Placental-Specific Overexpression of sFlt-1 Alters Trophoblast Differentiation and Nutrient Transporter Expression in an IUGR Mouse Model

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ABSTRACT
Since it is known that placental overexpression of the human anti-angiogenic molecule sFlt-1, the main candidate in the progression of preeclampsia, lead to intrauterine growth restriction (IUGR) in mice by lentiviral transduction of mouse blastocysts, we hypothesize that sFlt-1 influence placental morphology and physiology resulting in fetal IUGR. We therefore examined the effect of sFlt-1 on placental morphology and physiology at embryonic day 18.5 with histologic and morphometric analyses, transcript analyses, immunoblotting, and methylation studies. Interestingly, placental overexpression of sFlt-1 leads to IUGR in the fetus and results in lower placental weights. Moreover, we observed altered trophoblast differentiation with reduced expression of IGF2, resulting in a smaller placenta, a smaller labyrinth, and the loss of glycogen cells in the junctional zone. Changes in IGF2 are accompanied by small changes in its DNA methylation, whereas overall DNA methylation is unaffected. In addition, the expression of placental nutrient transporters, such as the glucose diffusion channel Cx26, is decreased. In contrast, the expression of the fatty acid transporter CD36 and the cholesterol transporter ABCA1 is significantly increased. In conclusion, placental sFlt-1 overexpression resulted in a reduction in the differentiation of the spongiotrophoblast into glycogen cells. These findings of a reduced exchange area of the labyrinth and glycogen stores, as well as decreased expression of glucose transporter, could contribute to the intrauterine growth restriction phenotype. All of these factors change the intrauterine availability of nutrients. Thus, we speculate that the alterations triggered by increased anti-angiogenesis strongly affect fetal outcome and programming. J. Cell. Biochem. 118: 1316–1329, 2017. © 2016 Wiley Periodicals, Inc.

KEY WORDS: DIFFERENTIATION; HUMAN sFlt-1; INTRAUTERINE GROWTH RESTRICTION; MOUSE PLACENTA; MOUSE TROPHOBLAST; NUTRIENT TRANSPORTER

Intrauterine growth restriction (IUGR) is characterized by insufficient growth of the fetus during pregnancy, resulting from an adverse maternal environment, such as poor nutrition or reduced oxygen supply at the fetomaternal interface [Devaskar and Chu, 2016]. IUGR is associated with a high incidence of perinatal morbidity and mortality, short-term and long-term metabolic and...
cardiovascular alterations and neurological disorders [Zohdi et al., 2015; Cohen et al., 2016]. At least 60% of the 4 million neonatal deaths that occur worldwide each year are associated with low birth weight, caused by IUGR or preterm delivery [Lawn et al., 2005].

Mice and rats have been extensively used in various models of bilateral uterine artery and vein ligation or maternal protein restriction in studies of the effects of IUGR and the “fetal programming” of adult diseases [van Straten et al., 2010; Zhang et al., 2015]. In these experimental models, changes have been demonstrated in a range of factors that regulate placental growth, such as various nutrient transporters and proteins of the insulin-like growth factor (IGF) family or molecules involved in adequate vascularizity in the placenta [Zhang et al., 2015]. In human placentas of IUGR pregnancies and in IUGR rodent models of protein restriction, reduced expression of some placental nutrient transporters of glucose and amino acids has been demonstrated [Malandro et al., 1996; Jansson et al., 2002, 2006; Roos et al., 2007]. In an IUGR rat model of bilateral uterine ligation, we recently could show that the expression of placental fatty acid transporters was increased, but the expression of glucose and amino acid transporters was decreased when blood flow was reduced, and these changes in expression may modify intrauterine programming [Nüsken et al., 2016]. Thus, the appropriate transport of nutrients from the maternal circulation to the fetus is important for adequate fetal growth.

One of the most prominent causes of IUGR in clinical practice is preeclampsia, a disease characterized by hypertension and proteinuria. It is still one leading cause of maternal and neonatal mortality [Verlhoren et al., 2012; Jardim et al., 2015]. Preeclampsia is primarily based on a shallow invasion of the extravillous trophoblast during placental development and is associated with an overexpression of placental soluble fms-like tyrosine kinase 1 (sFlt-1), the soluble VEGF receptor 1. The anti-angiogenic sFlt-1 is described as the main candidate in the progression of the maternal endothelial dysfunction in preeclampsia, because sFlt-1 binds and reduces free circulating levels of the proangiogenic factors VEGF and placental growth factor (PIGF) [Maynard et al., 2003].

In humans sFlt-1 is expressed in vascular endothelial cells, monocyte-macrophage-lineage cells, hypoxia-stressed smooth muscle cells and in the placenta, particularly in the syncytiotrophoblast and the invasive cytotrophoblast [Shibuya, 2011]. A placental overexpression of sFlt-1 leads to an imbalance of this angiogenic/anti-angiogenic system, the level of VEGF and PIGF decreases and consequences placental dysfunction, preeclampsia, and IUGR [Fan et al., 2014]. The increased level of sFlt-1 in serum leads to “glomerular endotheliosis,” a variant of thrombotic microangiopathy with glomerular endothelial swelling, loss of endothelial fenestrae, and occlusion of capillary lumens and finally leads to cellular injury and disruption of the filtration apparatus with proteinuria, edema, and hypertension as a consequence [Maynard et al., 2003].

