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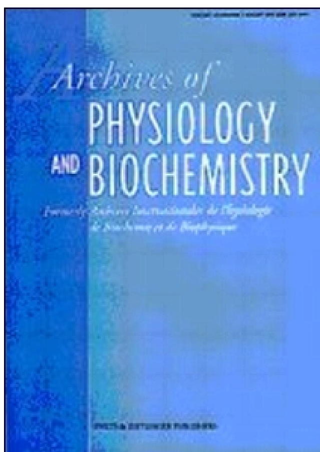

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Influence of murine maternal diabetes on placental morphology, gene expression, and function

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ORIGINAL ARTICLE

Influence of murine maternal diabetes on placental morphology, gene expression, and function

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Abstract
Maternal diabetes causes placental and foetal abnormalities in both rat and humans; however, its effect is less well documented in the mouse. We used a standard approach to induce manifest diabetes in pregnant mice and assessed morphology, function and gene expression in the placentas isolated from these females. We found that diabetic placentas exhibit a consistent abnormal phenotype characterized by increased junctional zone cross sectional area. Lipid profiling of diabetic foetuses and placentas showed that the placental phenotypes do not compromise the lipid transport function of this organ. In a genome-wide survey of mRNA expression by using cDNA micro-arrays, we identified 118 ESTs, corresponding to 59 annotated genes, with differential expression in the diabetic placentas. A significant proportion of these known is involved in metabolism, immunity and defence, and signal transduction. In addition, we found two imprinted genes, *Igf2* and *Gatm*, which exhibited altered expression. The expression of other imprinted genes, *Peg1, Gtl2, Peg3, Igf2r* and *Grb10*, was determined by quantitative RT-PCR. For all of these genes, slight changes in gene expression were observed between diabetic placentas and control placentas. Our study thus provides the basis for future work that will address gene action in the diabetic mouse placenta.

Key words: *Mouse placenta, maternal diabetes, morphology, function, gene expression.*

Introduction

In humans, maternal diabetes is still a major cause for birth defects and perinatal mortality, even though the rate of most other complications associated with diabetes has been strongly reduced (Reece and Homko, 1993; Garner, 1995; Eriksson *et al.*, 2003; Zhao *et al.*, 2005). Thus, the expected incidence for congenital malformation may be five to six times higher in diabetic compared to non-diabetic pregnancy (see Eriksson *et al.*, 2003 and references therein). Given the magnitude of the problem, it is not surprising that embryonic and foetal development in diabetic pregnancy has been studied extensively in both clinical (Mølsted-Pedersen *et al.*, 1964; Ray *et al.*, 2001) and experimental (Lazarow *et al.*, 1960; Wentzel *et al.*, 2001) studies. In brief, there is conclusive evidence that hyperglycaemia per se acts as primary teratogen (Styrud *et al.*, 1995; Moley *et al.*, 1998a, 1998b), but that other factors, such as inositol depletion (Baker *et al.*, 1990), alterations of arachidonic acid (Goldman *et al.*, 1985), and oxidative stress (Baynes, 1991) are also causative in abnormal embryonic and foetal development. Especially the sustained production of reactive oxygen species (ROS) caused by maternal hyperglycaemia seems to be a major additional teratogenic factor, as treatment with antioxidants, such as vitamins E and C, or addition of scavenging enzymes such as superoxide dismutase, catalase, or glutathione peroxidase, to embryo culture media reduce embryonic malformations in the diabetic rat model (reviewed in Eriksson *et al.*, 2003).

Maternal diabetes is known to target not only foetal but also placental development in humans (Casson *et al.*, 1997; Evers *et al.*, 2000; Evers *et al.*, 2003). Frequent placental abnormalities associated

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with maternal diabetes are chorangiosis and increased presence of nucleated foetal red blood cells, a sign of chronic foetal hypoxia (Fox, 1967; Green and Mimouni, 1990; Benirschke and Kaufmann, 1998). Another frequently observed abnormality is placental immaturity and extensive villous immaturity coupled with compromised function due to increased diffusion distance between intervillous space and foetal capillaries (Laurini *et al.*, 1987), which may ultimately lead to antenatal foetal aspyxia and foetal death (Fox *et al.*, 1978; Laurini *et al.*, 1987; Greco *et al.*, 1989). Increased placental weight may be one mechanism to compensate for villous immaturity and functional deficits (Evers *et al.*, 2003). Abnormally increased placental weight is also observed in a standard animal model of diabetes, streptozotocin (STZ)-induced diabetes in the rat (Gewolb *et al.*, 1986; Padmanabhan and Shafiullah, 2001). Like the human diabetic placenta, the rat diabetic placenta exhibits consistent and specific morphological alterations, apart from increased weight. One characteristic feature is a strikingly increased abundance of glycogen-containing cells (GlCs) in the decidua basalis, the spongiotrophoblast, and the labyrinth, and of giant cells in the junctional zone (Padmanabhan and Shafiullah, 2001). Interestingly, accumulation of glycogen is also a feature of the human diabetic placenta (Jones and Desoye, 1993; Desoye *et al.*, 2002). Furthermore, the spongiotrophoblast layer is thickened in the diabetic rat placenta as compared to controls. Of direct functional relevance is the finding that the trophoblast layers of the interhemal membrane are significantly thicker in diabetic placentas (Gewolb *et al.*, 1986; Padmanabhan and Shafiullah, 2001). Together with the distortion of the labyrinth, caused by ingrowth of spongiotrophoblast pegs, cystic changes of the spongiotrophoblast, and reduced arborization of the foetal vasculature, this could contribute significantly to compromised materno-foetal exchange and thus to foetal growth restriction (Gewolb *et al.*, 1986; Padmanabhan and Shafiullah, 2001). Thus, the increased weight of the diabetic rat placenta, like that of the human diabetic placenta, may again be a compensatory mechanism to alleviate its functional deficits (Gewolb *et al.*, 1986; Padmanabhan and Shafiullah, 2001).

