apical and basal surface areas of the interhemal membrane are also reduced (50% and 53% of wild-type, respectively) (Table 3). Total capillary length was reduced in the *Igf2*P0$^{+/-}$ placenta to 51% of wild-type, whereas there was no change in mean capillary diameter between genotypes (Table 3). In contrast to decreases in various components, the harmonic mean membrane thickness in the *Igf2*P0$^{+/-}$ placenta was increased by 128% of wild-type (Table 3). Hence, decreased surface area and increased membrane thickness results in a lower theoretical diffusion capacity in *Igf2*P0$^{+/-}$ placentas to 40% of that found in wild-type littermates (Table 3). Surface areas, thickness and the theoretical diffusion capacity are all comparable to the results we previously reported for the *Igf2*P0$^{+/-}$ placenta (Sibley *et al.* 2004).

Direct comparison between the *Igf2*null$^{+/-}$ and *Igf2*P0$^{+/-}$ labyrinthine zones reveals that the *Igf2*null$^{+/-}$ has significantly less volume of trophoblast, maternal blood spaces and fetal capillaries (Table 3). However, volume fractions of the same components are similar in both genotypes (Table 3). Surface areas are significantly reduced to 40% in the *Igf2*null$^{+/-}$ and capillary length is reduced to 60% compared to the *Igf2*P0$^{+/-}$ (Table 3). Moreover, harmonic mean thickness of the interhemal membrane is significantly less (88% of *Igf2*P0$^{+/-}$) in the *Igf2*null$^{+/-}$ compared to the *Igf2*P0$^{+/-}$ (Table 3). However, a favourable reduction in membrane thickness, combined with an unfavourable smaller surface area of the *Igf2*null$^{+/-}$ interhemal membrane results in a significant reduction in theoretical diffusion capacity compared to the *Igf2*P0$^{+/-}$ (49% of *Igf2*P0$^{+/-}$) (Table 3).

Discussion

The results show that the growth, morphology and diffusional characteristics of the mouse placenta depend on *Igf2* and differ in mice lacking all *Igf2* transcripts and those with deletion of the placental specific *Igf2*P0 transcript alone. In particular, it is development of the labyrinthine zone of the placenta that appears most sensitive to changes in *Igf2* availability. The volume of this zone was reduced in proportion to the other regions in the placenta of the *Igf2*P0$^{+/-}$ mutant, whereas, it was disproportionately reduced in the complete *Igf2*null$^{+/-}$ placenta. The length of the capillaries, the thickness of the interhemal membrane and the absolute volume and surface area of trophoblast within the labyrinthine zone also differed between the two *Igf2* mutants. Similarly, transplacental transfer of the three hydrophilic tracers was affected differently in these two mutants compared to their respective wild-type littermates. Placental phenotype therefore depends on the degree of *Igf2* gene ablation in the mouse. These findings, together with our previous studies, show that interplay between placental and fetal *Igf2* regulates placental morphology and transfer of substances by simple diffusion, facilitated diffusion and active transport (Sibley *et al.* 2004; Constancia *et al.* 2005).

In common with previous findings (Constancia *et al.* 2005), the current data on placental and fetal weights show that, by the end of gestation, growth restriction is less severe in the *Igf*P0$^{+/-}$ mutant than complete *Igf2*null$^{+/-}$ and that the *Igf2*null$^{+/-}$ placenta is less efficient whilst the *Igf2*P0$^{+/-}$ placenta is more efficient in terms of grams of fetus produced per gram of placenta, than their respective wild-type placentas. The data presented here indicate that the decreased efficiency of the complete null placenta may be partly due to the changes in placental morphology and, in particular, to the disproportionate reduction in the labyrinthine zone responsible for nutrient transfer to the fetus in late gestation. However, increased placental efficiency of the *Igf2*P0$^{+/-}$ placenta, at E19, cannot be explained morphologically and probably reflects, in part, the enhanced transfer of glucose observed previously in these placentas. (Constancia *et al.* 2005). Futhermore, the reduced passive permeability of the *Igf2*P0$^{+/-}$ placenta to all solutes will lower backflux of small molecules down their concentration gradient from the fetal to maternal circulation. This will thus increase the net flux of amino acids actively transported across the *Igf2*P0$^{+/-}$ placenta towards the fetus and consequently contribute to fetal tissue accretion and the increased fetal to placental weight ratio of these mutants.

