Leptin is differentially expressed and epigenetically regulated across monochorionic twin placenta with discordant fetal growth

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ABSTRACT: Severely growth-discordant monochorionic (MC) twins offer a unique opportunity to study fetal and placental growth based on a similar genetic background and maternal host environment where the healthy twin serves as an ideal control. Differences in development of MC twins may therefore be due to differential epigenetic regulation of genes involved in placental development and function. Growth-discordant twins are known for abnormal angio-architecture in the placenta of the smaller twin. Since the reasons for this phenotype are mostly unknown this study was aimed to investigate the expression and regulation of genes known to be involved in angiogenesis. We studied 10 severely growth-discordant MC twin placentas (birthweight difference \( \geq 20\% \)) without twin-twin-transfusion syndrome and 5 growth-concordant MC twin placentas. Growth-discordant twin placenta were phenotyped by histology. Placental mRNA expression of 88 angiogenesis-related genes was measured by PCR array. ELISA assay and immunohistochemistry were used to confirm PCR results. EpiTYPTER for DNA methylation was used to determine if methylation ratios were responsible for differential gene expression. The PCR array analysis showed significant mRNA up-regulation in the placental share of the smaller twin for several genes. These included leptin (24.6-fold, \( P = 0.017 \)), fms-like tyrosine kinase 1 (Flt1, 2.4-fold, \( P = 0.016 \)) and Endoglin (Eng, 1.86-fold, \( P = 0.078 \)). None of the other 84 angiogenesis-related genes showed significant differences. ELISA confirmed significantly increased leptin protein expression (49.22 versus 11.03 pg/ml, \( P = 0.049 \)) in the smaller twin of the discordant growth cohort. Leptin expression in smaller twins’ placentas was associated with elevated DNA methylation of the leptin promotor region suggesting the inhibition of binding of a transcriptional activator/inhibitor in that region. We attempted to overcome the limitation of sample size by careful patient selection. We minimized any bias in placental sampling by random sampling from two different sites and by avoiding sampling from areas with grossly visible abnormalities using a standardized sampling protocol. In conclusion, the smaller twin’s placenta is characterized by differentially increased gene expressions for Flt1 and Eng mRNA that may be causally associated with the villous pathology driven by abnormal feto-placental angiogenesis. The substantial up-regulation of leptin mRNA may be epigenetically conferred and relevant to the post-natal risk of metabolic syndrome in intrauterine growth restriction offspring with placental pathology. Growth-discordant MC twins offer unique insights into the epigenetic basis of perinatal programming.

Key words: multiple pregnancy / leptin / angiogenesis / placenta / discordant growth

Introduction

Monochorionic (MC) twin pregnancies have a 2.5-fold increased risk of significant morbidity and perinatal mortality compared with dichorionic twins (Bejar et al., 1990). This difference is mainly attributed to the twin–twin-transfusion syndrome (TTTS) and severe discordant intrauterine growth (Duncan et al., 1997; Lopriore et al., 2009). Both of these complications are conventionally explained by the characteristic gross anatomical differences and angio-architecture of each side of the MC twin placenta (Fick et al., 2006), while TTTS is classically explained by an imbalance in blood flow between the twins through surface arteriovenous placental vascular anastomoses (De Paepe et al., 2010; Baschat et al., 2011; Lopriore et al., 2011). Current opinion favors a central role for abnormalities in inter-twin vascular connections in discordant
growth andTTTS in MC twins, especially since these diseases often overlap; however, more fundamental factors must operate to mediate differences in fetal growth for the following reasons: first, the difference in placental share in discordant MC twins does not always reflect the difference in growth between the twins (Kusanovic et al., 2008). Secondly, inter-twin anastomoses are present in all MC twin placentas, but only ≏15% will develop TTTS (Kusanovic et al., 2008). Angiogenesis is known to play an important role in placental villous development (Mayhew, 2002; Charnock-Jones et al., 2004; Kaufmann et al., 2004). Dysregulation of angiogenic factors such as soluble fms-like tyrosine kinase (sFlt-1) and placental growth factor has been demonstrated in pregnancy complications associated with abnormal placental function. These include the severe forms of pre-eclampsia, intrauterine growth restriction (IUGR) and mirror-syndrome, indicating that aberrant expression of placentally derived angiogenic factors is involved in the pathophysiology of these conditions (Mayhew et al., 2004). Recent data suggest that alterations in angiogenesis and placental development are involved in abnormal MC twin pregnancies (Galea et al., 2008; Nevo et al., 2008). For example, the anti-angiogenic soluble receptor of vascular endothelial growth factor (VEGF), sFlt-1 mRNA and protein are up-regulated in IUGR twins compared with their healthy co-twins. In TTTS, differences in the expression of some angiogenic factors between donor and recipient placenta have been demonstrated, including a significant increase of VEGF mRNA in donor placentas (Galea et al., 2008). The role of angiogenic factors in MC twins is widely unknown.