Thus, the way in which sFlt-1 impairs endothelial cell function is clear, but the way in which sFlt-1 leads to IUGR and affects the placenta remains unknown. Therefore, this study was aimed at gaining mechanistic insights into the ways in which placental overexpression of sFlt-1 results in IUGR of the fetus.

In this study, we used a mouse model of preeclampsia and IUGR, recently developed by Kumasawa et al. [2011], which exhibits placenta-specific overexpression of human sFlt-1 (hsFlt-1) brought about by lentiviral transduction of blastocysts. With this model, we evaluated alterations in placental morphology and changes in the expression of various placental transporters and growth factors.

MATERIALS AND METHODS

LENTIVIRAL VECTORS AND LENTIVIRAL PARTICLE PRODUCTION

The following lentiviral vector (LV) plasmids (HIV-1 based, self-inactivating) were used for the transduction of 293T human embryonic kidney cells: pLV-EGFP/pLV-hsFlt-1 (EGFP, enhanced green fluorescent protein), the packaging plasmids pMDLg pRRE and pRSV Rev, and the VSV-G-expressing plasmid pVSVG. All of the lentiviral plasmids were kindly provided by M. Ikawa, Osaka, Japan. The 293T cells were transfected with lentiviral plasmids by Lipofectamine 2000 reagent according to the manufacturer’s instructions (Thermo Fisher, Waltham, MA) and as described by Okada et al. [2007] and Kumasawa et al. [2011]. Induction of CMV promoter was performed by addition of sodium butyrate (500 mM). After transduction, lentiviral particles were harvested and concentrated by ultracentrifugation.

Lentiviral particle concentration was determined by measuring p24 gag antigen with enzyme-linked immunosorbent assay (ELISA; Retrotek; Zepto Metrix, Buffalo, NY). Lentiviral efficiency was tested by transducing 293T cells with pLV-EGFP or pLV-hsFlt-1 lentiviral particles and cell lysates. Forty-eight hours after infection, supernatants were analyzed for green fluorescent protein (GFP) or sFlt-1 expression by immunohistochemistry, Western blotting, or ELISA, after which the transduction of blastocysts was performed as previously described [Kumasawa et al., 2011].

GENERATION OF MICE BASED ON LENTIVIRAL TRANSDUCTION

B6C3F1 mice (hybrids of BL/6 and C3H mice) were housed in the animal facility in a specific pathogen-free environment and were exposed to cycles of 12 h of light and 12 h of dark. They were provided with food and water ad libitum. The generation of mice based on lentiviral transduction was performed as described by Kumasawa et al. [2011].

Briefly, after mating and observation of the vaginal plug, female mice were killed on day 1.5 post-coitum (pc). Blastocysts were obtained by dissecting the uterus, washing it in phosphate-buffered saline (PBS), and flushing it with M2 medium (M7167; Sigma–Aldrich, St. Louis, MO) to obtain blastocysts. The zona pellucida was removed with Tyrode’s solution (Sigma–Aldrich). The blastocysts (without the zona pellucida) were incubated overnight in potassium-supplemented simplex optimized medium (KSOM; MR-121-D; Millipore, Darmstadt, Germany) and overlaid with mineral oil (Sigma–Aldrich). Next, 6μl of lentiviral particle solution was added to the drop, followed by incubation for another 4h. After being washed in KSOM medium, the transduced blastocysts were implanted into B6C3F1 pseudopregnant mice.

All animal procedures were performed in accordance with the German laws for animal protection.
ANALYSIS OF BLOOD SAMPLES
On embryonic day 18.5, blood was collected from anesthetized pregnant mice by cardiopuncture. Serum samples were prepared by centrifuging clotted blood. Total hsFlt-1 concentrations were measured with the human sVEGFR-1 Quantikine ELISA kit (SVR100B) according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN).

TISSUE PREPARATION AND HISTOLOGICAL ANALYSIS
At embryonic day 18.5, anesthetized pregnant females were killed by cervical dislocation. Embryos were dissected in PBS, and the amniotic membrane was removed from the placenta. Embryos and placentas of each experimental group (LV-hsFlt-1, n = 15; LV-EGFP, n = 22; no-LV, n = 8) were weighed, fixed in 4% formalin, embedded in paraffin (periodic-acid-Schiff [PAS] staining and morphology) or liquid nitrogen, and stored at –80°C (for RNA and protein isolation).

Placental sections (7 μm) were mounted on Super Frost slides (R. Langenbrinck Labor und Medizintechnik, Emmendingen, Germany). For morphological analysis, sections were stained with hematoxylin and eosin (H&E). Glycogen stores were detected with the PAS reaction. Sections were deparaffinized, rehydrated once in 96% and 70% ethanol, treated with 0.5% periodic acid, washed in tap water, incubated in Schiff’s reagent, and counterstained with hematoxylin.