Unfortunately, little is known to date about the effects of gestational diabetes on placental development and function in the mouse, as compared to rat and human. This deficit has become the more deplorable, as the mouse, due to the facility of gene targeting, has become the standard mammalian model to assess gene function. We therefore used a standard method to induce diabetes in pregnant mice and then assayed placental morphology, function and gene expression. Our results show that severe diabetes induces mild histological changes in the mouse placenta, which however do not seem to interfere with its transport function. Finally, we have identified a considerable number of genes with altered expression in diabetic placentas, which are therefore candidate genes for further analysis.

Materials and methods

Experimental animals

All experiments with mice were conducted according to the guidelines issued by Uppsala University. Four mouse strains, selected for availability, were used in our experiment, C57BL/6, BALB/c, DBA/1 and CBA/J (B&K, Stockholm), to rule out the possibility that effects of gestational diabetic differ between different mouse strains. Diabetes in female mice was experimentally induced by streptozotocin (STZ, Sigma). After 6 hours of starving, a single dose of STZ (200 mg/kg of body weight) dissolved in sterile saline was administrated by intraperitoneal injection. Control mice were injected with an equal volume of saline. On days 3 and 7 after injection, blood glucose concentration was measured in blood from tail vein using a glucose meter (Accu-Check Sensor, Roche). Mice with glucose concentration exceeding 11.1 mmol/l (200 mg/dl) were regarded as diabetic, with glucose concentrations between 11.1 and 16.7 mmol/l (300 mg/dl) defined as moderate diabetes and concentrations above 16.7 mmol/l defined as severe diabetes (Padmanabhan and Shafiullah, 2001). Such diabetic female mice were mated either on day 3 or day 7, depending on blood glucose concentration, with healthy males of the same strain. In case of BALB/c and C57BL/6 each, 15 mice were in the diabetic group and 5 in the control group. For both DBA/1 and CBA/J, 5 females were included in each group. All females were killed on E18 of gestation [counting the day of the vaginal plug as day 1 (E1) of gestation]. Mice were sacrificed by cervical dislocation and conceptuses were dissected out. Maternal and foetal blood glucose levels were determined. For the determination of placental and foetal wet weights, conceptuses from both moderately and severely diabetic females were used, however, for all further studies, only foetuses and placentas isolated from females with blood glucose levels of $\geq$16.7 mM were used. Foetuses and placental halves were frozen in liquid nitrogen and stored at -80°C until further processing. For histomorphological study, the other half of the placenta was fixed in cold Serra's fixative (60% ethanol, 30% formalin and 10% glacial acetic acid) at 4°C overnight and processed for paraffin embedding.

Histology and semi-quantitative morphometry

For visual assessment of placentas, hematoxylin-eosin (HE) and isolectin B4 staining were performed on paraffin-embedded sections (7μm) from placentas of control and diabetic mice. Altogether 80 sections from 10 control and 27 diabetic placentas were used: from C57BL/6 15 diabetic, four control placentas;

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from BALB/c 5 diabetic, two control; from DBA/1 4 diabetic, two control; and from CBA/J 3 diabetic, two control. Both male and female placentas were used for this analysis. A maximum of two placentas per litter were selected randomly and in most cases two sections per placenta derived from the midline were measured. Isolectin B4 staining was performed as described by Singh *et al*. (2005). Biotinylated isolectin B4 and DAB substrate kit were obtained from Vector Labs and Streptavidin-HRP conjugate was purchased from Perkin Elmer. Semi-quantitative morphometry on 38 sections stained with isolectin B4 was performed as previously described by Salas *et al.* (2004) using Adobe Photoshop CS. These 38 sections were derived from all different mouse strains and encompassed male and female placentas. Percentage of total section area occupied by the spongiotrophoblast, including the glycogen cells, and labyrinthine layer was calculated. Giant cells per section were counted on the same 38 sections. Quantification of glycogen cells was performed on 20 isolectin B4 stained sections from normal and diabetic placentas; glycogen cells were identified by their characteristic appearance, that is, their translucent cytoplasm and small, densely stained nuclei. 10 placentas were selected from each group, using both C57BL/6 and DBA/2 genotypes, and one section per placenta, derived from the midline, was assessed.

RNA extraction, micro-array hybridizations and data analysis

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Two pairs of purified RNA samples, derived from male placentas (Abs 260nm/Abs 280 nm $>$ 1.7) (Appendix, Table A1) were used for micro-array based expression profiling. Only male placentas were used to avoid possible sex specific effects of maternal diabetes on placental development. No pooling of samples was performed. Commercially available Mouse 15K (M15K) micro-arrays were obtained from University Health Network (UHN), Ontario Cancer Institute, Canada. Detailed information about these micro-arrays is available at the following link: http://www.microarrays.ca/products/types.html. The TSA-MICROMAX kit (NEN, Perkin Elmer, USA) was used for synthesizing labelled target cDNA. All steps of the protocol were done according to the manufacturer's instructions with some modifications. The hybridizations were performed using the DIG Easy Hyb (Roche). We used 1 μg of Cot1 DNA per hybridization. Tween 20 in the TNT buffer was used at a concentration of 0.1% v/v instead of 0.05% v/v described in the kit. Dye swaps for all hybridizations were performed simultaneously. The list of expressed sequence tags (ESTs) on the micro-array was supplied by the UHN as a clonetracker file and a Gene Array List (GAL) file was generated according to the format described for ScanArray Express, version 2.1.0. Hybridized and labelled micro-arrays were scanned immediately after washings using the ScanArray 4000 micro-array scanner (Packard BioChip Technologies) and ScanArray Express version 2.1.0 was used for generating the GPR files.

The data were stored and analyzed at the micro-array data storage and analysis facilities BASE and Data Ware House (DWH) respectively, run by the Linnaeus Centre for Bioinformatics (LCB), BMC, Uppsala University, Uppsala, Sweden (detailed information at http://www.lcb.uu.se, http://base.lcb.uu.se and http://dw.lcb.uu.se). Briefly, the data in the GPR files was subjected to background subtraction and within array Print-tip Lowess normalization implemented in R, filtered for different ranges of M values and for occurrences of a reporter in selected number of assays. Signal on control spots with 3 $\times$ SSC and Arabidopsis DNA were used to remove spots with non-specific hybridization. However, no flags were applied. Significance of variation of expression was calculated for each filtered reporter, by using the B statistics implemented in R Bioconductor. The LCB BASE and DWH facilities comply with the MIAME guidelines.

