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Placental Up-Regulation of Leptin and ARMS2 is Associated with Growth Discordance in Monochorionic Diamniotic Twin Pregnancies

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Fetal growth discordance is a relatively common complication of monochorionic diamniotic (MCDA) twin pregnancies and is caused by a combination of maternal and placental factors. The aim of the study was to survey placental gene expression patterns and identify genes associated with growth discordance. Clinical samples comprised eight growth-discordant MCDA twin placentas (31^{+3}–34^{+4} weeks gestational age) and six growth-concordant twin placentas (31^{+2}–37 weeks gestational age). Gene expression libraries were constructed from placental biopsy samples and analyzed by RNA-sequencing. The distribution and relative abundance of mRNA transcripts expressed in the smaller and larger placentas from growth-discordant and concordant MCDA twins was remarkably similar. However, leptin (*LEP*) and age-related maculopathy susceptibility 2 (*ARMS2*) mRNA levels were exclusively up-regulated in all of the eight smaller growth-discordant twin placentas. Quantitative real-time PCR of independent biopsy samples confirmed the levels of differential mRNA expression for both genes. Immunohistochemical analysis of tissue sections from matching twin placentas showed increased leptin expression in 5–10% of blood vessel cells of the smaller placenta and marginally higher levels of *ARMS2* expression in the microvillous membrane of the smaller placenta. Based on these findings, we speculate that up-regulation of leptin and ARMS2 forms part of an important survival mechanism to compensate for placental growth discordance. Since, leptin and ARMS2 are both expressed as soluble proteins, they may have clinical potential as measurable biomarkers for predicting the onset of growth discordance in MCDA twin pregnancies.

Keywords: monochorionic diamniotic, placenta, growth discordance, RNA sequencing, leptin, ARMS2

Fetal growth restriction (FGR) is a failure to fulfill the genetically predetermined growth potential during the course of pregnancy. The causes of FGR can be broadly subdivided into fetal, maternal, and placental etiologies. Identification of the contributing environmental and genetic factors, combined with an understanding of the pathological changes preceding development of FGR, is essential so that future care can be targeted toward developing potential therapies to alleviate adverse perinatal outcomes. Based on a plethora of studies, placental insufficiency has been consistently shown to be involved in the majority of FGR cases (Simonazzi et al., 2013). However, in previous studies (Cosmi et al., 2013; McCarthy et al., 2007; Sitras et al., 2009) where a singleton fetus was used as a model to study the role of placental inefficiency in the pathogenesis of FGR, contributions of maternal, genetic, and metabolic factors were still evident.

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TABLE 1

Clinical Characteristics of MCDA Pregnancies and Twin Placentas

	RNA and qPCR studies		qPCR studies
Clinical data	Growth-discordant MCDA twin placentas ($n = 8$)	Growth-concordant MCDA twin placentas ($n = 3$)	Growth-concordant MCDA twin placentas ($n = 3$)
Maternal age (years)	28.3 ± 3.62	28.0 ± 5.0	30.0 ± 3.5
Estimated fetal weight <10th percentile for smaller fetus (*n*)	8	0	0
Absent/reverse end diastolic flow in umbilical artery doppler (*n*)	8	0	0
Birth weight (g)	1747.6 ± 370.4	2712.2 ± 169.5	1869.2 ± 338.6
Large (g)	2030.1 ± 204.3	2813.3 ± 132.0	1930.0 ± 404.5
Small (g)	1465 ± 264.1	2611.0 ± 153.9	1808.3 ± 334.5
Birth weight discordance (%)	28.2 ± 6.9	7.1 ± 5.0	5.9 ± 2.8
Male/female	10/6	6/0	2/4
Placental weight (g)	756.3 ± 127.4	920.0 ± 70.7	683.3 ± 112.4
Average gestational age (weeks)	33.4 ± 1.21	36.8 ± 0.3	33.0 ± 1.5
Gestational age weeks (range)	31^{+3}–34^{+4}	36^{+3}–37	31^{+2}–34^{+1}

