Published in final edited form as:

Placenta. 2009 August ; 30(8): 693–699. doi:10.1016/j.placenta.2009.05.004.

Placental glycogen stores are increased in mice with *H19* null mutations but not in those with insulin or IGF type 1 receptor mutations

Diego R. Esquiliano, Weihui Guo, Li Liang, Pieter Dikkes, and Mary Frances Lopez

Endocrine Division, Neurology Children's Hospital and Harvard Medical School, Boston, MA 02115

Abstract

The function of glycogen in the placenta remains controversial. Whether it is used as a source of fuel for placental consumption or by the fetus in times of need has yet to be determined. Two imprinted genes, insulin-like growth factor 2 (*Igf2*) and *H19* are highly expressed in the placenta. We have previously demonstrated that mice with *Igf2* deficiency have lower levels of placental glycogen. In this study, we used mice with targeted disruption of the *H19* gene (*H19*^{-/-}) to determine the importance of *Igf2* overexpression in placental growth and glycogen stores. In addition, since *Igf2* mediates most of its functions by signaling through the insulin and/or IGF Type 1 receptors, we determined whether gene deletions to these receptors could affect placental glycogen stores. Our data demonstrate that placentas from *H19*^{-/-} mice are heavier, have higher number of glycogen cells, and contain larger glycogen concentrations than those of *H19*^{+/+} mice. No differences in GSK-3, ERK, or total Akt expression or phosphorylation were found between genotypes; however, Akt1 protein expression levels were significantly increased in *H19*^{-/-} placentas. Results obtained from insulin receptor or IGF Type 1 receptor mutant mice did not show differences in placental glycogen content compared to their wild-type littermates, supporting the notion of a specific placental *Igf2* receptor. Taken together, these results support a role for *Igf2* and Akt1, but not the insulin nor the IGF Type 1 receptors, in the regulation of placental growth and glycogen metabolism.

INTRODUCTION

One crucial function of the placenta is to transfer glucose from the maternal circulation into the fetus [1]. This glucose is used as a source of energy by the placenta and the fetus or stored as glycogen [1]. While the glycogen in the fetal liver is used during the neonatal period to maintain levels of glycemia, the role of glycogen in the placenta remains to be fully determined. Some studies support the idea that placental glycogen serves as a source of energy for its own needs [2], while others have raised the possibility that it is also available for fetal needs [3-5]. In their studies, Barash and collaborators demonstrated that placental

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

glycogen could be mobilized in response to hormonal stimuli; however, they postulated that glucose could not be produced in the placenta due to the absence of glucose-6-phosphatase (G6Pase), the key enzyme in glucose homeostasis [6]. Recent studies, however, have demonstrated the existence of a specific G6Pase gene present in the placenta [5]. This gene is unrelated to the hepatic G6Pase gene, thus suggesting that the placenta could generate glucose that potentially play a role in maintaining fetal blood glucose concentrations [5]. The placenta accumulates high glycogen concentrations during early gestation but these levels decrease near term [1]. This drop in glycogen is thought to be caused by the fetus placing high demands on maternal resources needed for growth during the final stages of gestation and thus using available placental glycogen reserves to meet those demands [7].

Although insulin plays an important role in postnatal glycogen metabolism [8], this hormone does not appear to play a role in placental glycogen metabolism [9]. Insulin promotes glycogen synthesis by increasing the activity of glycogen synthase and decreasing the activity of glycogen phosphorylase [8]. These two enzymes are present in the placenta but do not seem to be affected by insulin [9]. Insulin-like growth factor II (Igf2) is a hormone that is highly expressed in both the placenta and fetus [10,11]. It plays an important role in placental growth [12,13], nutrient transfer [14], and glycogen synthesis [13]. Placentas from Igf2 knockout (*Igf2*^{-/-}) mice are significantly smaller than those of their wild-type (*Igf2*^{+/+}) littermates [12,13], have disproportionately fewer numbers of glycogen cells, and have lower glycogen concentrations than those of *Igf2*^{+/+} mice, [13], thus supporting the role of Igf2 in placental glycogen metabolism.

Another gene involved in fetal and placental growth is *H19* [15,16]. *H19* is an unusual gene that does not encode a protein. *H19* is abundantly expressed in the developing embryo and the placenta [15, 17, 18]. Both, *Igf2* and *H19* are present within the same chromosome, only 90 Kb apart, and are part of a cluster of genes that are imprinted. They have similar expression in the fetus but they are reciprocally imprinted; while *Igf2* is paternally transcribed, *H19* is maternally expressed [19,20]. Mice with targeted deletion of the *H19* gene (*H19*^{-/-}) have increased Igf2 expression and are born 30% larger than their wild-type (*H19*^{+/+}) littermates [15]. The increased size is thought to be due to increased Igf2 expression rather than a loss of *H19* function. The maternal Igf2 allele, which is usually silent, becomes transcriptionally active leading to an increase in Igf2 mRNA expression from both paternal alleles and consequently to fetal and placental overgrowth [15,16].