Quantitative RT-PCR (qRT-PCR)

RNA was treated with RNase-free DNase (Promega) and reverse transcribed using M-MLV reverse transcriptase and random primers (Promega) as described previously (Shi *et al*., 2004a). Primers were designed using online freeware Primer3. qRT-PCR was performed using QuantiTect SYBR green PCR mix (Qiagen) as described (Shi *et al*., 2004b). The primers used in qRT-PCR are listed in Appendix, Table A2. PCR conditions were as follows: initial denaturation 95°C for 15 min followed by 40 cycles of 95°C for 20 s, 58°C for 20 s and 72°C for 30 s. Melting curve analysis were conducted between 65°C and 95°C. PCR reactions were run on a Rotorgene 3000 (Corbett Research). All samples were analysed in duplicate. The ratio of the expression of genes to *Actb*, many ESTs of which had shown no differential expression between diabetic and normal placentas in the micro-array hybridization, was calculated by averaging the folds change (calculated by $\delta\delta$Ct method) value for each duplicate sample. A minimum PCR efficiency of 1.7 was considered for analysis. Seven sample pairs, all derived from C57BL/6 and including the 2 pairs used in the micro-array hybridizations were used for qRT-PCR. This sample contained 4 male and 3 female pairs.

In situ hybridization

Non-radioactive *in situ* hybridization was carried out on the placentas as described (Anson-Cartwright

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et al., 2000). Briefly, *in situ* probes were generated by using RT-PCR. These were then cloned into pGEMT Easy vector (Promega). Digoxigenin (Roche) labelled sense and anti-sense riboprobes were synthesized by *in vitro* transcription using SP6 or T7 RNA polymerases. Sections were hybridized with anti-sense or sense probes at 50–53°C overnight. After high-stringency washes, sections were incubated with alkaline phosphatase conjugated anti-Digoxigenin antibody (Roche) overnight. Colour reaction was carried out as required for clear staining. Primers used for generating probes were as follows: *Igf2*, 5′-CGGGGATCCGAGAGGGACG TGTCTACCTCTC-3′ and 5′-CCGGAATTCGAG CTCTTTGGCAAGCATGCGACCCC-3′; *Gatm*, 5′-ATCCTGATGGTTGTGGGAAA-3′ and 5′-GG TCCATCCTGCTTTCTTGA-3′. Sections used for *in situ* hybridization were derived from 2 male C57BL/6 placentas.

Immunohistochemistry

Sections were deparaffinized and incubated with rabbit anti-rat decidual/trophoblast prolactin-related protein (DTPRP) at 1:4000 dilution in phosphate buffered saline containing 0.1% Triton X-100 (Rasmussen *et al.*, 1996; Orwig *et al.*, 1997). Immunolocalization of DTPRP was determined using Histostain SP AEC kit (Zymed, South San Francisco, CA). Immunostained sections were counterstained with hematoxylin. For DTPRP immunostaining, sections from two diabetic and one control placenta, all male, were used.

Lipid extraction and analysis

Total lipid was extracted and purified from 16 C57BL/6 E18 fetuses and their placentas, eight diabetic and eight controls, derived from two diabetic and two control pregnancies using methanol/chloroform reagent according to a standardized protocol (Bligh and Dyer, 1959; Wuhrer *et al.*, 2000). Both diabetic and control groups contained four males and four females. Lipids were treated with 500 μl of 1 M HCl and 10 M H_2O in methanol for 16 h at 100°C according to Gaver and Sweeley (1965). Fatty acids, released as their methyl esters, were recovered by a three-fold phase partition using *n*-hexane. Fatty acid methyl esters were analysed by gas chromatography/mass spectrometry using a VF 5ms capillary column (60 m, 0.25 mm inner diameter, 0.1 μm film thickness; Varian, Darmstadt Germany) and 2.5 ml/min helium as carrier gas. Temperature was maintained at 50°C for 2 min, raised to 130°C with 40°C/min and to 300°C with 6°C/min and was finally maintained at 300°C for 5 min. Fatty acid compounds were registered by mass spectrometry in the positive ion mode after chemical ionization with methanol using an PolarisQ instrument (ThermoQuest Analytical Systems).

Statistical Analysis

Statistical analysis was carried out by *t*-test using GraphPad Prism 3.0 (San Diego, CA). A value of $p < 0.05$ was considered significant. Data are presented as mean ± SD.

Results

Development of foetuses and placentas of diabetic mouse females

No significant difference was observed in litter-size between normal (7.8 ± 4.1, $n = 11$) and diabetic (7.4 ± 2.3, $n = 14$) pregnancies ($p > 0.05$). From these 25 litters, altogether 182 placentas were collected, 80 from controls and 102 from diabetic females. The latter were further sub-divided into two groups, originating either from moderately diabetic females, with blood glucose levels between 11.1 and 16.7 mM (200 and 300 mg/dl), or from highly diabetic females, with blood glucose levels ≥16.7 mM (300 mg/dl). As shown in Table I, placental weights were not significantly reduced at E18 of gestation in diabetic pregnancies. In contrast to this, foetal weights were significantly reduced in highly diabetic females, independent of maternal strain (Table II) and foetal sex (data not shown). Foetuses from these pregnancies also exhibited increased blood glucose levels (18.4 ± 12.4 mM), when compared to control pregnancies (0.9 ± 0.2 mM). While there was a tendency for reduced foetal weight in moderately diabetic pregnancies, this was not significant (Table II). However, higher resorption rates were observed in diabetic pregnancies (2.2 ± 0.7/litter) as compared to normal pregnancies (0.7 ± 0.5/litter).

Placental morphology

To determine if placentas from highly diabetic pregnancies exhibited altered morphology compared

Table I. Placental weights in non-diabetic and diabetic mice.