MORPHOMETRIC ANALYSIS
Morphometric analysis of placentas, placental compartments (labyrinth and spongiosiphoblast layer), and placental glycogen cells was performed on nine serial sections of the central region of at least three placentas from each experimental group (pLV-hsFlt-1-treated, pLV-EGFP-treated, and not treated with LV [no-LV]) (n = 3 placentas of experimental group no-LV, n = 6 placentas of experimental group LV-hsFlt-1, and n = 3 placentas of experimental group LV-EGFP). Data were analyzed with the Wallis test with Dunn’s post-test to compare the results obtained with pLV-hsFlt-1, pLV-EGFP, and no-LV.

GENOMIC DNA ISOLATION AND PYROSEQUENCING
Genomic DNA was isolated with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The quality and quantity of DNA were verified with Nanodrop 2000c (Thermo Scientific, Pittsburgh, PA). Bisulfite conversion of 500 ng genomic DNA was performed with the EZ DNA methylation gold kit (Zymo Research, Leiden, The Netherlands), according to the manufacturer’s protocol. Pyrosequencing was performed as previously described by Freitag et al. [2014]. The sequences of bisulfite-specific primers for long interspersed element-1 (LINE-1), IGF2 differentially methylated region 2 (IGF2-DMR2), and H19 imprinting control region (H19-ICR) were published earlier by Freitag et al. [2014]. The polymerase chain reaction (PCR) product was analyzed for the extent of methylation per selected CpG position on a Pyromark Q24 sequencer (Qiagen). Data were analyzed with PyroMark Q24 analysis software 2.0 (Qiagen). The level of DNA methylation (LV-hsFlt-1, n = 6 placentas; LV-EGFP, n = 4 placentas, and no-LV, n = 2 placentas) was given as a percentage.

PROTEIN ISOLATION AND WESTERN BLOTTING
Frozen placenta was homogenized in radioimmunoprecipitation assay (RIPA) protein extraction buffer (50 mM Tris/HCl, 150 mM NaCl, 1% NP-40, 0.25% Na-Deoxycholate, 1 mM ethylenediaminetetraacetic acid [EDTA]). The protein content was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). For protein detection, a total of 20 μg was separated on 8%, 12%, and 14.5% polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes for 120 min at 70 mA per membrane (Hybond-C Extra; Amersham Biosciences, Freiburg, Germany). Membranes were blocked for 1 h with 5% non-fat dried milk in Tris-buffered saline containing 0.15% (v/v) Tween20 (Sigma–Aldrich), followed by incubation overnight with primary antibodies in blocking buffer at 4°C, such as IGF2 (rabbit anti-mouse, ab9574; Abcam, Cambridge, UK), CD31 (rat anti-mouse, DIA 310, Dianova, Hamburg, Germany), ABCA1 (rabbit anti-mouse, ab 7360; Abcam), Cx26 (rabbit anti-Cx26, bs-1715R, BIOSS, Woburn, MA), h-FABP (rabbit anti-cardiac FABP, ab133585, Abcam), CD36 (mouse anti-CD36, NB110-59724, Novus Biologicals, Littleton, CO), SNAT2 (rabbit-anti-SNAT2, H-60, sc-67081, Santa Cruz Biotechnology, SNAT1 (mouse anti-SNAT1, sc-137032, Santa Cruz Biotechnology) and GLUT-1 (rabbit anti-GLUT-1, NB110-39113; Novus Biologicals, Littleton, CO) as described previously in Nüsken et al. [2016].

After being washed and incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies (donkey anti-rabbit HRP, 711-036-152; Jackson IR Laboratories, West Grove, PA; donkey anti mouse HRP, 715-035-150, Dianova; donkey anti-goat HRP, 711-035-147, Jackson IR Laboratories), membranes were developed on radiography film (Kodak, Stuttgart, Germany) with an enhanced chemiluminescence (ECL) kit (Pierce, Rockford, IL) according to the manufacturer’s protocol. Anti-β-actin (A3854; Sigma–Aldrich) was used to normalize protein expression. Protein isolation and Western blotting was carried out on n = 6 placentas of experimental group LV-hsFlt-1, n = 3 placentas of experimental group LV-EGFP and n = 3 placentas of experimental group no-LV.

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RNA EXTRACTION, cDNA SYNTHESIS, QUANTITATIVE RT-PCR
Total RNA was extracted from frozen placenta samples with the E.Z.N. A Total RNA Kit (Omega Bio-tek, Norcross, GA) according to the manufacturer’s protocol. Complementary DNA (cDNA) was synthesized with 1 μg RNA as previously described [Kaiser et al., 2015].

Gene expression was measured with the quantitative PCR Master Mix of SYBR green (Applied Biosystems, Darmstadt, Germany) and the GeneAmp 5700 Sequence Detection System (Applied Biosystems). For quantitative measurement, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. PCR analyses were carried out in triplicate from n = 8 placentas of experimental group LV-hsFlt-1, n = 4 placentas of experimental group LV-EGFP and n = 3 placentas from experimental group no-LV, as previously described [Kaiser et al., 2015]. The amount of cDNA in each sample was normalized to GAPDH. Primer sequences are listed in Table I.

STATISTICAL ANALYSIS
Statistical analysis was performed with GraphPad Prism 6.0 software, using the Kruskal–Wallis test with Dunn’s post-test to compare the results obtained with pLV-hsFlt-1, pLV-EGFP, and...
no-LV. Statistical significance was set at the level of \( P \leq 0.05 \). Outliers were excluded with the help of Grubbs’ test (significance level, \( P < 0.05 \)).