In the present study we chose to evaluate the role of placental dysregulation of angiogenesis- and metabolism-related gene expression in the development of discordant growth in MC twin pregnancies. As MC twins share the same genetic background and uterine environment, the study of MC twins (concordant and discordant) provides a unique model to investigate placental gene and epigenetic regulation.

### Materials and Methods

#### Patients and samples

The study was approved by the Mount Sinai Hospital Institutional Review Board (REB # MSH 04-0018-U and REB # MSH 10-0128-E). Potential discordant MC twin pregnancies as candidates for placental sampling were identified in the antenatal period and recruited for tissue sampling immediately following delivery. Growth discordance was defined as a difference in birthweight of ≏20% of the weight of the larger twin. The exclusion criteria were any fetal abnormalities, syndromes or infections, maternal pre-eclampsia or (gestational) diabetes and placental tumors (e.g. chorangioma) on pathological examination. Written informed consent was obtained from each participant. The clinical data of the patients is summarized in Table I. Qualified Research Associates from the Research Center for Women’s and Infants’ Health BioBank, Toronto collected two random and unbiased snap-frozen placental samples from each twin at delivery using a standardized and published sampling protocol (http://biobank.lunenfeld.ca). The placental areas belonging to each of the twins were identified by chorionic plate surface fetal vessel distribution avoiding decidual contaminations. Pooled samples containing one piece of tissue from each of the two cores were generated and snap frozen in liquid nitrogen. The remaining placenta was sent to pathology for routine examination including surface vessel injection studies to verify correct sampling of each twin.

Birthweight standard deviation scores (SDSs) were calculated as previously described by Tzschoppe et al. (2011). Canadian population-based birthweight percentiles were used as a reference (Kramer et al., 2001).

#### Histopathology and injection studies

For pathological analysis, placentas were sent to the pathology laboratory with differential cord clamping identifying Twin ‘A’ or Twin ‘B’ portions of the placenta. After fixation, the placentas were sectioned at 1 cm intervals, for selection of tissue for histological examination. Minimum sections examined, included three sections of parenchyma, and two sections of cord and a membrane roll for each twin. In addition, a section of the T-zone and dividing membrane were selected for documentation of chorionicity. All placental sections were reviewed by a staff perinatal pathologist (B.F.). Standard pathological features were documented as well as patterns of villous dysmaturity and abnormal morphology (Redline, 2008).

In four discordant and one concordant MC twin pair, injection studies were performed prior to fixation to demonstrate the distribution of placental tissue and types of inter-twin anastomoses. The cord length and coiling were measured before the cords were divided so that 5–8 cm of cord remained attached to the placenta. The vein and one artery in each cord were cannulated and the surface vasculature was flushed with saline and emptied by gentle pressure. Dyes were then injected to highlight the surface vasculature pattern, the position of the vascular equator and to help identify anastomoses. Where a large artery to artery connection or vein-to-vein connection existed, one color was used to outline the arterial or venous systems of both placental territories. Photographs were taken before and after injection studies.