Insulin-like growth factor 2 binds to three receptors: the IGF type 1 receptor (Igf1R), the IGF type 2 receptor (Igf2R), and the insulin receptor (InsR) [21,22]. These receptors may compete for Igf2 binding during placental development since they are all expressed in the placenta. The Igf1 and Igf2 receptors are abundantly expressed in the labyrinth zone of the placenta and are present in small amounts in the basal zone [11]. The InsR is expressed in both the labyrinth and basal zones [23]. The InsR and Igf1R are distinct, but related receptors that can mediate responses to any of the three ligands [24]. In addition to these three receptors, Efstratiadis and collaborators have postulated an additional placental receptor, which they named XR_p [25]. This receptor is thought to mediate some, if not all of the functions of Igf2 in the placenta [25].

In summary, the present study shows that Igf2 over-expression in *H19*^{-/-} placentas results in higher levels of placental glycogen stores. Since Igf2 binds to the Igf1R and InsR, we also measured glycogen levels in placentas of *Igf1R* and *InsR* deficient mice to determine the importance of these receptors in placental glycogen regulation. In addition, we also began to elucidate potential mechanisms that may be involved in regulating the over-growth phenotype observed in the *H19*^{-/-} placentas. Understanding placental glycogen metabolism will help us to better understand carbohydrate metabolism during gestation and its effects on fetal growth.

MATERIALS AND METHODS

Animals and tissue collection

Mice carrying the inactivated *H19*, *Igf1R*, and *InsR* genes [15, 25, 26] were maintained as separate colonies. Knockout (KO) and wild-type (WT) mice from each mutation were derived from the same progenitors, using heterozygous matings. Mice were kept on a 12 h light, 12 h dark schedule (lights on at 7:00 a.m.) and allowed free access to food and water. Eight to 10 week old females were housed with adult male mice and examined daily for vaginal plugs. The presence of a vaginal plug on the morning after introduction of the female into the male cage was designated as day 0 of pregnancy. Placentas were collected from mice on days 15 and 18 of gestation for morphological analysis and determination of glycogen levels. Experiments were performed using at least three litters per genotype and were limited to litters containing 7-10 conceptuses and their respective placentas. The mice were housed and cared for according to National Institutes of Health guidelines and all experiments were approved by the Animal Care and Use Committee of Children's Hospital, Boston.

Mice Genotyping

The genotype of *H19*^{-/-} and *Igf2*^{-/-} mice, and their respective wild-type (WT) littermates was determined by Southern blot analysis as described previously [12,15], whereas the genotype of mice carrying the *Igf1R* or *InsR* deletions were genotyped by PCR analysis [25,26].

Histological analysis

Placentas were fixed by immersion in either Carnoy's (for H&E staining) or Rossman's (for Best's carmine staining) fixative for 24 h. All tissues used in this study were embedded in paraffin using an automated tissue processor. Serial 5 µm sections were cut using a Reichert-Jung Biocut rotatory microtome. Prior to staining, paraffin was removed from the sections with three changes of xylene, followed by rehydration in descending concentrations of ethanol (100%, 90%, 70%, 50%) and water. Sections were stained with hematoxylin-eosin for morphological analysis or with Best's carmine for glycogen content [27]. Diastase treatment was used before or after Best's carmine staining to show that staining was specific to glycogen.

Counting of Placental Cells

Cells were counted in representative sections using an ocular grid in a Zeiss Axioskop as described previously [13]. Micrographs of three sections of each placenta were used to count and compare cell number in morphologically equivalent areas of *H19^{+/+}* and *H19^{-/-}* placentas. The most lateral section, that contained both the basal and labyrinth zones, was chosen as the first section. The second section was halfway to the midline of the placenta. The third section was at the middle of the placenta. Three sections from each of three different placentas harvested from different litters were used for the analysis of each phenotype [13].

Glycogen Assay

Glycogen in placental tissues was measured as described previously [28]. Briefly, placentas were digested with 30% KOH saturated with Na₂SO₄ at 95 °C for 30 minutes. The glycogen was precipitated with 1.1 volumes 95% ethanol. The glycogen precipitates were dissolved in water and analyzed by the phenolsulfuric acid colorimetric method [28].