Mouse strain	Placenta weight (mg)		
	Control group	Moderate diabetes[a]	Severe diabetes[b]
Balb/c	95.1 ± 8.4 ($n = 18$)	98.5 ± 11.3 ($n = 11$)	88.2 ± 14.9 ($n = 9$)
C57BL/6	114.6 ± 17.4 ($n = 26$)	118.4 ± 7.6 ($n = 18$)	107.5 ± 22.4 ($n = 25$)
CBA/J	116.2 ± 7.8 ($n = 13$)	117.4 ± 13.4 ($n = 9$)	104.1 ± 16.3 ($n = 10$)
DBA/1	125.7 ± 7.3 ($n = 23$)	126.3 ± 16.5 ($n = 8$)	112.6 ± 24.8 ($n = 12$)

Values are given as means ± SD. Differences between non-diabetic, moderately diabetic and severely diabetic placentas were not significant. [a]maternal blood glucose 11.1–16.7 mM; [b]maternal blood glucose > 16.7 mM.

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with control placentas, paraffin histology was performed. Visual analysis of 80 sections from 10 control and 27 diabetic placentas suggested that severe maternal diabetes in the mouse results in moderately enlarged spongiotrophoblast and increased numbers of giant cells. This impression was confirmed by semi-quantitative morphometric analysis of 38 isolectin B4 stained sections, which clearly showed significant spongiotrophoblast enlargement in all diabetic placentas (Figure 1B). The spongiotrophoblast, including glycogen cells, constituted $20.9 \pm 0.5\%$ of the section area of control placentas but $28.5 \pm 0.9\%$ of the section area of diabetic placentas ($p < 0.05$). Semi-quantitative morphometry showed that glycogen cell differentiation was strongly increased in the diabetic placentas, where they formed $16.7 \pm 2.1\%$ of section area, as compared to $7.5 \pm 1.3\%$ in control placentas. In diabetic placentas, but not control placentas, glycogen cells were frequently seen in the labyrinth of diabetic placentas (Figure 1D,F; Figure 2G). In addition, diabetic placentas had significantly more giant cells per section, 41.9 ± 6.4, than control placentas, 29.4 ± 7.0 ($p < 0.05$; Figure 1C–F). Thus, E18 mouse placentas from type I diabetic females exhibit some of the phenotypes that were seen in late-gestation rat placentas from diabetic pregnancies.

Table II. Fetal weight in non-diabetic and diabetic mice.

Mouse strain	Control (mg)	Moderate diabetes (mg)	Severe diabetes (mg)
Balb/c	852 ± 78.4 ($n = 18$)	825 ± 90.3 ($n = 11$)	716 ± 41.3^{a} ($n = 9$)
C57BL/6	845 ± 48.3 ($n = 26$)	833 ± 39.4 ($n = 18$)	665 ± 54.6^{b} ($n = 25$)
CBA/J	816 ± 68.7 ($n = 13$)	799 ± 37.6 ($n = 9$)	684 ± 42.9^{a} ($n = 10$)
DBA/1	856 ± 87.3 ($n = 23$)	820 ± 93.1 ($n = 8$)	609 ± 29.2^{c} ($n = 12$)

Values are given as means ± SD. Moderate and severe diabetes defined as for table I. $^{a}p < 0.05$, $^{b}p < 0.005$ and $^{c}p < 0.001$.

