Developmental adaptations to increased fetal nutrient demand in mouse genetic models of Igf2-mediated overgrowth

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transporters in response to a normal fetal growth demand (14, 15). These placentas are highly "efficient" because they are able to support normal fetal growth until near term, when fetal growth restriction finally ensues. When the fetal demand is reduced, as seen in the total *Igf2* knockout, this up-regulation is no longer observed, which strongly suggests that fetal demand influences placental phenotype (15). Similar adaptations in the nutrient transfer capacity of the placenta are seen when fetal demand exceeds the placental supply of nutrients during natural variations in placental growth (18). So far, these studies have been focused on models in which the potential for fetal growth is compromised by placental growth restriction. However, nothing is known about fetal-placental signaling in response to genetically determined overgrowth, despite the importance of these mechanisms to fully understanding the causes and consequences of growth-related complications of human pregnancy.

Here, we investigate placental adaptations in response to increased fetal growth demands in the imprinted $H19^{\Delta 13}$ mouse model of overgrowth (19). *H19* is a noncoding RNA, which is exclusively expressed from the maternal allele. Deletion of 13 kb, including the *H19* gene and the *H19/Igf2*-imprinting control region IC1 (also known as *H19* DMD), when transmitted maternally, leads to increased levels of *Igf2* and fetal overgrowth (19, 20). Excess of *Igf2* caused by disruption of IC1 has been implicated in the etiology of the overgrowth disorder Beckwith-Wiedemann syndrome (BWS), which is characterized by macroglossia, organomegaly, predisposition to embryonal tumors, and endocrine dysfunction (10, 21). Placentomegaly is commonly observed in mothers carrying BWS fetuses (22). In the $H19^{\Delta 13}$ mouse model, placental weight is increased as a result of the doubling of all *Igf2* transcripts, including the placental-specific P0 transcript (20, 23). The $H19^{\Delta 13}$ placentas are more overgrown than the fetus (20) and relatively "inefficient" as they produce fewer grams of fetus per gram of placenta compared to wild type (WT). We set out to test the hypothesis that $H19^{\Delta 13}$ placentas are less efficient because nutrient transfer to the fetus is reduced, perhaps in response to mechanisms that avoid excess drainage of maternal resources that might otherwise compromise fetal viability and future reproductive success.

MATERIALS AND METHODS

Mice

$H19^{\Delta 13}$ and *Igf2P0* mutant mice were generated as described previously (19, 24), and bred into an inbred C57BL6/J line for >10 generations. In experiments involving the single $H19^{\Delta 13}$ knockout, the mutant alleles were transmitted by a heterozygous mother, giving the genotypes $H19^{\Delta 13}$ (−/+) and WT (+/+; further information in Supplemental Fig. S1). In experiments involving crosses between $H19^{\Delta 13}$ and *Igf2P0* mice, the mutant alleles were transmitted by a homozygous $H19^{\Delta 13}$ mother and a heterozygous *Igf2P0* father, giving the genotypes $H19^{\Delta 13-}Igf2P0^{-}$ ($H19^{\Delta 13}$/*Igf2P0* double mutant) and $H19^{-}Igf2P0^{+}$ ($H19^{\Delta 13}$ single mutant; further information in Supplemental Fig. S1). Pregnant females were killed by cervical dislocation, and the fetuses were dissected at embryonic day (E)16 and E19 (E1 was defined as the day of vaginal plug detection).

Genotyping

Transmission of the $H19^{\Delta 13}$ allele was identified by PCR. The primer pair used to amplify an 895-bp fragment across the deletion was as follows: *H19*F, 5′-TGCCACAGAGGAAGAAACCAG-3′; *H19*R, 5′-AGTCATAGCCGAATAGCC-3′. A third primer was used as an internal positive control for the PCR reaction amplifying a 494-bp fragment: *H19*WT, 5′-TTCAGTCACTTCCCTCAGCCTC-3′. The transmission of the *Igf2P0* allele was identified by PCR, as described previously (15).