Western blot analysis

Placentas were homogenized in 3 ml of lysis buffer (54.6 mM Hepes, 28.6 mM NaF, 2.69 mM Na₄P₂O₇, 360 mM NaCl, 4.8 mM EDTA, 10% glycerol, 1% NP-40, 2mM PMSF, 1mM vanadate, 10µg/ml apoptotin, 10µg/ml leupeptin. Eighty micrograms of homogenate were electrophoresed on an 8% SDS-PAGE, and electroblotted with a miniTrans-Blot transfer cell system (BioRad, Hercules, CA) at room temperature for 1 h. After decanting the blocking buffer, the blots were incubated with either anti-IGF-II (1:200, Santa Cruz Biotechnology Inc, Santa Cruz, CA) or anti-Akt, Akt1, glycogen synthase kinase-3 (GSK-3), p44/42 extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) (all diluted 1:1000 with exception of Akt1 which was diluted 1:400, Cell Signaling, Danvers, MA) overnight at 4 °C. To detect levels of phosphorylation, phospho-Akt, phospho-GSK3, or phospho-p44/42 ERK1/2 antibodies were used (1:1000, Cell Signaling, Danvers, MA). The membranes were washed with TBS (0.1% Tween-20, 20 mM Tris, 150 mM NaCl pH7.4) and incubated for 1h with respective immunoglobulin G horseradish peroxidase (1:5000, Santa Cruz, CA). The immunoreactive proteins were visualized using the enhanced chemiluminescence kit (ECL, Amersham, Arlington Heights, IL). The ECL-exposed films were digitalized and densitometric quantification of the bands was carried out using NIH Image 1.6. Experiments were performed using placental extracts from 3 mice from each genotype.

Immunohistochemistry

Placentas were fixed in Bouins fixative for 24 hours, embedded in paraffin, and cut at 10 microns. Sections were deparaffinized, and analyzed by immunohistochemistry for Akt1 and Igf2 using a Vectastain ABC-Elite kit (Vector Laboratories, Burlingame, CA). Antigen retrieval was used by exposing the sections to boiling 10mM citrate buffer (pH 6), for 10 minutes. Slides were allowed to cool at room temperature, washed in water and placed in 3% hydrogen peroxide for 10 min. Sections were washed, blocked for 1 h at room temperature, and then incubated with primary antibody (Igf2, 1:100, Santa Cruz

Biotechnology, Santa Cruz, CA and Akt1 1:400, Cell Signaling, Danvers, MA) overnight at 4 °C, rinsed in PBS, incubated in biotinylated secondary antibody for 30 minutes, followed by avidin-biotin complex for 30 minutes. Labeling was visualized with 3, 3'-diaminobenzidine and H₂O₂ (DAB kit, Vector Laboratories, Burlingame, CA).

Statistical Analysis

Data were analyzed using the unpaired, two-tailed Student's *t*-test for comparison of the two groups and ANOVA for comparison of more than two groups followed by post-hoc multiple comparison tests. Significance was accepted at $P < 0.05$. Data were expressed as means $\pm$ standard error of the mean (SEM).

RESULTS

By day 15 of gestation, *H19*^{-/-} fetuses weighed approximately 30% more than *H19*^{+/+} controls ($P < 0.01$), 20% more on day 18 of gestation ($P < 0.01$), and 24% more at birth ($P < 0.01$; Figure 1A). Placentas from *H19*^{-/-} mice were also significantly heavier than those obtained from *H19*^{+/+} mice on days 15 and 18 of gestation ($P < 0.01$; Figure 1B), implicating the role of *H19* deficiency or Igf2 overexpression in fetal and placental growth. Notice that by the end of gestation, *H19*^{+/+} placentas were lighter than those collected at embryonic day 15 ($P < 0.01$, Figure 1B). This is to be expected, as it is known that the mouse placenta loses weight towards the end of gestation (29). However, no significant decrease was observed in *H19*^{-/-} placentas at the end of gestation ($P = 0.06$), suggesting that *H19* deficiency or Igf2 over-expression may inhibit signals that lead to the gestational placental decline that normally occurs in the placenta. We then examined placentas from *H19*^{-/-} mice to determine the extent and location of Igf2 overexpression in this tissue by western blot and immunohistochemistry experiments. Our data show stronger Igf2 antibody immunoreactivity in *H19*^{-/-} placental sections than in those of *H19*^{+/+} controls (Figure 2A-D). Most of the Igf2 staining was detected in the glycogen cells of the basal zone and in the glycogen cell clusters from the labyrinth zone. Western blot data demonstrated a significant higher ratio of Igf2/ β -actin in *H19*^{-/-} than in *H19*^{+/+} placentas ($P < 0.05$, Figure 2E), suggesting significantly higher levels of Igf2 protein expression in *H19*^{-/-} placentas than in *H19*^{+/+} controls.