### Table I Clinical characteristics of growth-discordant and -concordant twin pairs.

<table>
<thead>
<tr>
<th></th>
<th>Growth-discordant twin pairs (n = 10)</th>
<th>Growth-concordant twin pairs (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA at delivery (weeks)</td>
<td>33.8 ± 2.4 (28.7–37.3)</td>
<td>36.5 ± 0.49 (36.1–37.3)</td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>1812 ± 592</td>
<td>2505 ± 383</td>
</tr>
<tr>
<td>Sm</td>
<td>1470 ± 448</td>
<td>2438 ± 308</td>
</tr>
<tr>
<td>Lg</td>
<td>2154 ± 517</td>
<td>2572 ± 413</td>
</tr>
<tr>
<td>Growth discordancea</td>
<td>34.0 ± 8.1 (22.5–46.9)</td>
<td>4.1 ± 1.87 (2.2–7.4)</td>
</tr>
<tr>
<td>Sm &gt; 5th and ≤ 10th percentile</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Sm ≤ 5th percentile</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>AEDV/REDV (sm)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>PI &gt; 95th percentile (sm)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Male</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Female</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>PROM</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vag</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CS</td>
<td>9</td>
<td>5</td>
</tr>
</tbody>
</table>

GA, gestational age; Sm, smaller twin; Lg, larger twin; AEDV, absent end-diastolic velocity in umbilical artery; REDV, reversed end-diastolic velocity in umbilical artery; PI, pulsatility index; PROM, prolonged rupture of membranes; Vag, vaginal delivery; CS, Caesarean section.

*a Growth discordance calculated in % of larger twin’s birthweight.*
Tissue preparation and RNA isolation

Placental samples were pulverized under liquid nitrogen on dry ice and homogenized in Trizol® using a rotor stator homogenizer. Total RNA was extracted using Trizol and purified using the RNeasy kit (Catalogue #74104, Qiagen). Purity and quantity of RNA were assured with NanoDrop 1000 Spectrophotometer (NanoDrop Products, Wilmington, DE, USA) and integrity with Experion™ RNA High Sense Analysis kit (BioRad Laboratories, Inc.).

Reverse transcription and targeted qRT–PCR array

We performed qRT–PCR array on seven of the growth-discordant MC twin pairs. DNase-treated total RNA (600 ng) was reverse transcribed using the RT2 First Strand Kit (C-03/330401SABiosciences). A 384 well setup was used for targeted real-time PCR array for 88 angiogenesis- and metabolism-related genes (four samples per plate) according to the manufacturer’s recommendations (Qiagen, SABiosciences). Each well was loaded with 10 μl of experimental cocktail containing cDNA and PCR Mastermix (SABiosciences) (using a Perkin Elmer pipetting robot). Housekeeping genes were measured in quadruplicates for comparative analysis after assessing the expression stability between the groups. Quality was assured by quantification of potential genomic DNA contamination; reverse transcription control and positive PCR control were included in each assay. The PCR array was performed on a BioRad CFX 384 RT System. The PCR program was initiated at 95°C for 10 min, followed by 40 thermal cycles of 15 s at 95°C, 1 min at 60°C. Fold changes in gene expression between the smaller and the larger twins were calculated using the comparative delta delta Ct method. The data were normalized to the geometric mean of four housekeeping genes (B2M, HPRT1, RPL13A and ACTB) using the manufacturer provided analysis software (SABiosciences).

Protein expression analysis for leptin and leptin receptor

Placental leptin protein expression was quantified using Human Leptin Quantikine ELISA kit (Catalogue # DLP00; R&D Systems, Inc.). Total protein was extracted from snap-frozen tissue using rotor homogenizer and RIPA buffer containing protease and phosphatase inhibitors (Pierce, Thermofisher) at 4°C. Samples were incubated on ice for 1 h, centrifuged (14,000 g) at 4°C for 15 min and the supernatant was transferred to a microtube and stored at −80°C. Protein content was quantified using the Bradford assay (Biorad). Leptin receptor protein expression was quantified using Human Leptin Receptor Quantikine kit (Catalogue #DOBR00; R&D Systems, Inc.) using 30 μg of total protein per assay.