Growth discordance is one of the most common complications of monochorionic diamniotic (MCDA) twin pregnancies (Bejar et al., 1990; Valsky , Eixarch, Martinez, Crispi et al., 2010), with a prevalence of 10–19% (Acosta-Rojas et al., 2007; Lewi et al., 2014; Valsky, Eixarch, Martinez, & Gratacós, 2010). The mechanisms and progressive development of growth discordance largely depend on the relative effects of unequal placental sharing and inter-twin blood transfer through placental anastomoses (Chang et al., 2009; Hack et al., 2008; Lewi et al., 2007). MCDA twins share the same genetic background and maternal host environment. Thus, the differences in intrauterine growth of MCDA twins may be due to differential regulation of genes at the transcriptional and/or post-transcriptional level that are involved in placental development and function. On this basis, growth-discordant MCDA twins represent ideal models to study the underlying mechanisms of placental insufficiency independent of confounding genetic factors and maternal host environment.

Previous twin studies have demonstrated that phenotypic placental variation is associated with epigenetic factors (Bell & Saffery, 2012; Gordon et al., 2011; Kaminsky et al., 2009; Yu et al., 2012). In a recent study, that specifically examined placental mRNA expression of angiogenesis-related genes, significant up-regulation in the placental share of the growth-discordant MCDA twin for leptin (*LEP*) and several other angiogenic factors were reported (Schrey et al., 2013). In other studies, differential expression of osteopontin (Li et al., 2013) and the human endogenous family W, Env(C7), member 1 protein (Gao et al., 2012) were both found to be strongly associated with growth discordance. While there have been several studies focused on global gene expression in growth-discordant MCDA twin placentas (Gordon et al., 2011; Nishizawa et al., 2011; Sitras et al., 2009), cross-study analysis of the differentially expressed genes identified found no consensus genes strongly associated with growth discordance (Marr et al., 2016).

In order to shed further light on the key placental genes associated with growth discordance, our study compared the placental mRNA expression profiles between eight sets of growth-discordant MCDA twins using RNA sequencing (RNA-seq), which is now a well-established methodology to detect and quantitate the levels of genome-wide expressed transcripts in two closely related samples (Mortazavi et al., 2008). Thus, the identification of any differentially expressed placental genes between growth-discordant MCDA twin placentas would, therefore, be a proxy measure of epigenetic discordance. In this study, we identified two placental proteins, namely *LEP* and age-related maculopathy susceptibility 2 (*ARMS2*), which were strongly associated with growth discordance in MCDA twins.

Material and Methods

Ethics Statement

The research study was approved by the Human Research Ethics Committee of the Shanghai First Maternity and Infant Hospital (KS1677, June 2016–June 2019). Prior to delivery, patients provided written informed consent for the research studies conducted on their placental tissue collected after delivery.

Clinical Characteristics of Placental Samples

The selected study samples (Table 1) consisted of eight growth-discordant MCDA twin placentas and six growth-concordant MCDA twin placentas without complications (Table 1). MCDA twins were selected by chorionicity confirmation from ultrasound during the first trimester and placental pathology after birth. Growth-discordant MCDA twins selected for the study were defined by more than a 20% birth–weight discordance, according to the formulae (A-B)×100/A, where A is the birth weight of the larger twin, and B is the birth weight of the smaller twin (Lewi et al., 2014). Conversely, growth-concordant MCDA twins were defined as having a birth–weight discordance

less than 10% (Lewi et al., 2014) and normal umbilical artery doppler for both fetuses (Gratacós et al., 2007; Valsky, Eixarch, Martinez, Crispi et al., 2010). Other MCDA complications, such as preeclampsia, twin-to-twin transfusion, twin anemia-polycythemia sequence, fetal structural abnormalities, genetic syndromes, or chromosomal abnormalities were excluded. All pregnant women recruited to the study had no maternal or pregnancy complications.