To exclude differences in cell size between the *H19*^{+/+} and *H19*^{-/-} placentas, we measured the diameters of placental cells. Measurements were taken of the long and the short axis of each cell as previously described [13], and found no significant cell size difference between genotypes suggesting that differences in placental size were not due to cell size. We next compared the number of cells in *H19*^{-/-} and *H19*^{+/+} placentas by counting cells in morphologically equivalent areas of the basal zone of the placenta [13]. We found no significant differences the number of giant or spongiotrophoblast cells between *H19*^{-/-} and *H19*^{+/+} placentas. However, we found a significantly higher number of glycogen cells in *H19*^{-/-} placentas than in those of *H19*^{+/+} controls ($P < 0.02$, Table 1). As a consequence, the mean ratio of glycogen to spongiotrophoblast cells was significantly higher in the *H19*^{-/-} placentas ($P < 0.01$), suggesting the importance of Igf2 in the development of placental glycogen cells.

Because glycogen cells produce and store glycogen [7,30], we examined the glycogen content in *H19*^{-/-} and *H19*^{+/+} placentas using Best's carmine, a glycogen-specific stain. We found higher glycogen staining in placentas from *H19*^{-/-} mice compared to those of *H19*^{+/+} placentas on day 15 of gestation (Figure 3A). Most glycogen-producing cells were located in the basal zone of the placenta; however, some clusters of cells were also found in the labyrinth zone. To quantify differences in glycogen concentrations, we used a biochemical assay [28]. We found that by day 15 of gestation, *H19*^{-/-} placentas contained significantly higher glycogen concentrations than those of *H19*^{+/+} placentas ($P < 0.01$); however, no significant differences were found between genotypes by day 18 of gestation ($P = 0.067$, Figure 3B).

Since the *InsR* is known to mediate some of the actions of Igf2 in the fetus [22], we wanted to determine whether placentas from insulin receptor knockout mice (*InsR*^{-/-}) mice had any weight or glycogen concentration alterations compared to those placentas of *InsR*^{+/+} littermates. We found no differences in placental weight between genotypes. The average *InsR*^{-/-} placenta on day 15 of gestation weighed 0.092 ± 0.005 g ($n=6$) vs. 0.095 ± 0.005 g ($n=8$) in *InsR*^{+/+} mice; 0.067 ± 0.004 g ($n=9$) and 0.071 ± 0.004 g ($n=10$), respectively, on day 18 of gestation. When compared the basic morphology of *InsR*^{-/-} and *InsR*^{+/+} placentas, no obvious morphological alterations were observed. The levels of glycogen concentrations were also determined in placentas from both genotypes and we found no differences. By day 15 of gestation, glycogen concentrations in *InsR*^{-/-} placentas were 10.3 ± 0.7 mg/g tissue and 11.9 ± 1 mg/g tissue in *InsR*^{+/+} placentas ($n=6$ /genotype, $P=0.13$). On day 18 of gestation, glycogen concentrations in *InsR*^{-/-} placentas were 7.9 ± 0.8 versus 6.8 ± 0.4 mg/g tissue for *InsR*^{+/+} placentas ($n=3$ /genotype, $P=0.3$). In addition, no obvious morphological differences in the basic placental morphology were observed between *InsR*^{-/-} and *InsR*^{+/+} placentas. Because the *Igf1R* is also known to mediate some of the Igf2 actions and this receptor is also present in the placenta [11], we compared glycogen concentrations from placentas collected from *Igf1R*^{-/-} and *Igf1R*^{+/+} mice on day 18 of gestation and no differences were found (13.98 ± 1 and 15.99 ± 2 mg/g placenta, respectively, $P = 0.5$, $n=3$ /genotype, Figure not shown). Placental weights in *Igf1R*^{-/-} mice are known to be similar to those of WT littermates [25], suggesting that neither the insulin nor the Igf1 receptors are essential for growth or glycogen synthesis to take place in the placenta.