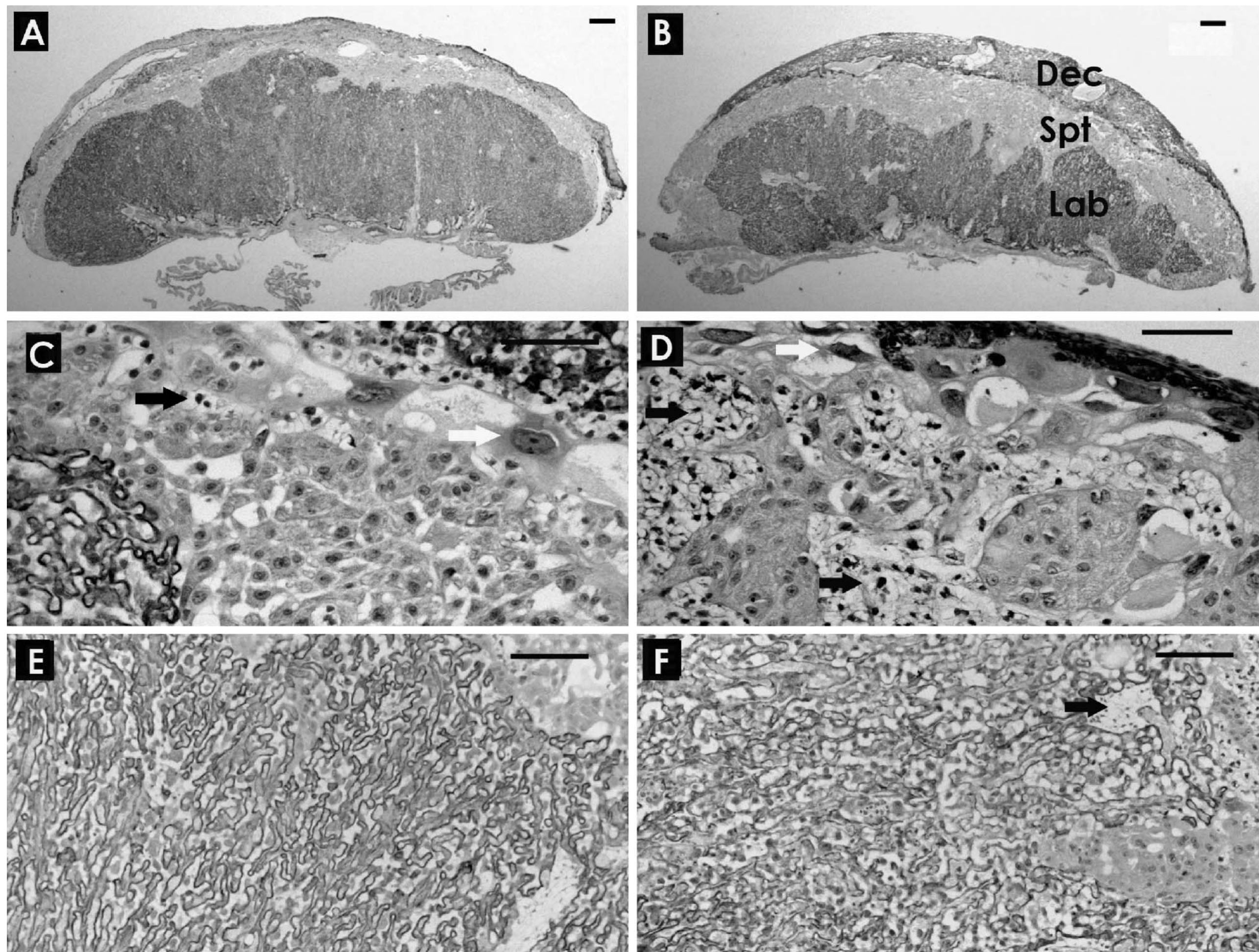

Figure 1. Morphological analysis of diabetic and control placentas. A, B, C, D, E and F: isolectin B4 staining. A: control placenta. B: diabetic placenta. C and D: spongiotrophoblast layer in control and diabetic placenta. E and F: labyrinth layer in control and diabetic placenta. Black arrows show glycogen cells, white arrows show giant cells. Scale bar: 1 mm. Dec, decidua; Spt, spongiotrophoblast; Lab, labyrinth.

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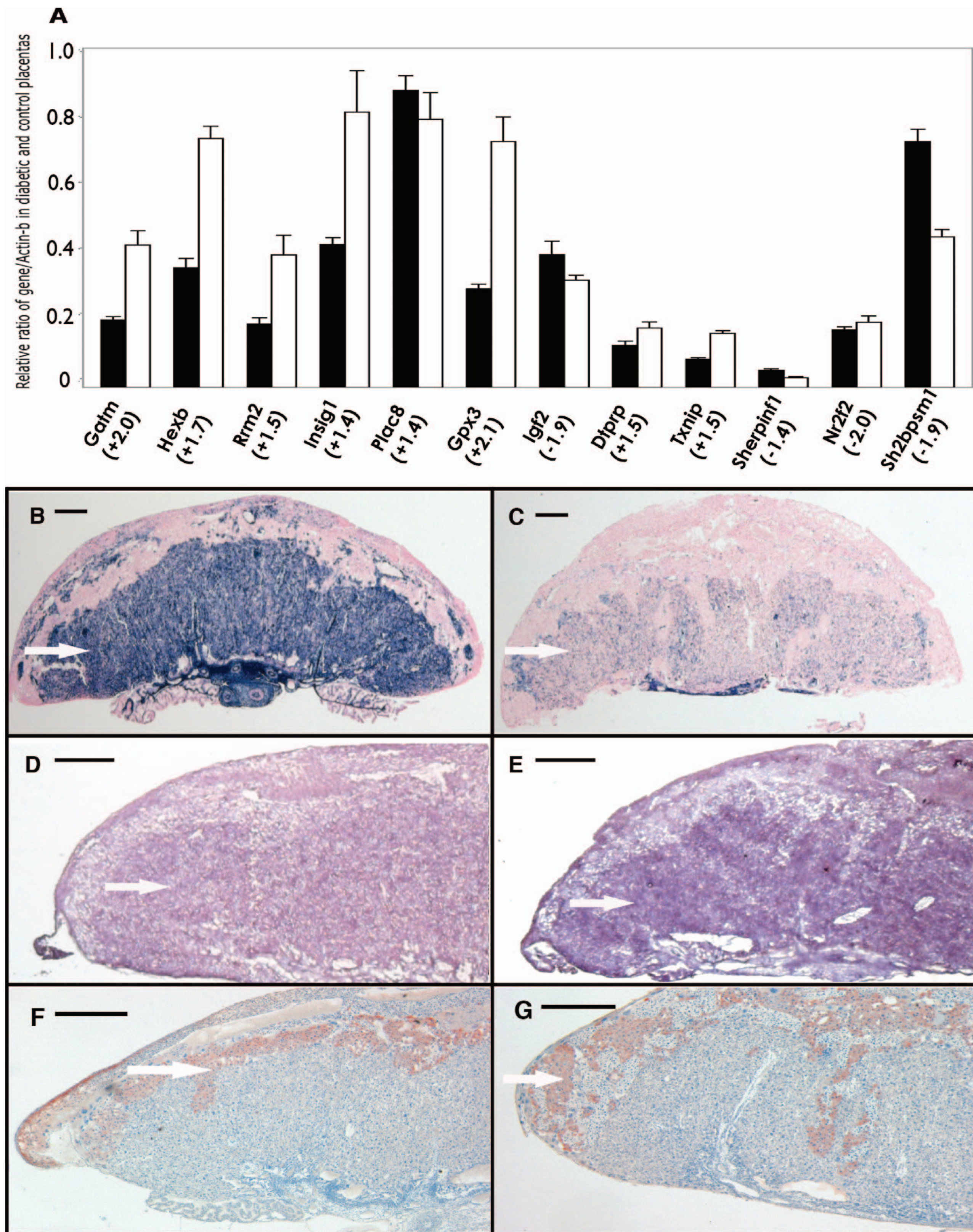

Figure 2. Quantitative RT-PCR, ISH and IHC analysis of diabetic and control placentas. A: quatitative RT-PCR. Black bar: gene expression level in control placentas; White bar: gene expression level in diabetic placentas. Gene names and fold changes obtained in the microarray hybridizations are shown below the *X*-axis. B and C: *Igf2* in situ hybridization in control and diabetic placentas. D and E: *Gatm* in situ hybridization in control and diabetic placentas. F and G: DTPRP immunohistochemistry staining in control and diabetic placentas. Arrows show positive signal. Scale bar: 1 mm.

Function of placentas from diabetic pregnancies

The over-representation in diabetic placentas of spongiotrophoblast at the expense of the labyrinth, which is responsible for nutrient transfer, suggested that placental transport functions could be compromised. Thus, one parameter of placental transport

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was assessed by determining the accumulation of the essential fatty acids (EFAs) linoleic acid (18:2), arachidonic acid (20:4), and docosahexaenoic acid (22:6), and the non-essential fatty acids (NEFAs) palmytic acid (16:0) and stearic acid (18:0). EFAs are obtained through the mother's diet and are therefore transported to the foetal organism via the placenta. NEFAs are synthesized in the foetal organism. With this approach, compromised placental transport function is indicated by distorted ratios of EFAs and NEFAs in favour of the latter (Wu *et al.*, 2003). Our lipid measurements provided no evidence for defective lipid transport function of diabetic placentas. Thus, in control E18 foetuses the EFAs made up $31.5 \pm 10.0\%$ ($N = 8$) of all FAs. In diabetic fetuses this value was 29.0 ± 11.9 ($N = 8$; $P = 0.327$). In the placentas from the same conceptuses, these values were $20.4 \pm 5.1\%$ and $17.3 \pm 9.8\%$, for control and diabetic placentas, respectively ($P = 0.07$). However, this finding does not exclude the possibility that other transport systems are affected in the diabetic placentas.