**RESULTS**

**VALIDATION OF THE MOUSE MODEL OF PLACENTAL OVEREXPRESSION OF HUMAN sFlt-1**

Human sFlt-1 (hsFlt-1) was expressed specifically in the murine placenta by the transduction of blastocysts with an LV expressing hsFlt-1 (LV-hsFlt-1), followed by transplantation of the blastocysts into pseudopregnant females. Immunoblot analysis of two representative LV-hsFlt-1-transduced mice demonstrated that the LV-hsFlt-1 transgene was exclusively integrated and expressed in the placenta and not in the embryo (Supplementary Fig. S1).

At E18.5, hsFlt-1 protein was detectable only in the serum of mice transduced with pLV-hsFlt-1 (485.7 ± 603.1 pg/ml). In contrast, neither mice transduced with LV-EGFP nor those without lentiviral transduction (no-LV) exhibited a measurable quantity of hsFlt-1 in the serum.

**OVEREXPRESSION OF HUMAN sFlt-1 IN MICE RESULTED IN LOWER FETAL AND PLACENTAL WEIGHTS**

At E18.5, placentas and embryos (after cervical dislocation) of pregnant mice in all three experimental groups were dissected.

Fetal weight at day 18.5 pc was significantly lower in the group of mice with LV-hsFlt-1 transduction (670.1 ± 45.9 mg, \( n = 15 \)) than in no-LV control group with no transduction of the trophoderm (1004.6 ± 84.0 mg, \( n = 8 \)) (Fig. 1A).

However, also fetuses in the LV-EGFP group, supposed to be a control group, weighed significantly less (805.7 ± 47.0 mg, \( n = 22 \)) than did fetuses in the no-LV control group (1004.6 ± 84.0 mg, \( n = 8 \)). At day 18.5 pc, placentas from the LV-hsFlt-1 group weighed significantly less than those from the no-LV control group (Fig. 1B). Placental weight of mice in the LV-EGFP group was also lower than that of mice in the control group, but the difference was not statistically significant.

As shown in Table II, the rate of resorption at E18.5, as determined by the number of transferred embryos, was higher in LV-hsFlt-1 mice (17.4%) than in LV-EGFP mice (6.2%) and in no LV mice (4%), but the difference was not statistically significant.

Thus, we confirmed that this mouse model of placenta-specific sFlt-1 overexpression results in the IUGR phenotype, as previously shown by Kumasawa et al. [2011].

**OVEREXPRESSION OF HUMAN sFlt-1 IN MICE AFFECTED PLACENTAL SIZE AND PLACENTAL COMPARTMENT AREA**

To investigate the effect of hsFlt-1 expression and the resulting IUGR on placental morphology, we analyzed placental sections at E18.5. Overall, we detected altered placental morphology, with a change in the size of the placenta caused by changes in the size of various placental compartments, as shown in Figure 2A. The total placental area was significantly smaller in LV-hsFlt-1 mice (9826.4 ± 316.2 mm²) than in LV-EGFP mice (10460 ± 74.6 mm²) and in no LV mice (4%) (Fig. 2B). Placentas from LV-EGFP mice were slightly but not significantly smaller in area and size than those from the no-LV control group.

The labyrinthine layer, comprising the transporting trophoblast, the fetal blood vessels, and the maternal sinusoids, was significantly smaller in LV-hsFlt-1 placentas (5495.7 ± 199.9 mm²) than in the no-LV placentas (7009.7 ± 155.6 mm²) and the LV-EGFP placentas (6974.1 ± 126.7 mm²) (Fig. 2D).

The ratio of labyrinth to spongiotrophoblast (L/S ratio) (Fig. 2C) is calculated by dividing the labyrinthine area (Fig. 2D) by the spongiotrophoblast area (Fig. 2E), a placental layer rich in endocrine...
and energy-storing glycogen cells (Fig. 2C). The L/S ratio of placentas from LV-hsFlt-1 mice was smaller (1.3 ± 0.07) than that of placentas from LV-EGFP mice (2.1 ± 0.11) and that of placentas from no-LV mice (1.7 ± 0.05). Interestingly, the spongiosarblast area in LV-EGFP mice was significantly smaller than that of LV-hsFlt-1 mice and that of no-LV mice (Fig. 2E). Thus, overexpression of hsFlt-1 exclusively in the murine placenta changed placental differentiation by significantly reducing the placental labyrinthine area. In contrast, compared to LV-hsFlt-1 and no-LV placentas, LV-EGFP placentas exhibited a smaller spongiosarblast area but no change in the area or size of the labyrinthine compartment.