Immunohistochemistry

Parafomaldehyde fixed and embedded in wax tissue slices were used for hematoxylin and eosin (H&E) histology and immunohistochemistry. High-temperature sodium citrate antigen retrieval, pH 6.0, was used for all antibodies. Immunohistochemistry for leptin antibody was used at a dilution of 1:100 (Ob/A20) rabbit polyclonal antibody IgG 200 μg/ml, sc-842; Santa Cruz Biotechnologies, Inc.). Immunohistochemistry was performed on rehydrated sections with streptavidin–biotin staining procedure, using Peroxidase Dako LSAB kit (Dako Canada, Inc., Mississauga, ON, USA) as previously described (Baczyk et al., 2004). Light microscopy images were viewed using 20 × regular and 40 × and 100 × oil immersion objective lens (NA 1.35) using an Olympus BX61 microscope with Olympus DP color camera.

DNA extraction and methylation of leptin promoter region

Total DNA was extracted using the DNasey blood and tissue kit (# 69504; Qiagen). Quality and quantity of DNA were assured with a NanoDrop 1000 Spectrophotometer. DNA samples of eight growth-discordant twin pairs were sent for Sequenom EpiTYPER analysis to the Analytic Genetics Technology Centre at Princess Margaret Hospital/University Health Network, Toronto to determine methylation ratios for the 62 CpG sites within the proximal region of the leptin promoter (chr7:127667987-12766861). Analysis was done in triplicates.

Data analysis

Data are presented as mean ± SEM. PCR array was analyzed using the manufacturer provided analysis software as described above. Paired Student’s t-tests were used to test for significant differences between smaller twins and larger co-twins. Data were analyzed for normal distribution and the Wilcoxon matched pair test applied as necessary. Linear regression analysis was used to evaluate the correlation between leptin mRNA expression and mRNA expression of other genes, as well as analysis of correlation between leptin protein expression and birthweight SDS. Statistical analysis was performed using Prism 5.0a software and P-values of 0.05 were considered significant.

Results

Clinical characteristic of growth-discordant and -concordant MC twin pairs are presented in Table I. All growth-discordant twin pairs had a difference in birthweight of ≥20% of the larger twin’s weight. All larger twins of discordant growth twin pairs were normally grown, and all smaller twins were small for gestational age with a birthweight below the 10th percentile. None of the growth-discordant twin pairs showed signs of twin anemia–polycythemia sequence (TAPS), but there was one case of TAPS in the group of growth-concordant twins. None of the mothers showed any clinical signs of pre-eclampsia.

Smaller placental share and abnormal placental morphology in the smaller twins

Placental tissue distribution between the twin pair was evaluated by injection studies in four cases: 33.3, 35, 40 and 60% of placental territory were assigned to the smaller twins (Table II). In the other six growth-discordant twin pairs, injection studies were not performed and therefore placental tissue distribution could only be grossly evaluated by T-zone division.

In 9 of 10 cases of discordant growth, histopathological analysis identified more pathological features in the IUGR twin’s placental share when compared with the normally grown twin’s side (Fig. 1A and B). Pathological features included either abnormal villous maturation, parenchymal lesions and/or abnormal cord implantation (Table I). The normally grown twin’s placental share, in contrast, did not show significant signs of abnormal placental development or maturation and were comparable to what would be expected for the according gestational age in a normally grown fetus.

In growth-discordant twins, placentas appeared ‘normal’ in three of the four cases. In the fourth case the placental side of the slightly smaller twin (birthweight difference 2.2%) was largely abnormal with multiple foci of villous agglutination, accelerated villous maturation, increased syncytiot knots, persistently muscularized vessels in the basal plate decidua and focal chronic villitis of unknown origin; most of these features
would be in keeping with evidence of maternal vascular under perfusion (Redline et al., 2004). This placenta also showed multiple inter-twin anastomoses (arterial–arterial, veno-venous and arteriovenous anastomoses going from the smaller to the larger twin).