Gene expression in placentas from diabetic females

Expression profiles of two male placentas each from severely diabetic and control E18 C57BL/6 pregnancies were compared by cDNA micro-array hybridization. At thresholds of B value > -4 (Appendix, Figure A1), 118 ESTs exhibited differential expression consistently in all four hybridizations and also for duplicate spots representing each EST on the micro-arrays with a minimum fold change of 1.3. Of these differentially expressed ESTs, 28 were up-regulated and 90 were down-regulated. As inferred from NCBI UniGene and Gene Ontology databases, for 59 of these 118 ESTs, no precise functional annotation is available. The remaining 59 ESTs represented 10 biological process clusters as defined by using http://www.pantherdb.org/. The differentially expressed genes could be classified into the following major functional groups: metabolism ($n = 20$), signal transduction ($n = 13$), and immunity and defence ($n = 8$). A list of functional categories, accession numbers, and folds changes of all annotated, differentially expressed ESTs is given in Table III. Two imprinted genes, *Igf2* and *Gatm* were also found deregulated in the diabetic placentas compared to controls. While *Igf2* was decreased in expression, *Gatm* exhibited an increase in expression. Both of these genes are implicated in placental growth. Whereas for *Igf2* a direct role in placental growth has been proven (Constância *et al.*, 2002), *Gatm* was found under-expressed in some other models of placental hyperplasia (Singh *et al.*, 2004). For 12 genes, *Gatm*, *Hexb*, *Rrm2*, *Insig1*, *Plac8*, *Gpx3*, *Igf2*, *Txnip*, *Sh2bpsm1*, *Sherpinf1*, *Nr2f2* and *Dtprp*, micro-array hybridization results were controlled by qRT-PCR, and, for *Igf2*, *Gatm*, and *Dtprp*, by in situ hybridisation (ISH) or immunohistochemistry (IHC) analysis as well. As only two pairs of E18 diabetic/control placentas had been used for micro-array analysis, an additional five pairs were used in qRT-PCR. With exception of *Plac8* and *Nr2f2*, qRT-PCR largely supported the results of the micro-array hybridizations (Figure 2A). However, for some genes such as *Igf2* and *Rrm2*, fold-change values differed considerably. Down-regulation of *Igf2*, specifically in the labyrinth of diabetic placentas and in glycogen cells, was also clearly visible by ISH (Figure 2B, C). The fact that signal strengths were comparable in yolk sac of diabetic and normal placentas demonstrated that reduced *Igf2* expression was specific for the placenta. *Gatm* up-regulation was also clearly visible by ISH (Figure 2D, E). DTPRP immunostaining intensity also seemed to be stronger in spongiotrophoblast of diabetic than of control placentas (Figure 2G, F), however, the difference was not as strong as with *Igf2* and *Gatm*. As imprinted genes are important in placental development (Coan *et al.*, 2005), relative transcript levels were determined by qRT-PCR for the imprinted genes *Peg1*, *Gtl2*, *Peg3*, *Igf2r* and *Grb10*, which are not present on the micro-array, in the same seven pairs of diabetic and control placentas. For *Peg1*, *Gtl2*, *Peg3*, and *Igf2r* a tendency towards up-regulation was observed, with fold changes of 1.21 ± 0.22, 1.30 ± 0.29, 1.38 ± 0.33, and 1.42 ± 0.45, respectively. *Grb10* exhibited a tendency to down-regulation in diabetic placentas with a fold-change of 0.82 ± 0.25.

Discussion

While it is known that maternal diabetes causes abnormal placental development in humans and in the rat, much less information is available on placental development and function in the mouse. This lack of information is the more deplorable, as in many aspects, such as accessibility to genetic manipulation, the mouse is a far superior animal model compared to the rat. We therefore performed a first analysis, encompassing histo-morphological, functional, and gene expression approaches, on late gestation placentas isolated from female diabetic mice.

Our results show conclusively that even severe maternal diabetes causes a relatively mild placental phenotype characterized by spongiotrophoblast enlargement, and increased numbers of glycogen and giant cells. These phenotypes are comparable to those observed in placentas of diabetic rats (Gewolb *et al.*, 1986; Padmanabhan and Shafiullah, 2001), albeit much less severe. Namely, the significant placental over growth, which is observed in the rat at a comparable gestational age to mouse E18 (Robinson *et al.*, 1988) was not seen by us in any of the mouse strains that were used to induce diabetes. It is at present not clear why the rat and the mouse placenta react differently to maternal diabetes; however, the weak phenotypes of the diabetic mouse placenta explain the apparent absence of functional deficits as

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Table III. Genes differentially expressed in diabetic placentas.