OVEREXPRESSION OF HUMAN sFlt-1 IN MICE ALTERED THE EXPRESSION OF PLACENTAL MARKER GENES

To understand in more detail the effect of the observed changes in placental morphology on hsFlt-1 overexpression, we focused on marker genes in the various placental compartments. To examine in particular the changes that occur in the labyrinthine layer, we investigated the transcript levels of Gcm1, which is important for the formation of labyrinthine trophoblasts [Janatpour et al., 1999], and those of CD31, a marker of fetal endothelial cells. As shown in Supplementary Figure S2, we found no clear differences in the expression levels of either Gcm1 (Supplementary Fig. S2A) or CD31 (Supplementary Fig. S2B) in any of the experimental conditions. To investigate in general the alterations in the morphology of the junctional zone, we analyzed the expression of Tbpba, which is specific for diploid spongiosarblast cells and glycogen cells [Plum et al., 2001; Cross, 2005; Hu and Cross, 2010]. There was no significant difference between LV-hsFlt-1 mice and no-LV mice in the expression of Tbpba (Fig. 3A). The reduced spongiosarblast area in LV-EGFP placentas, however, was correlated with the slightly reduced expression of Tbpba by spongiosarblast cells. Moreover, on E18.5 we found no differences between the groups in the size of fetal and maternal blood spaces in hematoxyline and eosin stained placental sections (data not shown).

We also evaluated the mRNA expression profiles of four trophoblast giant cell subgroups: the parietal trophoblast giant cells (P-TGCs) surrounding maternal decidua; the spiral artery-associated TGCs (SpA-TGCs) located in the decidua; the canal-associated TGCs (C-TGCs) in the decidua and the junctional zone; and the sinusoidal trophoblast giant cells (S-TGCs) lining the maternal sinusoids in the labyrinth [Simmons et al., 2007; John and Hemberger, 2012]. These TGC subtypes can be defined by various markers.

S-TGCs express primarily prolactin family 3, subfamily b, member 1 (Prl3b1), and cathepsin Q (Ctsq). Prl3b1 and Prl2c2 genes are expressed by C-TGCs (SpA-TGCs) located in the decidua; the canal-associated TGCs (C-TGCs) in the decidua and the junctional zone; and the sinusoidal trophoblast giant cells (S-TGCs) lining the maternal sinusoids in the labyrinth [Simmons et al., 2007; John and Hemberger, 2012]. These TGC subtypes can be defined by various markers.

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We found that the expression of prolactin Prl3b1 and Prl2c2 genes was significantly lower in LV-hsFLT-1 placentas than in no-LV control placentas, whereas there was no difference between the experimental groups in the expression of prolactin Prl3d1 and Ctsq genes (Fig. 3B–E). A similar but not statistically significant result was observed for LV-EGFP. Thus, the TGC markers may indicate a reduction in TGC differentiation to the invasive TGC subtypes SpA-TGCs and C-TGCs.
OVEREXPRESSION OF HUMAN sFlt-1 IN MICE REDUCED THE 
PLACENTAL EXPRESSION OF IGF2 AND ALSO REDUCED THE NUMBER 
OF GLYCOGEN CELLS

To find reasons for the reduced fetal and placental weights in the LV-
hsFlt-1 and LV-EGFP groups on E18.5 we examined the expression 
level of paternally expressed IGF2, which acts as a direct regulator of 
fetomaternal development by promoting placental growth and 
transport capability and, accordingly, fetal growth [DeChiara et al., 
1991; Constància et al., 2002; Sferruzzi-Perri et al., 2011; Gao et al., 
2012]. Interestingly, we found that IGF2 protein expression was 
significantly lower in LV-hsFlt-1 placentas than in control placentas 
(no-LV) (Fig. 4A). The expression of IGF2 protein by LV-EGFP 
placentas was slightly but not significantly lower than the 
expression of this protein by no-LV placentas (Fig. 4A).
The transcript level of maternally expressed IGF2 receptor (Igf2r), which is believed to restrain fetal growth [Constância et al., 2002], was not significantly higher in LV-hsFlt-1 placentas than in the LV-EGFP group and the no-LV group (Fig. 4B).

Spongiotrophoblasts and glycogen cells form the junctional zone of the murine placenta (endocrine compartment), which also supplies energy to the embryo. Huge amounts of glycogen are stored in glycogen cells and can be released as glucose for the energy supply,
particularly late in gestation [Coan et al., 2006]. We measured the area of glycogen cell clusters to identify differences between the experimental groups (Fig. 5A). The proportion of glycogen cells in the junctional zone was significantly lower in LV-hsFlt-1 placentas (4.75%) than in LV-EGFP placentas (7.28%) and no-LV placentas (6.86%) (Fig. 5B).

In summary, LV-hsFlt-1 placentas exhibited a lower IGF2 expression level and a smaller number of IGF2-producing glycogen cells in the junctional zone.

OVEREXPRESSION OF HUMAN sFlt-1 AFFECTS IGF2 BUT NOT GENERAL DNA METHYLATION

IGF2, an imprinted, epigenetically regulated gene, expresses lower levels of protein in hsFlt-1 placentas. Consequently, we analyzed the gene-specific DNA methylation pattern of Igf2. We found that, compared to LV-EGFP placentas and no-LV placentas, LV-hsFlt-1 placentas were significantly hypomethylated at two of the five CpG positions studied (Fig. 6A).

Igf2 is co-localized with H19 on chromosome 11p15 as part of an imprinted cluster; thus, Igf2 transcription is also dependent on the methylation status of the H19 imprinting control region (H19 ICR), which is located upstream of the H19 promoter [Gebert et al., 2016]. Accordingly, we investigated six CpG positions in this area and found that the methylation percentages were not significantly different between the groups, although the LV-EGFP group exhibited a trend toward hypermethylation (Fig. 6B).