Flt-1, endoglin and leptin expression are up-regulated in the IUGR twin placental tissue

Gene PCR array of seven growth-discordant MC twin pairs showed significantly increased mRNA expression in the smaller twins when compared with their normal co-twins for total Flt-1 (2.4-fold increase, \( P = 0.004 \)), endoglin (Eng) (1.86-fold increase, \( P = 0.049 \)), and leptin (24.59-fold increase, \( P = 0.045 \)). Flt-1 and Eng have been associated with abnormal angiogenesis and placental dysfunction in other studies and we confirmed here these findings. All other 84 angiogenesis-related genes did not show a significant difference between the smaller and their normally grown co-twins (Supplementary data, Table SI).

ELISA confirmed elevated leptin protein levels in 9 of 10 smaller twins compared with their co-twins (average leptin expression in IUGR twins \( 49.22 \pm 59.9 \) versus \( 11.03 \pm 5.7 \) pg/ml in co-twins, \( P = 0.048 \), Fig. 2A). Interestingly, growth-concordant twins showed the same tendency of increased leptin levels in the smaller twins placenta allocation, although this observation was not significant (\( 78.5 \pm 59.5 \) versus \( 20.2 \pm 11.22 \) pg/ml \( P = 0.1 \), Fig. 2A). The measured difference in leptin

### Table II Abnormal placental morphology in growth discordant twins.

<table>
<thead>
<tr>
<th>Twin pair</th>
<th>Twin share (%)</th>
<th>Abnormal villous development or maturation</th>
<th>Parenchymal pathological lesions</th>
<th>Abnormal cord insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>Sm 40(^a)</td>
<td>DVH, AM</td>
<td>N</td>
<td>Ma CI, rare avasc. villi</td>
</tr>
<tr>
<td></td>
<td>Lg N</td>
<td></td>
<td>CVUE, CDdec</td>
<td>Vel CI, CFVO/FTV</td>
</tr>
<tr>
<td>D2</td>
<td>Sm 33.3(^a)</td>
<td>N</td>
<td>N</td>
<td>UA aneurysm, CFVO</td>
</tr>
<tr>
<td></td>
<td>Lg N</td>
<td></td>
<td>Inc Kn, Vil Ag</td>
<td>Hyp C</td>
</tr>
<tr>
<td>D3</td>
<td>Sm 50(^b)</td>
<td>DVH</td>
<td>N</td>
<td>Hyp C</td>
</tr>
<tr>
<td></td>
<td>Lg N</td>
<td></td>
<td>RIH, mild fibrinoid, Inc Kn, CVUE</td>
<td>Int CI</td>
</tr>
<tr>
<td>D4</td>
<td>Sm 35(^a)</td>
<td>Regional DVH</td>
<td>N</td>
<td>Hyp CI</td>
</tr>
<tr>
<td></td>
<td>Lg N</td>
<td></td>
<td>N</td>
<td>Hyp CI</td>
</tr>
<tr>
<td>D5</td>
<td>Sm 60(^a)</td>
<td>DVH</td>
<td>N</td>
<td>Hyp CI</td>
</tr>
<tr>
<td></td>
<td>Lg N</td>
<td></td>
<td>Infarct</td>
<td>Ma CI, CFVO</td>
</tr>
<tr>
<td>D6</td>
<td>Sm 50(^b)</td>
<td>DVH, AM</td>
<td>N</td>
<td>Ma CI</td>
</tr>
<tr>
<td></td>
<td>Lg N</td>
<td></td>
<td>Area of increased fibrinoid</td>
<td>Ma CI</td>
</tr>
<tr>
<td>D7</td>
<td>Sm 33(^b)</td>
<td>Hyp Dev (regional)</td>
<td>N</td>
<td>Ma CI</td>
</tr>
<tr>
<td></td>
<td>Lg N</td>
<td></td>
<td>Velamentous vessel</td>
<td>Ma CI</td>
</tr>
<tr>
<td>D8</td>
<td>Sm 40(^b)</td>
<td>N</td>
<td>N</td>
<td>Vel CI, CFVO</td>
</tr>
<tr>
<td></td>
<td>Lg N</td>
<td></td>
<td>IVT</td>
<td>Hyp C</td>
</tr>
<tr>
<td>D9</td>
<td>Sm 25(^b)</td>
<td>DVH (small areas)</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Lg N</td>
<td></td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>D10</td>
<td>Sm 30(^b)</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Lg N</td>
<td></td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