Category	Numbers (*N*)	Genes	Accession number	Folds change
Angiogenesis	1	Serine (or cysteine) proteinase inhibitor clade F) member 1 (Serpinf1)	BG 074697	−1.4
Apoptosis	2	Mitochondrial carrier homolog 1 (Mtch1)	BG 075113	−1.5
		Programmed cell death protein 7 (Pdcd7)	BG 066710	−2.2
DNA repair	1	RAD1 homolog (S. pombe) (Rad1)	BG 063699	−1.4
Immunity	2	Tumor rejection antigen gp96 (Tra1)	BG 065045	−1.5
		Histocompatibility 2 D region locus 1 (H2D1)	BG 071219	1.6
Metabolism	15	Ribonucleotide reductase M2 (Rrm2)	BG 064808	1.5
		Glutathione peroxidase 3 (Gpx3)	BG 073718	2.1
		Phospholipase A2 group VII (platelet activating factor acetylhydrolase plasma) (Pla2g7)	BG 073623	−1.8
		Thioredoxin interacting protein (Txnip)	BG 073888	1.5
		Peroxiredoxin 4 (Prdx4)	BG 075325	−1.6
		Aldo keto reductase (aldehyde reductase) family 1 member A4 (Akr1a4)	BG 075244	−1.5
		Glutamate oxaloacetate transaminase 2 mitochondrial (Got2)	BG 076244	−1.3
		Spastic paraplegia 4 homolog (human) (Spg4)	BG 074537	−1.8
		Glycine amidinotransferase (L arginine glycine amidinotransferase, Gatm)	BG 074311	2
		Acyl CoA monoacylglycerol acyltransferase 2 (Loc2)	BG 073465	1.4
		Cytochrome P450 family 11 subfamily a polypeptide 1 (Cyp11a1)	BG 071067	1.4
		Hexosaminidase B (Hexb)	BG 069642	1.5
		Insulin induced gene 1 (Insig1)	BG 067488	1.4
		ADP ribosylarginine hydrolase (Adprh)	BG 064935	−1.6
		SH2 B domain containing signaling mediator 1 (Sh2bpsm1)	BG 073987	−1.9
Oxygen transport	2	Hemoglobin alpha adult chain 1 (Hbaa1)	BG 066536	−1.5
		Hemoglobin beta adult major chain (Hbbb1)	BG 072639	−2.1
Peptide hormone	2	Insulin like growth factor 2 (Igf2)	BG 064815	−1.9
		decidual or trophoblast prolactin related protein (Dtprp)	BG 064136	1.5
Protein metabolism	7	Ribosomal protein L3 (Rpl3)	BG 066297	−1.5
		Ribosomal protein S25 (Rps25)	BG 073074	−1.3
		Calnexin (Canx)	BG 064003	−1.4
		DNA segment Chr 5 Wayne State University 45 expressed (D5Wsu45e)	BG 074700	−2.0
		Serine protease inhibitor 1 (Serpina1e)	BG 070053	1.9
		Protein disulfide isomerase related (Pdir)	BG 070874	−1.3
		Eukaryotic translation initiation factor 1A (Eif1a)	BG 072709	−1.4
Protein transport	2	Sorting nexin 2 (Snx2)	BG 073785	−1.7
		Vacuolar protein sorting 45 (yeast) (Vps45)	BG 074740	−1.4
RNA interference	1	DNA segment Chr 12 ERATO Doi 7 expressed (D12Ertd7e)	BG 065647	−2.0
Signal transduction	3	Nuclear receptor subfamily 2 group F member 2 (Nr2f2)	BG 073706	−2
		Prostaglandin E receptor 2 (subtype EP2) (Ptger2)	BG 073859	−1.6
		Lobe homolog like (Drosophila) (Lobel)	BG 063209	1.4
Structural protein	4	Adducin 3 (gamma) (Add3)	BG 073394	−2.1
		Kelch like 2 Mayven (Drosophila) (Klhl2)	BG 074165	−1.9
		Cadherin 1 (Cdh1)	BG 069329	1.4
		Epithelial protein lost in neoplasm (Eplin)	BG 066187	−1.7
Transcription	1	Ankyrin repeat and FYVE domain containing 1 (Ankfy1)	BG 073858	1.4
Unclassified	75	data not shown		

assessed by measurement of EFA transport. In contrast to this, it has been argued that uteroplacental haemodynamic disturbances observed in the diabetic rat are a major cause of foetal growth restriction (Chartrel *et al.*, 1990). It was also suggested that the drastic over growth of rat diabetic placentas is a mechanism to compensate for the reduced nutrient transfer to the foetus caused by haemodynamic defects (Padmanabhan and Shafiullah, 2001). If correct, this hypothesis may possibly explain the differences between rat and mouse diabetic placentas. In the rat, the generation of the invasive trophoblast lineage is critical to vascular remodelling and the spongiotrophoblast/junctional zone is the source of progenitors for invasive trophoblast. In the mouse, trophoblast invasion is modest and likely to be less

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relevant to vascular remodelling (Ain *et al.*, 2003; Caluwaerts *et al.*, 2005). Alternatively, damage to the foetal rat islet cells, caused by maternal and subsequently foetal hyperglycaemia, has been invoked in intrauterine growth restriction associated with maternal diabetes (Kervran *et al.*, 1979; reviewed in Holemans *et al.*, 2003). While it should be stressed that lack of changes in EFA transport, used by us and others (Wu *et al.*, 2003) as paradigm for placental transport, does not exclude effects of maternal diabetes on other placental transport systems, our results support the idea that foetal growth retardation is caused directly by foetal hyperglycaemia.