The labyrinthine zone of both *Igf2* mutant placentas is growth restricted. *Igf2* deficiency may be leading to elongation of the labyrinthine trophoblast cell cycle and perturb the normal rate of proliferation, limiting growth of the labyrinthine zone (Baker *et al.* 1993). Another possibility may be that there is a higher turnover in the labyrinthine zone of the mutants. *Igf2* is a known survival factor and ablation of this gene may lead to a tip in the balance between proliferation and apoptosis in the placenta (Burns & Hassan, 2001). However, structurally there are also striking differences between the *Igf2* mutant placentas. The greater volume of labyrinthine zone in the *Igf2*P0$^{+/-}$ placenta may be associated with *Igf2* expressed from the junctional zone. Furthermore, *Igf2* from the fetus or from fetal endothelium may also influence labyrinthine trophoblast growth though there is currently no published evidence of these specific interactions. Interestingly, junctional zone volume in both mutants

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is similar suggesting that *Igf2* expression, from spongiotrophoblasts and glycogen cells, may not be responsible for growth in this compartment. Thus in the complete absence of *Igf2*, junctional zone growth is likely to be modulated by various factors including *Activin*, *Cdkn1c*, *Egfr*, *Follistatin*, *Grb10*, *Nodal* and *Peg3* (Li & Behringer, 1998; Rossant *et al.* 1998; Takahashi *et al.* 2000; Hiby *et al.* 2001; Ma *et al.* 2001; Charalambous *et al.* 2003; Candeloro & Zorn, 2007; Dackor *et al.* 2007). However, in the wild-type situation, *Igf2* transcribed from the *Igf2*P0 transcript may be providing growth stimulation to the junctional zone. On the other hand, *Igf2* could be acting as a survival factor in order to maintain the balance between apoptosis and proliferation in the junctional zone (Waddell *et al.* 2000).

Labyrinthine zone architecture as well as growth was affected by *Igf2* deficiency and differently in the two *Igf2* mutants. Total fetal capillary volume, surface area and length differ significantly between genotypes and may well indicate that *Igf2* plays an important role in angiogenesis. Moreover it has been suggested that IGF-II exerts angiogenic effects through binding IGF2R (Herr *et al.* 2003). *Igf2* deficiency also results in dramatically diminished volumes and surface areas concurrent with an increase in thickness of the interhemal membrane. Deficiency in *Igf2*P0 alone results in a less pronounced decrease in volumes and surface areas but a greater interhemal membrane thickness than in the total absence of *Igf2*. Therefore, *Igf2* plays a critical role in the development and increased sophistication of the interhemal membrane's architecture. One explanation for the increased interhemal thickness in the *Igf2*P0$^{+/-}$ placenta may be a by-product of fetal (from the endothelium or from the fetus itself) or junctional zone *Igf2* which is compensating *Igf2*P0 transcription by increasing labyrinthine zone growth and nutrient transporter gene expression. The increase in membrane thickness in the *Igf2*null$^{+/-}$ placenta may be connected to a membrane maturation problem, whereby *Igf2* could promote the stretching and thinning of labyrinthine trophoblast syncytial layers, and in its absence, the syncytial layers are less stretched and so remain thicker. This could be compounded by the growth restricted *Igf2*null$^{+/-}$ with a potentially reduced fetal haematocrit which may exhibit less shear stress to promote capillary angiogenesis (Karimu & Burton, 1994; Resnick & Gimbrone, 1995).

In common with previous observations (Sibley *et al.* 2004), the passive permeability of the *Igf2*P0$^{+/-}$ placenta was significantly less for all three tracers than their respective wild-type placenta. The actual P.S values measured for the wild-type and mutant placentas of the *Igf2*P0$^{+/-}$ mouse in the present study were similar to those reported previously at E19 (Sibley *et al.* 2004). In contrast to the *Igf2*P0$^{+/-}$ mutants, the P.S values of the complete *Igf2*null$^{+/-}$ placenta, reported here for the first time, were only reduced compared to wild-type for inulin. The P.S values for mannitol and EDTA were similar in the wild-type and complete *Igf2*null$^{+/-}$ placentas, despite the even more severe reduction in labyrinthine zone growth than seen in the *Igf2*P0$^{+/-}$ placenta at E19. Indeed, the theoretical diffusion capacity of the two mutant placentas calculated from their surface areas and interhemal membrane thicknesses indicates that passive diffusion across the complete *Igf2*null$^{+/-}$ placenta should have been reduced to a greater extent than in the *Igf2*P0$^{+/-}$ mutant placenta.