\(^a\)Placental share verified by injection study.

\(^b\)Placental share grossly evaluated by vascular equator.

**Figure 1** Morphology of placental tissue of the smaller twin. The smaller twin’s side of the placenta demonstrates extended areas with abnormal tissue morphology (distal villous hypoplasia, Fig. 1A) in co-existence with areas with normal placental morphology (Fig. 1B). Both slides are from the same smaller twin to demonstrate that abnormal and normal placental tissue was not evenly distributed within the smaller twins’ placentas.

**Figure 2A** Altered leptin levels in IUGR twins. ELISA confirmed elevated leptin protein levels in 9 of 10 smaller twins compared with their co-twins (average leptin expression in IUGR twins \( 49.22 \pm 59.9 \) versus \( 11.03 \pm 5.7 \) pg/ml in co-twins, \( P = 0.048 \), Fig. 2A). Interestingly, growth-concordant twins showed the same tendency of increased leptin levels in the smaller twins placenta allocation, although this observation was not significant (\( 78.5 \pm 59.5 \) versus \( 20.2 \pm 11.22 \) pg/ml \( P = 0.1 \), Fig. 2A). The measured difference in leptin...
expression in growth-concordant twins was small (≤ 4.04-fold), only in one twin pair we saw a 10.15-fold difference, although this twin pair did not show any specific other abnormalities in clinical results or placental pathology when compared with the other growth-concordant twin pairs.

Linear regression analysis showed a significant positive correlation between sFlt-1 and total Flt-1 versus leptin expression in growth-discordant twins. Gene expression of VEGF-A, as well as total and soluble Eng, angiopoietin-1 and hypoxia-inducible factor 1 alpha (HIF1α) was not correlated with leptin expression.

Leptin protein expression was negatively associated with birthweight SDS ($P = 0.011$, Fig. 2B).

**Immunohistochemistry**

Positive staining for leptin protein was found in the syncytiotrophoblast and cytotrophoblast cells with highest staining in the microvillous membrane (Fig. 3).

**Placental leptin receptor expression did not differ between the smaller twins and their co-twins**

To test the hypothesis that leptin over-expression is a compensatory mechanism caused by reduced receptor expression, total leptin receptor was quantified in all placental samples. ELISA showed no significant difference in the expression of the leptin receptor between the twin pairs (smaller twins $14.45 \pm 8.1$ versus $17.52 \pm 7.8$ pg/ml in normal co-twins, $P = 0.313$), and neither in a sub-group analysis of growth-discordant ($12.78 \pm 5.9$ versus $17.51 \pm 10.7$, $P = 0.282$) or growth-concordant twins ($17.8 \pm 9.01$ versus $17.54 \pm 5.33$ pg/ml, $P = 0.935$).
Increased leptin expression in the smaller twins is associated with increased DNA methylation levels of leptin promoter region

The region encompassing the human leptin promoter (chr7:127667987-12766861) contains a dense CG-rich CpG island, suggesting a potential role for methylation in the regulation of its expression. Therefore, we compared the methylation status of 63 CpG sites extending over ~ 600 bp region upstream of the transcription start site in the smaller twins and their healthy twins.