To begin to elucidate a potential signaling mechanisms by which *H19* deficiency and/or *Igf2* over-expression could lead to higher placental glycogen stores, we examined some proteins known for their involvement in glycogen signaling. Akt (also known as protein kinase B) inhibits the activity of glycogen synthase kinase-3 (GSK-3), which then decreases glycogen synthase activity, the rate-limiting enzyme in glycogen synthesis, thus allowing for more glycogen to be produced [31]. Western blot data showed no significant differences in phosphorylation levels and/or total protein expression of Akt and/or GSK-3 or extracellular signal-regulated protein kinases 1 and 2 (ERK1 and ERK-2) between genotypes (Figure 5). However, when levels of Akt1, an Akt isoform associated with placental growth, were measured, *H19*^{-/-} placentas contained significantly higher levels than *H19*^{+/+} controls ($P < 0.01$, Figure 5A). These results were supported by immunohistochemistry data, which show

that *H19*^{-/-} placentas contain higher Akt1 immunostaining than *H19*^{+/+} placentas in both labyrinth and basal zones (Figure 5B).

DISCUSSION

The present study demonstrates that *H19* deficiency, or Igf2 over-expression, leads to placental overgrowth, higher numbers of placental glycogen cells, and larger placental glycogen concentrations. The higher number of glycogen cells in *H19*^{-/-} placentas appears to be an important difference between genotypes. Glycogen cells are primarily found throughout the basal zone of the placenta; however, some clusters of glycogen cells are also found in the labyrinth zone [13,30]; they are the main source of glycogen in the placenta [7,29], and also express high levels of Igf2 [30]. Since *Igf2* deficient placentas have lower number of glycogen cells [13], one can suppose that the increased glycogen concentrations found in the *H19*^{-/-} placentas could be a consequence of the higher number of glycogen cells or alternatively a reflection of an increase in glucose influx. Recent studies have shown the importance of placental *Igf2* expression in regulating placental nutrient supplies [33]. When *Igf2* transcription was abolished in the placenta due to a deletion of the P0 promoter, Constancia and collaborators observed a decrease in placental size, followed by a decline in fetal growth and nutrient transport capacity [32]. Therefore, it is possible that excess Igf2 in *H19*^{-/-} placentas may lead to increased transport of nutrients and glycogen stores.

Igf2 mediates most of its effects via the Igf1 and Ins receptors [22,34]. The InsR is expressed in the labyrinth and basal zones of the placenta [23], whereas the Igf1R is most abundantly expressed in the labyrinth zone [11]. In this study we wanted to determine whether these receptors played an important role in placental glycogen synthesis. We found that insulin receptor deficiency does not lead to any alterations in placental weight and/or morphology. In addition, placental glycogen concentrations were similar in both *InsR*^{-/-} and *InsR*^{+/+} placentas, suggesting that the insulin receptor is not essential for placental growth or glycogen synthesis to take place. This was to be expected, since it has been reported that insulin does not play a role in placental glycogen synthesis [9]. However, since Igf2 mediates some of its functions through the InsR [34], we wanted to determine whether excess Igf2 could drive placental growth and glycogen metabolism via this receptor, but our data does not support this view. Likewise, placentas of *Igf1R*^{-/-} mice did not show any alterations in glycogen stores, suggesting that placental glycogenesis is not mediated through this receptor either. Earlier studies have proposed the existence of a placenta Igf2-specific receptor [22,25]. This unknown receptor does not appear to be the Igf2R, since it appears to be mainly associated with Igf2 turnover in the placenta [35]. Louvi and collaborators have suggested that the InsR could be the unknown Igf2 receptor [22]. That may be the case in the fetus but not in placenta, since we observed no differences in placental weights, morphology or glycogen levels between null insulin receptor and WT placentas.

Glycogen synthesis is mediated by a cascade of intracellular signals that leads to an increase in the activity of glycogen synthase, the rate-limiting enzyme for glycogen [36]. Glycogen synthase is usually found in an active (dephosphorylated) and inactive (phosphorylated) state. Akt increases the activity of glycogen synthase by phosphorylating and inactivating