To investigate whether the changes in placental morphology are related to a generally altered epigenetic status of the placenta, we evaluated the DNA methylation of the long interspersed element–1 (LINE1). LINE1 is an important class of transposable elements, which compose approximately 17% of the human and mouse genome [O’Donnell et al., 2013] and can therefore serve as an indicator of the total DNA methylation levels [Yang et al., 2004]. We inspected five CpG sites in the promoter of the LINE1 element by pyrosequencing. We did not observe any significant differences between the groups in the methylation percentages (Fig. 6C).

OVEREXPRESSION OF HUMAN sFlt-1 IN MICE ALTERED THE EXPRESSION OF NUTRIENT TRANSPORTERS IN THE PLACENTA

To determine whether the reduced fetal and placental weights of LV-sFlt-1 mice are dependent on changes in the nutrient transport ability of the placenta, we analyzed protein expression levels of glucose, fatty acid, cholesterol, and amino acid transporters.

Expression of the glucose diffusion channel Cx26 was decreased in LV-hsFlt-1 placentas. Placental connexin 26 (Cx26) is strongly expressed and is localized between the two syncytial layers of the labyrinthine trophoblast and serves as a diffusion channel for glucose across the barrier [Gabriel et al., 1998]. Cx26 protein expression was significantly lower in LV-hsFlt-1 placentas than in the no-LV control group and the LV-EGFP group (Fig. 7A). The expression of the protein glucose transporter 1 (Glut1), which is also localized in the labyrinthine trophoblast [Hahn et al., 1999; Kibschull et al., 2008], was slightly but not significantly lower in LV-hsFlt-1 placentas than in control placentas (Fig. 7B).

Expression of placental fatty acid transporter CD36 is increased in LV-hsFlt-1 placentas. We found that the expression of the fatty acid translocase CD36 protein (Fig. 7C), but not that of heart fatty acid binding protein (hFABP) (Fig. 7D), was significantly higher in LV-hsFlt-1 placentas than in control placentas. CD36 and hFABP were clearly located in the placental labyrinth compartment (data not shown), as previously reported by others [Zschiesche et al., 1995; Islam et al., 2014].

Expression of the cholesterol transporter ABCA1 is increased in LV-hsFlt-1 placentas. The ABCA1 transcript expression was significantly higher in LV-hsFlt-1 placentas than in control placentas. Transcript expression of the ATP-binding cassette transporter member 1 (Abca1) (Fig. 7F) and of its corresponding protein (Fig. 7E) were significantly higher in LV-hsFlt-1 placentas than in control placentas.
Expression of placental amino acid transporters SNAT1 and SNAT2 was not altered in LV-hsFlt-1 placentas. Our analysis of protein expression of the sodium-coupled neutral amino acid transporter 1 (SNAT1) and SNAT2 found no obvious differences between the three experimental groups (Fig. 7G and H).

The various nutrient transporters investigated here were all located in the labyrinth compartment of the placenta, as previously shown by Jones et al. [2014]. However, immunohistochemical analysis of placental sections at E18.5 found no differences between the experimental groups in the expression or the location of these transporters (data not shown).

In summary, the LV-hsFlt-1 group exhibited a reduced placental labyrinthine layer as the nutrient transport unit, a finding that corresponds to the reduced fetal and placental weights caused by placental overexpression of hsFlt-1. Most important, the number of glycogen cells as an additional energy store and the number of IGF2 producers, which are important for fetal growth, were decreased. Moreover, placental overexpression of sFlt-1 also affected placental nutrient transport, with a decrease in the glucose diffusion channel Cx26 and an increase in fatty acid transport caused by elevated expression of CD36. We found that the LV-EGFP group, supposed to be a control group, exhibited lower fetal and placental weights, a smaller spongiotrophoblast area, and lower expression of IGF2, but no reduction in the number of glycogen cells and no changes in the expression of placental nutrient transporter.
DISCUSSION

IUGR is one of the main causes of the pregnancy disease preeclampsia, which is strongly associated with elevated levels of the anti-angiogenic factor sFlt-1, the soluble VEGF receptor-1, which is the main candidate believed to aggravate preeclamptic symptoms. The main aim of this study is to investigate how sFlt-1 influences placental morphology and physiology as well as fetal weight.

Our study demonstrates that placental-specific overexpression of hsFlt-1 alters placental morphology and trophoblast differentiation and interferes with IGF2 and nutrient transporter expression. Upon hsFlt-1 induction in the placenta and transfer into the blood system of dams, we observed reduced fetal and placental weights at E18.5. The affected placenta is characterized by a reduced placental area, a diminished labyrinth layer, and a decreased number of glycogen cells in the junctional zone. IGF2 protein expression, well established as an important regulator of fetal and placental growth [Constância et al., 2002], was decreased upon placental sFlt-1 induction, with minor changes in the DNA methylation pattern. Although the glucose diffusion channel Cx26 was downregulated in LV-sFlt-1 placentas, the fatty acid transporter CD36 and the cholesterol transporter ABCA1 were upregulated, but no changes were detected in amino acid transporter expression.