Collection of Placental Tissue and Isolation of RNA

Immediately following delivery, pieces of tissue (approximately 100 mg) were removed from the middle portion of the placenta, which was identified by inter-twin membrane, each cord insertion, and placental vessel distributions. The biopsied tissue was washed repeatedly in phosphate-buffered saline to remove all traces of contaminating blood, snap frozen in liquid nitrogen, and then stored at -80 degree Celsius. Frozen tissue (~100 mg sliced into smaller fragments) was placed directly into 1.5 ml siliconized eppendorf tubes containing 1 ml of Trizol solution (Qiagen). Total RNA was extracted according to the recommended protocol. In brief, tissue was homogenized and then tubes incubated at room temperature for at least 5 min to dissociate nucleoprotein complexes. Following addition of 0.2 ml of chloroform and vigorous vortexing for 15 s, tubes were further incubated at room temperature for 3 min. Tubes were centrifuged at 12,000x*g* for 15 min at 4 degree Celsius and the aqueous phase transferred to a fresh tube. Glycogen (10 μg) was added and RNA precipitated by addition of 1 ml absolute ethyl alcohol and incubation at -80 degree Celsius for 1 hour. Tubes were centrifuged at 12,000x*g* for 10 min at 4 degree Celsius, the RNA pellet washed with 75% ethanol, briefly air-dried, and then finally dissolved in RNase-free water. The concentration (260 nm absorbance) and quality of the purified RNA (260 nm/280 nm absorbance ratio) was determined by Nanodrop-c (Thermo Scientific).

RNA Sequencing and Data Analysis

Poly A^+ containing mRNA was isolated from total RNA using poly-T oligo-attached magnetic beads (Illumina, US). A total of 100 ng of poly A^+ mRNA was fragmented into an average size of 300 bp by incubation in 100 mM $MgCl_2$ at 95 degree Celsius for 3 min. Fragmented mRNA was reverse transcribed into first strand cDNA using random primers and reverse transcriptase, then copied to double stranded DNA using DNA polymerase and RNase H (Enzymatics, US). Following fragment end repair using DNA modifying enzymes T4 DNA polymerase, Klenow and T4 polynucleotide kinase and, addition of a single A base to the 5′ ends using Klenow Fragment (3′ to 5′ exo-), sequencing adaptors (Liang et al., 2013) were ligated to each end and then fragments finally amplified by PCR to generate the placental cDNA libraries. Libraries were then subjected to massively parallel sequencing on the Illumina HiSeq2500 platform to produce in the order of 20 million 2 × 100 bp paired end reads.

Paired end reads were uniquely mapped to the hg19 reference genome and contiguous sequences aligned using the TopHat2 algorithm (Kim et al., 2013) incorporated in the web-based analysis protocol http://ccb.jhu.edu/software/tophat/index.shtml. The relative abundance of each transcript was normalized by calculating the reads per kilobase per one million reads (RPKM values; Mortazavi et al., 2008) according to the formula RPKM = total exon reads/mapped reads (millions) × exon length (kb). The significance (*p* value) of the level of individual gene expression was calculated from the ratio of the RPKM values determined for the small and large placental samples, using the edgeR algorithm (Robinson et al., 2010). In a Microsoft excel sheet, significant transcripts (p value $< .05$) showing >2-fold change in gene expression between the eight small and large growth-discordant twin placental samples (columns) were ordered by gene name (rows), allowing identification of differentially expressed genes.

Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) to quantitate relative gene expression levels between placental RNA samples was performed using the Kapa SYBR qPCR kit (KAPABIOSYSTEMS) according to the recommended protocol. Briefly, 20 μl reactions were prepared in 200 μl PCR tubes comprising 10 μl of Kapa SYBR FAST qPCR master mix ABI Prism, 0.4 μl of 10 μM forward primer, 0.4 μl of 10 μM reverse primer, 2 μl of cDNA (20 ng) and 7.2 μl of molecular grade water. Reactions were heated at 95 degree Celsius for 3 min to activate the heat stable DNA polymerase and then subjected to 40 two-step PCR cycles of 95 degree Celsius for 3 s and 60 degree Celsius for 25 s. Melting curve analysis was performed and fold changes between comparative samples calculated by the delta delta C_T method, with data normalized using the expression levels of the house keeping gene β-actin.