The overall level of methylation was higher in the smaller twins (eight growth-discordant twin pairs, P < 0.0001) (Fig. 4). An in-silico analysis of this hypermethylated region revealed several highly conserved transcription factor-binding sites. This included binding sites for the Myb proto-oncogene, which can act as both a transcriptional activator and repressor. Furthermore, we identified other binding sites such as sequences for the transcriptional regulators HLF (Hepatic leukemia factor) and CEBPB (CCAAT/enhancer-binding protein beta) and TBP (TATA box-binding protein), Fig. 5.

Discussion

In this study we investigated the changes in placental gene expression involved in angiogenesis. We focused on MC concordant and discordant growth twin cases to investigate the differential expression of genes, which might contribute to the pathological angio-architecture found in these placentas. The large majority of angiogenetic genes examined in the PCR array in our study did not show any significant difference between the smaller and the larger twin. This finding is to be interpreted with caution, as the sample size was relatively small, and a larger sample size might have demonstrated differential regulation for other genes.

Our main findings showed that the angiogenic decoy proteins Flt-1, Eng and leptin mRNA expression were up-regulated in the placental territory of the smaller twins when compared with their normally grown co-twins. Leptin showed the most striking results and only little is known about its function in placentation.

Flt-1 and Eng have extensively been studied both in pre-eclampsia and IUGR. It was shown that these proteins play a role in repressing angiogenesis resulting in abnormal placental morphology in placentas of pre-eclamptic patients (Cerdeira and Karumanchi, 2012). We used this observation to confirm the pathological assessment of the samples used in this work. Some studies have correlated abnormal placental morphology with increased levels of Flt-1 and Eng (Levine et al., 2004; Nevo et al., 2008). Whereas in pre-eclampsia multiple studies agree that placental expression and maternal serum levels of these proteins are increased, results for IUGR remain contradictory (Tsatsaris et al., 2003; Shibata et al., 2005; Tessier et al., 2013). To some extent this can be explained by the fact that in singletons a clear distinction between pathological small infants that grow below their potential and healthy, constitutionally small infants is practically impossible (Tessier et al., 2013). This problem can be avoided in growth-discordant MC twins, as the larger fetus poses a (perfectly) matched control for his co-twin as it originates from the same parents and is exposed to a similar uterine environment. We would expect that the smaller twin’s growth potential would have been similar to the larger ones, if placental function had been normal.

Based on the observation that leptin gene expression was significantly up-regulated in all smaller twins compared with their larger co-twins, we decided to examine in more detail the role of leptin in this cohort. Leptin has been shown to be up-regulated in the placenta and in the maternal serum pregnancies with pre-eclampsia and insulin-dependent gestational diabetes. The significant up-regulation of leptin mRNA and protein in the smaller twin’s placental territory in the present study confirms previous data in singletons, where increased leptin mRNA levels were measured in small for gestational age and IUGR placentas (Sagawa et al., 2002).

The current data contradict work by Lea et al. (2000), where decreased leptin levels were found both in maternal serum and in the placenta of IUGR fetuses. Surprisingly, this group used a rather crude immuno-histochemical approach to quantify placental leptin, although an ELISA was available, as used in our study.

Previously, leptin has been studied in MC twin pregnancies with IUGR and TTTS by Sooranna et al. (2001a, b). Whereas our study focused on placental leptin levels, they examined leptin levels in fetal (cord) blood. This is in concordance with the hypothesis that only a minor part of leptin is released into fetal circulation, and fetal leptin levels are mainly influenced by leptin production in the fetal adipose tissue (Clapp and Kiess, 1998; Linnemann et al., 2000).

The mechanism of leptin regulation in IUGR placentas is only partially understood, but it has been hypothesized that impaired initial placental development, resulting in chronic placental hypoxia might cause leptin up-regulation. However, in our study, we did not see this correlation as the expression of HIF1α as a commonly used indicator of placental hypoxia was not correlated with the increase in leptin in the smaller twins’ placentas.