Interestingly, the foster dams with lentivirally infected EGFP blastocysts, supposed to be a control group, give also rise to growth-restricted fetuses with reduced IGF2 expression at E18.5. Surprisingly, these LV-EGFP placentas exhibited a different placental phenotype compared to LV-hsFlt-1 placentas, with a reduced spongiotrophoblast area but no reduction in glycogen cell numbers and no alterations in the expression of placental nutrient transporters. This discrepancy may be explained by the observation in numerous studies that GFP is known to be cytotoxic and leads to apoptosis when transfected into various cell lines, such as NIH/3T3 fibroblast cells [Liu et al., 1999; Krestel et al., 2004]. Baens et al. [2006] also demonstrated that EGFP inhibits polyubiquitination. Thus, caution is recommended using GFP as an appropriate control because GFP itself has an effect on placental development and fetal weight as shown here. As an alternative control we recommend to use either the lentiviral “empty” vector or the IgG2a Fc fragment previously used in an adenoviral sFlt-1 mouse model by Bytautiene et al. [2011].
Fig. 7. (A and B) Expression of placental glucose transporters. Protein samples were obtained from complete placentas at 18.5 dpc. Protein expression level of Cx26 (A) was significantly lower in the LV-hsFlt-1 placentas than in no LV placentas. The protein expression levels of Glut1 (B) were slightly but not significantly downregulated compared with control no LV. (C and D) Expression of placental fatty acid transporters. Protein expression level of the fatty acid translocase CD36 (C) was significantly higher in the LV-hsFlt-1 placentas than in no LV placentas. Protein expression of heart fatty acid binding protein hFABP (D) showed a not significant tendency of downregulation. (E and F) Expression of placental cholesterol transporters. RNA and protein samples were obtained from complete placentas at day 18.5 post-coitum (pc). The protein expression level of ATP-binding cassette transported member 1 (ABCA1) (E) was mildly but not significantly upregulated in LV-hsFlt-1 placentas than in LV-EGFP placentas and those not treated with LV (no LV). The mRNA expression level of ABCA1 (F) was significantly higher in LV-hsFlt-1 placentas than in LV-EGFP placentas. (G and H) Expression of placental amino acid transporters. The protein expression levels of sodium–coupled neutral amino acid transported 1 (SNAT1) (G) and SNAT2 (H) were not differently regulated between the three groups: LV-hsFlt-1, LV-EGFP, and placentas not treated with LV (no LV). Expression levels were normalized to the housekeeping genes Actin and GAPDH. Actin served as a housekeeping gene for Western blot analysis, and GAPDH for quantitative RT-PCR. *P < 0.05. Data are presented as means ± SEM.
Because neither LV-hsFlt-1 placentas nor LV-EGFP placentas exhibited the same alterations in placental morphology and nutrient transporter expression, we conclude that LV-hsFlt-1 and LV-EGFP placentas are two separate phenotypes and thus produce two separate IUGR mouse models.

In this study, we found that a growth-restricted labyrinth upon hsFlt-1 upregulation decreases the surface area of nutrient exchange and could contribute to the restricted growth of the developing fetus. A possible reason for the limiting growth of the labyrinthine zone caused by sFlt-1 overexpression may be a decrease in the labyrinthine trophoblast cell cycle; this decrease perturbs the normal rate of proliferation and may explain the reduced size of the placental area, as has already been shown for IGF2 in Coan et al. [2006].

The effect of sFlt-1 may be an indirect effect of reduced placental IGF2 expression. The decreased placental expression of IGF2 in LV-sFlt-1-treated mice was one of the most obvious changes but was probably due to several factors. IGF2 is expressed by various placental cells during pregnancy. Expression in the labyrinthine layer begins early, and from day 9.5 pc until day 12.5 pc IGF2 is also strongly produced by the spongiotrophoblast, whereas at the end of the pregnancy IGF2 expression is restricted to the glycogen cells located in the junctional zone [Redline et al., 1993; Coan et al., 2006].

In LV-sFlt-1 placentas, we found fewer glycogen cells and a reduced labyrinthine layer; these findings could explain the diminished level of IGF2 in LV-sFlt-1 placentas, resulting in the reduced weights of the offspring.

In mice, deficiencies in IGF2, either through deletion of the Igf2 gene from all fetoplacental tissues (complete null), or specifically from the labyrinthine trophoblast through deletion of the P0 promoter (Igf2P0 null), restrict placental and fetal growth [Coan et al., 2008]. Previous studies have shown that in several species placental expression of IGF2 is reduced by maternal undernutrition [Olausson and Sohlström, 2003]. Moreover, using P0 placentas (placenta-specific deletion of Igf2), Sibley et al. [2004] found a reduced labyrinthine layer and, as a consequence thereof, a 40% reduction in transport capability. The authors assume that the diffusional exchange in the mouse placenta is regulated by placental Igf2. These findings corroborate our findings of a reduced labyrinthine layer and a reduced expression of some nutrient transporters in the placental sFlt-1 mouse model, leading to IUGR.

In LV-sFlt-1 mice, the reason for fetal growth restriction seems to be the reduced labyrinthine compartment as the nutrient-supporting area and the reduced number of IGF2 producing glycogen cells, whereas in the LV-EGFP mice the reduction of the endocrine spongiotrophoblast, not a reduced labyrinthine layer, may be responsible for the lower fetal and placental weights. In the LV-EGFP, the significantly reduced spongiotrophoblast layer could be the reason for the IGF2 shortage because the glycogen cell number did not change. This finding is corroborated by the observed decrease in the expression of Tpbpa as a marker for spongiotrophoblasts.