Immunohistochemical Analysis

Paraffin-embedded placental tissue biopsies were cut into 5 μm sections, deparaffinized with xylene, and then gradually rehydrated with decreasing concentrations of ethyl alcohol. Boiled 10 mM citrate buffer (pH 6.0) was used for antigen retrieval and endogenous peroxidase activity was blocked in 3% hydrogen peroxidase prepared in PBS. After blocking further with 10% rabbit serum, sections were probed overnight at 4 degree Celsius with either rabbit anti-LEPTIN antibody (1:100, Proteintech, USA) or rabbit anti-ARMS2 antibody (1:200, Novus, USA). Specific protein expression was visualized using the EnVision Peroxidase/DAB detection system (Dako, Denmark) according to the manufacturer's instructions. Negative control sections were treated similarly, except the primary antibody, was excluded.

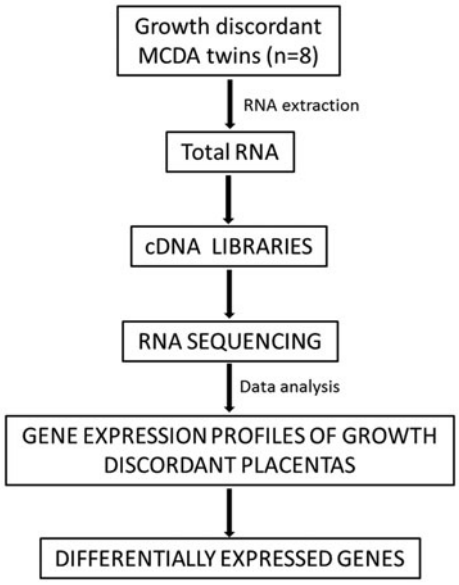

FIGURE 1.

Study design to identify placental genes associated with growth discordance.

Results

The overall study design to identify differentially expressed genes associated with growth-discordant twins is shown in Figure 1. We initially, compared gene expression patterns by RNA-seq between the larger and smaller placentas of eight growth-discordant MCDA twins (144, 149, 152, 166, 170, 197, 202, and 228) with an average gestational age of 33.4 ± 1.21 weeks, range 31^{+3}–34^{+4} weeks (Table 1). As controls, we also analyzed the gene expression patterns in the smaller and larger placentas of three MCDA growth-concordant twins (234, 262, and 272) with a slightly higher average gestational age of 36.8 ± 0.3 weeks, range 36^{+3} ± 37 weeks. We hypothesized that by comparing these two sets of clinically well-defined placental samples, any genes associated with growth discordance would be differentially expressed in smaller of the two growth-discordant placentas but not differentially expressed in the smaller of the two growth-concordant placentas.

Poly A^+ cDNA libraries were generated from growth-discordant and concordant placental pairs and subjected to deep sequencing. By Pearson correlation, the transcript profiles between the smaller and larger placenta pairs were highly concordant (range R^2 values of 0.832–0.949), indicating that high-quality gene expression data had been obtained from all placental samples for meaningful comparative analyses. Across all placental mRNA profiles, the top 16 expressed gene transcripts were *CSH1*, *CSH2*, *CGA*, *CGB*, *HBA1*, *HBA2*, *PSG1*, *PSG2*, *PSG3*, *HBG2*, *GH2,* and *CSHL1,* specifically associated with pregnancy, and *KISS1*, *H19*, *TFPI2*, and *FBLN1,* mainly known to be associated with oncogenesis and tumor suppression (Table 2). All these genes have been previously shown to be highly expressed in independent placental samples analyzed by both microarray (Sood et al., 2005) and RNA-seq (Kim et al., 2012) methodologies. In all cases, none of these genes were differentially expressed in the growth-discordant placenta pairs.

To identify differentially expressed genes in the smaller of the two growth-discordant placentas, the fold change for each transcript was plotted against its relative abundance (Figure 2, selected plots). The subset of the statistically significant transcripts ($p < .05$) with a fold change of >2 were compared across all growth-discordant placenta pairs. Two genes, leptin (*LEP*) and age-related maculopathy susceptibility 2 (*ARMS2*), were found to be specifically up-regulated to different degrees in all eight growth-discordant placentas (Table 2). Importantly, the expression levels of these two genes were relatively similar between the smaller and larger placentas of the three growth-concordant twins, suggesting that up-regulation of both *LEP* and *ARMS2* mRNA was associated with growth discordance.