GSK-3 [35]. IGFs are known to act via the phosphatidylinositol 3 kinase (PI-3K)/Akt signaling [20]. Soares and collaborators demonstrated that growth-restricted placentas are associated with a down-regulation of Igf2/Akt signaling [37], suggesting the importance of Akt in Igf2 signaling and placental growth. In mammals, there are three Akt isoforms: Akt1/ PKB α , Akt2/ PKB β , and Akt3/ PKB γ , each encoded by separate genes [38]. Akt1 is widely expressed throughout the placenta and *Akt1*^{-/-} placentas are significantly smaller than those of their WT controls, suggesting the role of Akt1 in placental growth [32]. Since *H19*^{-/-} placentas are significantly larger and express higher Igf2 levels than WT placentas [15], we wanted to determine whether there was any alteration in Akt and/or GSK-3 expression between genotypes. Our data show no significant differences in total Akt and/or GSK-3 expression between *H19*^{-/-} and *H19*^{+/+} placentas; however, significant higher levels of Akt1 were detected in *H19*^{-/-} placentas than in their *H19*^{+/+} controls. Since Akt1 is thought to be involved in placental growth [32], it is likely that higher Akt1 levels in *H19*^{-/-} placentas may contribute to the overgrowth phenotype. It is also possible that the higher concentrations of Akt1 may contribute to the higher levels of glycogen detected in the *H19*^{-/-} placentas. However, since no differences in GSK-3 phosphorylation were found between genotypes, it is possible that Akt1 may not be playing an essential role in placental glycogen synthesis. Akt1^{-/-} placentas, however, are known to have fewer glycogen expressing cells than Akt1^{+/+} placentas, therefore the higher levels of Akt1 found in the *H19*^{-/-} placentas may lead to increased numbers of glycogen cells and to higher glycogen concentrations. Moreover, the increase in placental glycogen stores in *H19*^{-/-} placentas may not due to an increase in glycogen signaling but rather to an increase in the number of glycogen-producing cells. Further *in vitro* studies will help elucidate potential mechanisms by which Igf2 regulates glycogen synthesis in placental cells.

In summary, the data presented in this study demonstrates that *H19* deficiency, or Igf2 over-expression, leads to placental overgrowth, higher numbers of glycogen cells, and greater glycogen concentrations in the placental. The increase in glycogen stores in *H19*^{-/-} placentas are likely to be due to the increase in Akt1 expression and/or to the higher number of glycogen-producing cells. Our data also demonstrate that neither the InsR nor the *InsR*^{-/-} play an important role in placental growth or glycogen regulation since mutant placentas for these genes have normal placental morphology, size and glycogen stores. Defining the role of *Igf2*, *H19*, and other related genes in the regulation of placental carbohydrate metabolism will further advance our knowledge regarding the role of placental glycogen and its effect on fetal growth.

ACKNOWLEDGMENTS

We thank Leva Jensen and Zamir Vrapı their technical assistance. We are particularly grateful to Drs. Shirley M. Tilghman, Mimmo Accili, Argrıs Efstratiadis for providing us with the *H19*, *InsR*, *Igf1R*, and *Igf2* knockout mice, respectively. This work was supported by a Harvard Scholar in Medicine Award and by a NIH grant to MFL [R01 GM071046].

REFERENCES

- Desoye G, Shafrir E. Placental metabolism and its regulation in health and Diabetes. Mol Aspects Med. 1994; 15:505–682. [PubMed: 7700120]