The explanation for the difference in passive diffusion predicted by morphological and functional measurements in the complete *Igf2*null$^{+/-}$ is not readily apparent but three possibilities need to be considered. First, there may be alterations in the absolute or relative blood flow through either the maternal or fetal circulation of the labyrinthine zone which alter the effective delivery of the tracers to the fetus. This would be consistent with the greater reduction in capillary length in the *Igf2*null$^{+/-}$. Furthermore, the reductions in the muscle volume and the ratio of radius^4/length of umbilical arteries of *Igf2*null$^{+/-}$ fetuses suggest that blood volume flow may be reduced by more than half in these mutants (Ahmad *et al.* 2005). However, unidirectional transfer of hydrophilic tracers such as those used here is not normally expected to be altered by flow (Atkinson *et al.* 2006).

Secondly, a single measurement of interhemal membrane thickness may underestimate the complexity of the membrane as a diffusion barrier. There are three layers of trophoblast in the interhemal membrane of the mouse placenta. The outer layer is composed of mononucleate labyrinthine giant cells that line the maternal blood spaces, with two underlying layers of syncytiotrophoblast. The outer layer is not complete, however, and large perforations within the cells will allow maternal plasma to percolate into the narrow and irregular intercellular space between layers 1 and 2 (Coan *et al.* 2005*b*). The rate of diffusion will therefore be influenced by the frequency and dimensions of the perforations, and the width of the intercellular space, all of which may vary between the mutants. The two syncytial layers are linked by gap junctions so functionally act as a single layer for diffusion purposes. However, there is again a potentially variable subsyncytial fluid-filled space between it and the basement membrane, which could affect diffusion characteristics. Further studies at the ultrastructural level, preferably of tissues fixed by perfusion at physiological pressures, are required to test whether differences in the microscopic morphology of the interhemal membrane could account for the results obtained.

Finally at an ultrastructural level, transfer of hydrophilic tracers by diffusion across the syncytiotrophoblast is presumed to take place through extracellular water

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filled channels or pores (Stulc, 1989). These pores have never been morphologically identified unequivocally in the trophoblast. Therefore, it could be that whilst changes in trophoblast membrane surface area and interhemal barrier thickness normally approximate to pore surface area and length, this may not always be the case; complete *Igf2* ablation might have differential effects on the trophoblast cell layer and paracellular diffusional pathway, which result in a leakier placenta than would be predicted from the morphological measurements alone. By considering all the morphological, P.S and P.S/D_w data, one could speculate that whilst the *Igf2*P0$^{+/-}$ interhemal membrane has a reduced number of pores, the *Igf2*null$^{+/-}$ interhemal membrane has a reduced number of larger pores, which offer less steric hindrance to the smaller tracer molecules. Consistent with this suggestion, our preliminary observations at E16 suggest that there is a small but significant increase in the inulin P.S in the complete *Igf2*null$^{+/-}$ placenta in contrast to the reduced inulin P.S values found previously in the *Igf2*P0$^{+/-}$ placenta (Sibley *et al.* 2004). These potential differences in pore size between mutants accord with the other differential effects of total *versus* partial ablation of the *Igf2* gene on placental structure reported here.

Although there were no significant differences in placental structure or function between the wild-types of the two mutants, the current data hint at the possibility that development of the wild-type placenta is affected by the genotype of its neighbouring conceptus and/or the total uteroplacental availability of IGF-II. If placental *Igf2* contributes to the pool of maternal circulating IGF-II, the mothers with *Igf2*null$^{+/-}$ placentas may have a lower IGF-II concentration than those with *Igf2*P0$^{+/-}$ placentas with consequences for resource allocation to growing feto-placental units.

Many of the features of both *Igf2* mutants have also been reported in human cases of IUGR. An early study on small-for-gestational-age (SGA) placentas found a significant reduction in the total exchange surface area despite being in the normal weight range (Teasdale, 1984). Other, more recent, IUGR studies have reported fewer arterial vessels per unit of stem villous, reduction in fetoplacental perfusion, reduction in volume of intervillous space, villi and capillaries, a smaller exchange surface area and thicker trophoblast epithelium (Jackson *et al.* 1995; Mayhew *et al.* 2003). However, the extent to which changes in *Igf2* gene expression are linked to IUGR in the human placenta remain unclear (Antonazzo *et al.* 2008).

In conclusion, total ablation of the *Igf2* gene from the fetoplacental unit in the mouse results in a disproportionate growth of the structural compartments of the placenta. These effects contrast to those of solely deleting the placental-specific transcript of *Igf2*, where there are proportionate effects on the compartments and diffusional exchange characteristics, and an ability of the placenta to increase its transport efficiency, leading to a less severe growth restriction. Future studies are required to elucidate the molecular networks by which IGF-II operates to regulate composition of the placenta and development of its exchange membrane.

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