Apart from these morphological and functional results, we also present results of a comparative global gene expression analysis in diabetic and normal placentas. The analysis of these results is somewhat complicated by the fact that diabetic placentas exhibit over growth of a specific tissue, the spongiotrophoblast and thus enhanced expression of a gene expressed in spongiotrophoblast could be just an outcome of shifted relative tissue contributions. The same obviously applies to reduced expression of genes expressed in the labyrinth. We have found that the contribution of spongiotrophoblast to total placental volume was increased in diabetic placentas by almost 1.4-fold over that in control placentas. Thus, as long as the spatial expression of the genes identified in the expression screen is not known, the possibility remains that moderate fold-changes between 0.7 and 1.4 are in fact caused by changed tissue distribution. On the other hand, this also suggests that over expression of, for instance, *Peg1*, an imprinted gene expressed in the labyrinth, is in fact more pronounced than shown by our analysis. The same is true for *Gatm*, which is expressed mainly in the labyrinth but which also exhibited up-regulation. In any case, the analysis has identified several interesting genes, for which a function in placental development is known or at least likely. Thus, *Igf2* and *Gatm*, which are both imprinted genes (DeChiara *et al.*, 1991; Sandell *et al.*, 2003), exhibited strong alterations in gene expression. *Igf2* has a well-established function in control of placental growth (DeChiara *et al.*, 1991; Constância *et al.*, 2002), as deletion of this gene causes strongly reduced placental growth. In contrast, very little is known about the function of the product of *Gatm*, the enzyme L-arginine:glycine amidinotransferase, in the placenta. This is the rate-limiting enzyme in creatine biosynthesis. The phosphorylated form of creatine, phosphocreatine, provides a reservoir for high-energy phosphate. It has been speculated that in the placenta phosphocreatine might serve to buffer the maternal organism against sudden high-energy demands from the foetus or foetuses (Sandell *et al.*, 2003). For some genes that were identified in the expression screen, a direct link to hyperglycaemia has been reported. Thus, the up-regulated gene *Txnip* codes for thioredoxin interacting protein (TXNIP), a protein primarily involved in response to oxidative stress. TXNIP was shown to be a central regulator of insulin secretion (Hui *et al.*, 2004). In addition, TXNIP acts at many branching points of gluconeogenesis, glycolysis, and lipogenesis (Hui *et al.*, 2004). Another interesting gene, with lowered expression in diabetic placentas, is *Sh2bpsm1*. Mice with targeted deletion of this gene develop hyperinsulinemia, hyperglycaemia, and glucose intolerance (Duan *et al.*, 2004). Consequently, SH2BPSM1 is believed to be a physiological enhancer of insulin receptor activation and to be essential for maintaining insulin sensitivity and glucose homeostasis. Further studies showed that SH2BPSM1 is also involved in determining leptin sensitivity and in maintenance of normal energy metabolism and body weight (Li *et al.*, 2006). While the precise functions of these genes in the mouse placenta are not known to date, their altered expression levels in diabetic placentas clearly demonstrate that the increased glucose levels cause drastic changes in gene pathways directly involved in glucose metabolism and insulin regulation.

In a recent study on gene expression in human placentas exposed to gestational diabetes, which shares many features of type 2 diabetes, more than 400 genes with altered expression were identified (Radaelli *et al.*, 2003). Interestingly, comparison of the two datasets provided no genes that were common. At present it is not clear whether different experimental procedures, comparison of different species, or fundamental differences between placentas from mothers with type 1 or gestational diabetes are mainly responsible for these differences. Further work will be necessary to address these questions.

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Appendix

Table A1. Placentas used in microarray analysis.

Sample	Placental weight (mg)	Fetal weight (mg)	Fetal blood glucose (mmol/l)	Maternal blood glucose (mmol/l)
Diabetes 1	136	739	19.5	> 33.3
Control 1	101	920	1.2	5.6
Diabetes 2	121	745	18.3	> 33.3
Control 2	96	816	0.8	5.6

All placentas were from male C57BL/6 conceptuses.

Table A2. Primer sequences for real-time RT-PCR.

Gene name	Forward primer sequence	Reverse primer sequence
Igf2	5′-ACAGAGGGTGGTCAGCAAAT-3′	5′-TGGAACATTGGACAGAAACTCA-3′
Gatm	5′-TGAAGGAAGGAGGGAAGGAC-3′	5′-TGACTTTCCATGAATGCCTTT-3′
Hexb	5′-ATTCTCCCCACAGGCAAG-3′	5′-GCCATCCTTTCTGCTCCTT-3′
Plac8	5′-TCTCTACCGAACCCGATACG-3′	5′-AGGCTGAAGGAGTGTGTGCT-3′
Insig1	5′-CAGGAAAGTGAGTGTGCTTG-3′	5′-AAAAGGTGAATCTGAAGGATGC-3′
Dtprp	5′-TGATCCATGCACCCATAAAA-3′	5′-AGCCAGAAATCACTGCCACT-3′
Gpx3	5′-TAGAGACACCACCCCTGACC-3′	5′-CAGCCTCCTTCTTCCTTCCT-3′
Txnip	5′-GATGGGTGGCAATCAGTAGG-3′	5′-TCCGAGAAAGTGGTCAGGTC-3′
Serpinf1	5′-CATTTGGACGAGGACAGGAC-3′	5′-AGGGGCAGGAAGAAGATGAT-3′
Nr2f2	5′-CGTGGCTGTTGAGAGGATTT-3′	5′-AAGATTCCCTTGACCTGTGG-3′
Sh2bpsm1	5′-GACAGTTGGTAGGGCATTGG-3′	5′-TCATTCCTGCTCCGTCTTTC-3′
Actb	5′-TGTTACCAACTGGGACGACA-3′	5′-GGGGTGTTGAAGGTCTCAAA-3′
Rrm2	5′-TGTGTGTGTGTGCAAAGTGC-3′	5′-GAGCAGTGACCATCAAGCAA-3′

Abbreviations: *Igf2*, insulin-like growth factor 2; *Gatm*, glycine amidinotransferase; *Hexb*, hexosaminidase B; *Plac8*, placenta-specific 8; *Insig1*, insulin induced gene 1; *Gpx3*, Glutathione peroxidase 3; *Actb*, beta actin; *Rrm2*, ribonucleotide reductase M2; *Dtprp*, decidual/trophoblast prolactin-related protein; *Txnip*, thioredoxin interacting protein; *Serpinf1*, serine (or cysteine) proteinase inhibitor clade F, member 1; *Nr2f2*, nuclear receptor subfamily 2 group F member 2; *Sh2bpsm1*, SH2 B domain containing signaling mediator 1.

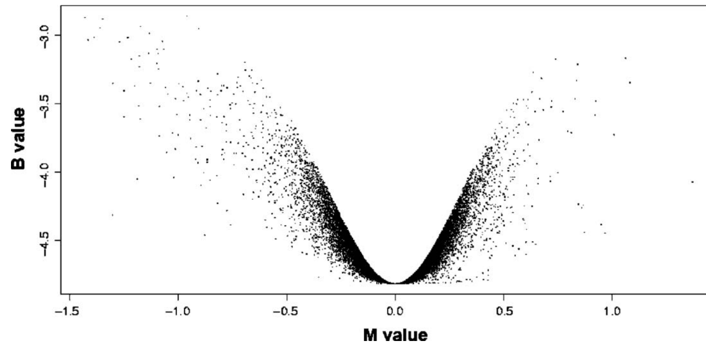

Figure A1. Volcano plot for all the reporters in the four microarray hybridizations performed on pairs (1) and (2). The X axis represents M value and the Y axis represents B value for each reporter.

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