2. Barash V, Shafrir E. Mobilization of placental glycogen in diabetic rats. *Placenta*. 1990; 11:515–21. [PubMed: 2149754]
3. Gabbe SG, Demers LM, Greep RO, Villee CA. Placental glycogen metabolism in diabetes mellitus. *Diabetes*. 1972; 21:1185–91. [PubMed: 4629070]
4. Gebbe SG, Demers LM, Greep RO, Villee CA. The effects of hypoxia on placental glycogen metabolism. *Am J Obstet Gynecol*. 1972; 114:540–5. [PubMed: 4631760]
5. Leonce J, Brockton N, Robinson S, Venkatesan S, Bannister P, Raman V, Murphy K, Parker K, Pavitt D, Teoh TG, Regan L, Burchell A, Steer P, Johnston DG. Glucose production in the human placenta. *Placenta*. 2006; 27(Suppl A):S103–8. [PubMed: 16618444]
6. Barash V, Riskin A, Shafrir E, Waddell ID, Burchell A. Kinetic and immunologic evidence for the absence of glucose-6-phosphatase in early human chorionic villi and term placenta. *Biochim Biophys Acta*. 1991; 1073:161–7. [PubMed: 1846754]
7. Coan PM, Conroy N, Burton GJ, Ferguson-Smith AC. Origin and characterization of glycogen cells in the developing placenta. *Dev Dyn*. 2006; 235:3280–94. [PubMed: 17039549]
8. Espinal, J. Understanding insulin action--principles and molecular mechanisms. Ellis Horwood Limited; West Sussex: 1989. p. 39
9. Shafrir E, Barash V. Placental glycogen metabolism in diabetic pregnancy. *Israel J Med Sci*. 1991; 27:449–60. [PubMed: 1835720]
10. Carter A, Nygard K, Mazzuca D, Han VKM. The expression of insulin-like growth factor and insulin like growth factor binding protein mRNAs in mouse placenta. *Placenta*. 2006; 27:278–290. [PubMed: 16338473]
11. Zhou J, Bondy C. Insulin-like growth factor-II and its binding proteins in placental development. *Endocrinology*. 1992; 131:1230–1240. [PubMed: 1380437]
12. DeChira TM, Efstratiadis A, Robertson EJ. A growth deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature*. 1990; 296:78–80.
13. Lopez MF, Dikkes P, Zurakowski D, Villa-Komaroff L. Insulin-like growth factor II affects the appearance and glycogen content of glycogen cells in the murine placenta. *Endocrinology*. 1996; 137:2100–8. [PubMed: 8612553]
14. Constancia M, Hemberger M, Hughes J, Dean W, Ferguson-Smith A, Fundele R, Steward F, Kelsey G, Fowden A, Sible C, Reik W. Placental-specific IGF-II is a major modulator of placental and fetal growth. *Nature*. 2002; 417:945–8. [PubMed: 12087403]
15. Leighton PA, Ingram RS, Eggenschwiler J, Efstratiadis A, Tilghman SM. Disruption of imprinting caused by deletion of the H19 gene region in mice. *Nature*. 1995; 375:34–9. [PubMed: 7536897]
16. Eggenschwiler J, Ludwig T, Fisher P, Leighton PA, Tilghman SM, Efstratiadis A. Mouse mutant embryos overexpressing IGF-II exhibit phenotypic features of the Beckwith-Wiedemann and Simpson-Golabi-Behmel syndromes. *Genes Dev*. 1997; 11:3128–42. [PubMed: 9389646]
17. Adam GR, Cui H, Miller SJ, Flam F, Ohlsson R. Allele-specific in situ hybridization (ASISH) analysis: a novel technique which resolves differential allelic usage of H19 within the same lineage during human placental development. *Development*. 1996; 839–47. [PubMed: 8631262]
18. Lustig O, Ariel I, Ilan J, Lev-Lehman E, De-groot N, Hochberg A. Expression of the imprinted gene H19 in the human fetus. *Mol Reprod Dev*. 1994; 38:239–46. [PubMed: 7917273]
19. Bartolomei MS, Tilghman SM. Parental imprinting of mouse chromosome 7. *Sem Dev Biol*. 1992; 3:107–17.
20. DeChiara TM, Robertson EJ, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell*. 1991; 4:849–59. [PubMed: 1997210]
21. Jones JI, Clemmons DR. Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev*. 1995; 16:3–34. [PubMed: 7758431]
22. Louvi A, Accili D, Efstratiadis A. Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. *Dev Biol*. 1997; 189:33–48. [PubMed: 9281335]
23. Wang LS, Raizada MK, Shiverick KT. Ontogeny of insulin receptors in the rat hemochorial placenta. *Dev Biol*. 1987; 123:487–93. [PubMed: 3308578]
24. Nakae J, Kido Y, Accili D. Distinct and overlapping functions of insulin and IGF-I receptors. *Endocr Rev*. 2001; 22:818–35. [PubMed: 11739335]

25. Baker J, Liu JP, Robertson EJ, Efstratiadis A. Role of insulin-like growth factors in embryonic and postnatal growth. *Cell*. 1993; 75:73–82. [PubMed: 8402902]
26. Accili D, Drago J, Lee EJ, Johnson MD, Cool MH, Salvatore P, Asico LD, Jose PA, Taylor SI, Westphal H. Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. *Nat Genet*. 1996; 12:106–9. [PubMed: 8528241]
27. Cook, HC. Carbohydrates.. In: Bancroft, JD.; Stevens, A., editors. *Theory and practice of histological techniques*. 2nd edition. Churchill Livingstone, Inc.; New York: 1982. p. 180-216.
28. Lo S, Russell JC, Taylor AW. Determination of glycogen in small tissue samples. *J Appl Physiol*. 1970; 28:214–36.
29. McLaren A. Placental weight loss in late pregnancy. *J Reprod Fertil*. 1965; 9:343–46.
30. Redline RW, Chernicky CL, Tan HQ, Ilan J, Ilan J. Differential expression of insulin-like growth factor-II in specific regions of the late (post day 9.5) murine placenta. *Mol Reprod Dev*. 1993; 36:121–9. [PubMed: 8257562]
31. Avruch J. Insulin signal transduction through protein kinase cascades. *Mol Cel Biochem*. 1998; 182:31–48.
32. Yang Z, Tschopp O, Hemmings-Mieszczak M, Feng J, Brodbeck D, Perentes E, Hemmings BA. Protein kinase B α / Akt1 regulates placental development and fetal growth. *J Biol Chem*. 2003;32124–31. [PubMed: 12783884]
33. Constancia M, Angiolini E, Sandovici I, Smith P, Smith R, Kelsey G, Dean W, Ferguson-Smith A, Sibley C, Reik W, Fowden A. Adaptation of nutrient supply to fetal demand in the mouse involves interaction between the Igf2 gene and placental transporter systems. *PNAS*. 2005; 102:19219–24. [PubMed: 16365304]
34. Rother KI, Accili D. Role of insulin receptors and IGF receptors in growth and development. *Pediatr Nephrol*. 2000; 14:558–61. [PubMed: 10912518]
35. Ludwig T, Eggenschwiler J, Fisher P, D'Ercole AJ, Davenport ML, Efstratiadis A. Mouse mutants lacking the type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in Igf2 and Igf1r null backgrounds. *Dev Biol*. 1996; 177:517–35. [PubMed: 8806828]
36. Devan P. Insulin signaling. *J Cell Sci*. 2001; 114:1429–40. [PubMed: 11282018]
37. Ain R, Canham L, Soares MJ. Dexamethasone-induced intrauterine growth restriction impacts the placental prolactin family, insulin-like growth factor-II and the Akt signaling pathway. *J Endocrinol*. 2005; 185:253–63. [PubMed: 15845918]
38. Brazil DP, Hemmings BA. Ten years of protein kinase B signaling: a hard Akt to follow. *Trends Biochem Sci*. 2001; 26:657–64. [PubMed: 11701324]