Placental transport assays of radiolabeled solutes

We performed placental transfer assays according to our previous publications (14, 15). Briefly, radiolabeled solutes were injected into the jugular vein of $H19^{\Delta 13}$ females either crossed with C57BL6/J males or heterozygous *Igf2P0* males.

Statistical analysis

Differences in mRNA expression levels between group means were evaluated by the 2-tailed unpaired *t* test. All other data were analyzed by means of 2-way analyses of variance, with "litters" and "genotype" as the two factors. Data are expressed as means ± ,. For data representing radioactive counts, a logarithmic transformation was carried out before statistical analysis. The summary data from these experiments were then represented as ratios, together with 95% confidence limits.

RESULTS

Fetal and placental overgrowth in *1* $^{\Delta 13}$ mutants

As described previously (19, 20), maternal inheritance of the *H19*$^{\Delta 13}$ mutation results in fetal and placental overgrowth. Fetal wet weight was increased in *H19*$^{\Delta 13}$ mutants by 12% at E16 and 23% at E19 compared with WT littermates (**Table 1**). We found that the overgrowth was more pronounced in the placenta at both gestational ages. Accordingly, *H19*$^{\Delta 13}$ placental wet weights were increased by 30% at E16 and 45% at E19 compared to WT. As a result of the disproportionate overgrowth of the placenta, *H19*$^{\Delta 13}$ mutants demonstrated a decreased fetal-placental weight ratio (85% of WT for both E16 and E19) (Table 1). This relative inefficiency of the large *H19*$^{\Delta 13}$ placenta in supporting fetal growth may have either a morphological and/or functional origin.

Increased surface area for nutrient exchange in the *1* $^{\Delta 13}$ placenta

To investigate the possible morphological causes of *H19*$^{\Delta 13}$ placental inefficiency, we studied the structural basis of maternal-fetal nutrient transfer using stereological analyses of mutant *vs.* WT littermate placentas. We found that the placental overgrowth in *H19*$^{\Delta 13}$ was global, affecting both the labyrinthine zone (Lz) and junctional zone (Jz) (Supplemental Fig. S2 and **Table 2**). The absolute volumes of the different components of the *H19*$^{\Delta 13}$ placenta and WT littermates are summarized in Table 2. The absolute volume of the Lz was significantly increased to 198 and 167% of WT littermate values at E16 and E19, respectively, simincya6d615 Td [(affe Td [(affie Td [(affeg6

TABLE 3. *Absolute quantities of measurements of Lz vasculature in the placenta of* H19$^{\Delta 13}$ *and WT mice at 2 gestational ages*

	E16			E19		
Measurement	WT	*H19*	*H19*/WT (%)	WT	*H19*	*H19*/WT (%)
MBS SA (cm^2)	18.81 ± 0.374	39.77 ± 3.051	211**	14.16 ± 1.018	25.08 ± 1.405	177**
FC SA (cm^2)	15.88 ± 1.084	30.97 ± 3.306	195*	16.17 ± 1.110	24.98 ± 1.871	154*
IMT (μm)	4.20 ± 0.187	4.18 ± 0.152	99	3.11 ± 0.148	3.29 ± 0.356	105
TDC ($mm^2 \cdot min^{-1} \cdot kPa^{-1}$)	7.2 ± 0.3	15.0 ± 1.9	208*	8.6 ± 0.8	13.6 ± 1.6	158*

Values are means ± ; $n = 6$ from 3 litters/group. MBS SA, maternal blood space surface area; FC SA, fetal capillary surface area; IMT, labyrinthine interhemal membrane thickness; TDC, theoretical diffusion capacity of the interhemal membrane. *$P < 0.05$; **$P < 0.01$.