Kumasawa et al. [2011], using the same mouse model, also found decreased fetal and placental weights in LV-sFlt-1-treated females at E18.5, combined with a reduction in maternal blood spaces and fetal vessels in the labyrinth. We found a smaller labyrinthine area, leading to a reduced surface area for fetomaternal exchange but not an obvious reduction in maternal or fetal blood vessels, as determined by the endothelial marker CD31. This discrepancy with the findings of Kumasawa et al. [2011] may be explained by the difference in the time points at which the placentas were analyzed: E13.5 versus E18.5.

Previous studies have suggested that disrupted epigenetic regulation in the placenta may contribute to altered placental morphology [Chou et al., 2015]. However, when we compared the global and IGF2-specific gene methylation levels between the groups, only small changes in the regulatory sequences of IGF2 were observed. Although differences in IGF2 methylation in IUGR murine placentas have not yet been extensively described, a human study reported hypomethylation at the IGF2/H19 locus [Bourque et al., 2010]. Our findings indicate that LV-sFlt-1 overexpression does not generate extensive changes in the general DNA methylation pattern, as measured by LINE1 element methylation, but is limited to only two CpG positions of the IGF2 locus. Thus, it is tempting to speculate that some other epigenetic mechanism, such as histone modifications [Lewis et al., 2004], may participate in the regulation of IGF2 in our model.

The reduced placental expression of the glucose diffusion channel Cx26 and the glucose transporter Glut1 by LV-sFlt-1 placentas could be due either to the reduced labyrinthine area or to the reduced IGF2 expression. Glucose, which is important for fetal and placental growth, must be taken up by facilitated transport via the glucose transporter Glut1 from the maternal compartment and across the placental barrier via Cx26 channels connecting the two spongiotrophoblast layers [Takata and Hirano, 1997]. Gabriel et al. [1998] found that the absence of Cx26 leads to embryonic death at approximately day E9.5 because of the lack of glucose supply to the fetus. Similar observations have been made by us in a rat model with bilateral ligation of the uterine arteries and veins; in this model IUGR is induced by reduced placental expression of Glut1 and Cx26 [Nüsken et al., 2016]. Moreover, Jones et al. [2014] found that adenoviral-mediated placental gene transfer of IGF1 corrects placental insufficiency via enhanced placental glucose transport mechanisms; this finding verified the association between impaired placental development and reduced glucose transport.

In contrast to the rat ligation model [Nüsken et al., 2016], in the LV-sFlt-1 mouse model both neutral amino acid transporter SNAT1 and SNAT2 were unchanged, and this finding could be explained by the difference in the causes of IUGR in both models: sFlt-1 overexpression with only one factor changed, versus a reduction in nutrient and oxygen supply caused by uterine ligation, representing a more complex situation in IUGR.

The increased expression of the fatty acid translocase CD36 in LV-sFlt-1 placentas suggests that fatty acid transport across the placenta may be enhanced as a compensatory reaction to decreased glucose transport. This reaction minimizes uteroplacental insufficiency and maintains and supports adequate brain development in late gestation. It is well known that the fetus depends primarily on maternal factors to ensure the supply of free fatty acids. Maximin et al. [2016] and Coti Bertrand et al. [2006] found that a maternal diet deficient in fatty acid during gestation negatively influences the early development of the rat brain.

Focusing on the findings in the junctional zone, we found that the decrease in the expression of the giant cell markers Prl2c2 and Prl3b1...
in LV-hsFlt-1 placentas indicates a shift in the differentiation of the invasive SpA-TGCs and C-TGCs, with less marker gene expression of both giant cell subtypes in the LV-sFlt-1 group. SpA-TGCs regulate spiral artery remodeling and thus are important for sufficient blood flow to the placenta; they produce angiogenic and anticoagulative factors and thereby regulate vasodilatation. Moreover, the canal-associated TGCs also adjust vasodilatation [John and Hemberger, 2012]. The significant change in differentiation of the invasive TGC subtypes SpA-TGCs and C-TGCs may reduce blood flow into the labyrinthine layer, and this reduction may lead to changes in nutrient transport capability and may contribute to fetal growth restriction.

In summary, placenta-specific overexpression of human anti-angiogenic sFlt-1 in our IUGR mouse model resulted in altered differentiation of the placental compartment, characterized by reductions in the placental labyrinthine area, as the nutrient exchange unit, and in the trophoblast giant cells C-TGCs and SpA-TGCs. In addition, our results indicate that the loss of glycogen cells as an energy store and producer of IGF2 for fetal and placental growth, the reduced expression of Cx26 and Glut1 for glucose uptake, and the increased expression of the fatty acid transporter CD36 could be the reason for IUGR.

Preeclampsia is a complex disease, caused by the interaction of multiple factors in humans. Therefore, the advantage of our IUGR mouse model here is that only one single factor of this multifactorial human disease, sFlt-1, the main candidate in the progression of preeclampsia, and its effect on placental differentiation and human disease, sFlt-1, the main candidate in the progression of mouse model here is that only one single factor of this multifactorial mouse model here is that only one single factor of this multifactorial disease. Placenta 31:197–202.


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