Figure 1.

H19 deficiency affects fetal (A) and placental weight (B). The mean values are shown in solid bars for the *H19*^{-/-} mice and hatched bars for *H19*^{+/+} mice. *H19*^{-/-} fetuses weighed significantly more on embryonic days 15 (n=9) and 18 (n=19), and at birth (n=34) compared to *H19*^{+/+} fetuses (n= 15, 11, 47, respectively). *H19*^{-/-} placentas also weighed significantly more on embryonic days 15 (n=24) and 18 (n=12) compared to *H19*^{+/+} controls (n= 15 and 12, respectively). Data are mean ± SEM. * P < 0.01. Notice that gestational decrease in weight occurred in *H19*^{+/+} but not in *H19*^{-/-} placentas on day 18 of gestation **P < 0.01.

Figure 2. Igf2 expression in *H19*^{+/+} and *H19*^{-/-} placentas determined by immunohistochemistry (A-B) and western blotting (E,F). Representative image of a placental section immunostained for Igf2 from *H19*^{+/+} (A) and *H19*^{-/-} (B) mice. Steady-state levels of Igf2 protein were also determined in both genotypes by Western blotting (C). Densitometric quantification of the bands was carried out using NIH Image 1.6. (D). Numerical values are presented as the mean $\pm$ SEM. * P < 0.05. B, basal zone; L, labyrinth zone

Figure 3.

Glycogen levels in *H19*^{+/+} and *H19*^{-/-} placentas. A. Best Carmine glycogen staining in the basal zone of *H19*^{+/+} and *H19*^{-/-} placentas B. Hatched bars represent the mean glycogen concentration for *H19*^{+/+} placentas and solid bars for *H19*^{-/-} placentas on days 15 and 18 of gestation. Numerical values are presented as the mean $\pm$ SEM (n= 7-8 placentas/genotype/gestational age). *P < 0.05. G, giant cells; Sp, spongiotrophoblast cells; Gl, glycogen cells; B, basal zone; L, labyrinth zone.

Figure 4.

Western blot analysis data show that *H19* deficiency does not result in significant differences in total Akt (A), GSK-3 (B) or ERK (C) protein expression or levels of phosphorylation. Densitometry data (arbitrary units) are means $\pm$ SEM of three placentas collected from three different litters.

Figure 5.

Akt1 expression in *H19*^{+/+} and *H19*^{-/-} placentas. A. Akt1 protein levels determined by Western blotting. Densitometric quantification of the bands was carried out using NIH Image 1.6. Numerical values are presented as the mean ± SEM. * *P* < 0.05. B. Representative image of placental sections immunostained with Akt1 obtained from *H19*^{+/+} and *H19*^{-/-} mice. Sp, spongiotrophoblast cells; Gl, glycogen cells; Ct, cytotrophoblast cells.

TABLE 1

Mean number of placental cells on day 15 of gestation

	H19 ^{+/+}	H19 ^{-/-}
Giant cells ^b	35 ± 4	70 ± 12
Spongiotrophoblasts ^b	2,528 ± 246	3,074 ± 440
Glycogen cells ^{a,b}	1,822 ± 412	4,667 ± 593
Glycogen/spongiotrophoblasts ^a	0.72	1.52

^a Statistical different

^b Numbers were derived from the basal zone