The *H19*$^{\Delta 13}$ overgrown placenta down-regulates nutrient supply

Despite the observed expansion in volume and surface area of the Lz trophoblast and the increase in TDC of the *H19*$^{\Delta 13}$ placenta, we found a pronounced reduction in its passive permeability relative to WT controls. The amounts of ^{14}C-mannitol and ^{14}C-inulin transferred per gram of placenta were significantly reduced to 65–80% of the WT values at both E16 and E19 (**Fig. 1**). This, combined with the larger size of the placenta, resulted in *H19*$^{\Delta 13}$ mutant fetuses receiving the same actual amount of the solute as WT littermates, except for ^{14}C-mannitol at E19 (Fig. 1*A*).

Next, we investigated the capacity of the placenta to transfer nutrients by facilitated diffusion (^{14}C-glucose) and active transport (^{14}C-MeAIB) *in vivo* in relation to placental expression of the glucose and system A amino acid transporters. We found that transfer of ^{14}C-glucose was significantly reduced per gram of placenta compared to WT at both gestational ages (Fig. 1*B*). Accumulation of ^{14}C-glucose per gram of fetus was significantly reduced in *H19*$^{\Delta 13}$ mutants, *i.e.*, fetuses were receiving less of the solute than WT. We found that mRNA levels of glucose transporter *Slc2a3* were reduced significantly in *H19*$^{\Delta 13}$ compared to WT placenta at E16 but not E19 (**Fig. 2**). There were no significant changes in mRNA levels of the glucose transporter *Slc2a1* at either gestational age. ^{14}C-MeAIB transfer per gram of placenta was also less in *H19*$^{\Delta 13}$ mutants than in their WT littermates (Fig. 1*B*). However, in contrast to ^{14}C-glucose, mutant fetuses were receiving an appropriate amount of ^{14}C-MeAIB for their size. We found that the levels of mRNA of the *Slc38a4* 2 kb isoform were reduced in *H19*$^{\Delta 13}$ relative to WT placenta at E19 but not at E16 (Fig. 2). None of the other isoforms of the system A amino acid transporters were altered in expression in the *H19*$^{\Delta 13}$ placenta.

Feto-placental signaling of nutrient demand in *H19*$^{\Delta 13}$/ P0 double mutants

Large placentas can, therefore, respond to resource allocation signals and down-regulate the nutrient transfer capacity when placental supply and fetal demand are genetically matched at a high level. To test whether large placentas can respond to fetal-placental signals when the genetic drive for fetal growth is higher than that for placental growth, we aimed to genetically reduce the degree of placental overgrowth. This was achieved by crossing *H19*$^{\Delta 13}$ homozygous females (−/−) with *Igf2* heterozygous P0 males (+/−) and examining the progeny at E19. Two possible combinations of *H19*$^{\Delta 13}$ and *Igf2P0* mRNA expression were represented among the conceptuses: *H19*$^{\Delta 13}$ single mutant (*H19*$^{\Delta 13-}$*Igf2P0*$^{+}$) and *H19*$^{\Delta 13}$/*Igf2P0* double mutant (*H19*$^{\Delta 13-}$*Igf2P0*$^{-}$; see Supplemental Fig. S1). *H19*$^{\Delta 13}$/*Igf2P0* placentas showed a specific reduction in P0 levels (~65% of *H19*$^{\Delta 13}$; **Fig. 3**), as expected from the deletion of one active allele. As a result of reducing P0 levels, the *H19*$^{\Delta 13}$/*Igf2P0* double-mutant placentas

response to fetal signals designed to reduce the uptake of substrates in line with the reduced passive diffusion of other key substances required for intrauterine growth. Alternatively, maternal signals may be constraining placental nutrient allocation to the large $H19^{\Delta 13}$ fetuses at the period of late gestation when the absolute demand for nutrients is at its greatest. By reducing expression of key transporters, these maternal signals may place an upper limit on nutrient transfer and avoid excess drainage of maternal resources into fetuses with an increased genetic drive for growth. However, little is known about the maternal metabolic

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Received for publication October 21, 2010.
Accepted for publication January 21, 2011.