As an alternative explanation, leptin has been demonstrated to be a strong pro-angiogenic effector in the cornea (Sierra-Honigmann et al., 1998), and might likely also be involved in the regulation of placental angiogenesis. This could have a ‘compensatory’ effect to maintain the function of the growth-restricted placenta. The qRT–PCR array data indicate a positive correlation between the pro-angiogenic leptin and the anti-angiogenic sFlt-1 expression. Furthermore, leptin might also be involved in the regulation of other placental and metabolic functions, such as nutrient transport across the placenta by amino acid transporters (Parrott et al., 2007) and regulation of cell proliferation (Cameo et al., 2003; Maymo et al., 2011).

Figure 4 The overall methylation rate of known CpG-rich sites in the leptin promoter region of the smaller and the larger co-twin placental territories. Numbers represent an average of the overall methylation rate in all the eight twin discordant-growth twin pairs, which were tested for DNA methylation.
As MC twins share the same genetic material, differential gene expression is likely epigenetically conferred, and therefore MC twins are a unique model to study epigenetic regulation such as DNA methylation of CpG-rich sites. Typically, gene expression is reduced/silenced by methylation of areas in the promoter region, but for some genes (in breast and prostate carcinoma) it has been suggested that increased methylation might be associated with an ‘atypical’ increase in gene expression (De Larco et al., 2003; Kelavkar et al., 2007). For leptin, so far, only the ‘classic’ form of epigenetic regulation with methylation, causing a decrease in gene expression, has been described in the white adipose tissue (Marchi et al., 2011). In the current study, we found a significant correlation between increased leptin promoter methylation and an increase in gene expression in the IUGR twins. This effect might be caused by inhibiting repressor binding to the promoter region resulting in over-expression of the leptin protein. We identified three putative transcription factor-binding sites in the leptin promoter, which were hypermethylated in the smaller twins, which are currently the subject of further investigation.

**Conclusion**

The smaller twins’ placentas in discordant growth MC twins demonstrated abnormal morphology, which was associated with a significant up-regulation of mRNA expression of Flt-1, Eng and leptin and leptin protein expression. These data indicate that these angiogenesis-related genes play an important role in the differential regulation of placental function between the smaller twin’s and the normal placental side. Altered leptin promoter methylation between the two sides of the growth-discordant twin placenta indicates an epigenetic regulation of these genes.

**Supplementary data**

Supplementary data are available at http://molehr.oxfordjournals.org/.

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**Figure 5** The graph shows the overall methylation of known CpG rich sites in the leptin promoter. The IUGR twins show significantly increased DNA methylation. The methylation status of leptin CpG island in MC discordant twin placentas. (A) A schematic depiction of leptin promoter CpG island spans the region from −640 bp to +20 bp with respect to the ATG start codon (yellow boxed arrow, leptin). This region contains 62 CpG dinucleotides (depicted by black vertical lines). (B) Methylation status of individual CpG sites in the DNA isolated from placental portions of the smaller twin and its larger co-twin was determined by bisulfate sequencing primers. The change in the percentage of methylation at each of the CpG sites in the smaller twin was calculated by normalizing the methylation status of its healthy twin (set to a baseline of 100%). The results from four sets of twins are depicted in the graph and a summary is shown in Fig. 3. Methylation status of the smaller twin portion of the placenta was increased compared with the matched control twin. The highest methylation variance was observed in the region of the promoter encompassing binding sequences of transcription factors such as MYB, HLF and CEBPB as well as containing TATA box-binding sequence (in silico data were obtained using the genome browser at http://genome.ucsc.edu/).
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Authors’ roles

S.S. executed experiments, analyzed data, drafted manuscript and critical discussion, J.K. designed study, analyzed data, reviewed manuscript and critical discussion, B.F. performed histological examinations, perfusion studies and critical discussion, S.K. performed histological examinations and critical discussion, D.B. performed immunohistochemistry, designed and analyzed DNA methylation analysis, reviewed manuscript, G.R. designed and analyzed clinical aspects of the manuscript, S.D. designed study, executed experiments, analyzed data and reviewed manuscript.

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Conflict of interest

None declared.

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