Independent confirmation of *LEP* and *ARMS2* mRNA differential expression was performed by qPCR on a second placental biopsy taken in close proximity to the first biopsy used for RNA-seq. Up-regulation of *LEP* and *ARMS2* mRNA was also exclusively observed in each of the smaller of the two growth-discordant placentas at levels, generally similar to those measured by RNA-seq (Figure 3). Levels of increased *LEP* mRNA expression in the smaller placenta varied from as low as 7.2-fold (sample 228) to as high as 1,774-fold (sample 170). In contrast, levels of increased *ARMS2* mRNA expression in the smaller placenta were generally lower, ranging from 3.5-fold (sample 228) to 53.4-fold (sample 170).

Given that the three control growth-concordant placenta pairs used in the RNA-seq and PCR studies were of a slightly higher gestational age compared to the growth-discordant pairs (Table 1), we further analyzed *LEP* and *ARMS2* mRNA expression levels by qPCR in three additional growth-concordant placentas (397, 424, and 480) with similar gestational ages to the eight growth-discordant MCDA twin placentas (Table 1). In all three cases, we confirmed that the levels of *LEP* and *ARMS2* mRNA were similar between the smaller and larger placentas in these independent control samples (Figure 3).

To further determine whether leptin and ARMS2 were also differentially expressed at the protein level, immunohistochemistry (IHC) was conducted on paraffin embedded tissue sections from selected growth-discordant placental samples (Figure 4). Strong leptin staining was consistently observed in approximately 5–10% of blood vessels and the surrounding chorionic villous cells of the smaller placenta. In contrast, all the blood vessels cells in the larger placenta showed relatively weak or no leptin staining. ARMS2 staining was observed in all placental cell types in both twin placentas, although marginally increased staining was also

TABLE 2

List of Highly and Differentially Expressed Placental Genes

Locus	Gene	RPKM value[a]	Gene name
A. Highly expressed genes in both growth-discordant and concordant MCDA twin placentas			
Chr17:61972267-61974021	*CSH1*	205841.7	Chorionic somatomammotropin hormone 1 (placental lactogen)
Chr17:61949371-61951089	*CSH2*	130560.8	Chorionic somatomammotropin hormone 2
Chr6:87795215-87804865	*CGA*	20432.75	Glycoprotein hormones, alpha polypeptide
Chr1:204159468-204165619	*KISS1*	14540.28	KiSS-1 metastasis-suppressor
Chr7:93885397-93890991	*TFPI2*	6019.564	Tissue factor pathway inhibitor 2
Chr22:45898718-45997014	*FBLN1*	4182.648	Fibulin 1
Chr19:49526125-49527632	*CGB*	3931.729	Chorionic gonadotropin, beta polypeptide
Chr19:43370612-43383871	*PSG1*	3716.856	Pregnancy specific beta-1-glycoprotein 1
Chr16:222845-223709	*HBA2*	3690.052	Hemoglobin, alpha 2
Chr11:2016405-2019065	*H19*	3627.351	H19,imprinted maternally expressed transcript (non-coding region)
Chr19:43064210-43082741	*PSG2*	3378.741	Pregnancy specific beta-1-glycoprotein 2
Chr19:42721642-42740516	*PSG3*	3186.883	Pregnancy specific beta-1-glycoprotein 3
Chr16:226678-227520	*HBA1*	3132.802	Hemoglobin, alpha 1
Chr17:63909605-63911258	*CSHL1*	2713.01	Chorionic somatomammotropin hormone-like 1
Chr11:5274420-5276011	*HBG2*	2679.881	Hemoglobin, gamma G
Chr17:63880212-63881942	*GH2*	2433.652	Growth hormone 2
Locus	Gene	Expression trend	Gene name
B. Differentially expressed genes in the smaller of the growth-discordant MCDA twin placentas			
chr7:127881330-127897682	*LEP*	Higher	Leptin
chr10:124214178-124216868	*ARMS2*	Higher	Age-related maculopathy susceptibility 2

Note: Note: [a]RPKM values represent the relative transcript abundance.

consistently observed in the microvillous membranes of the smaller placenta.

Discussion

In this pilot study of clinically defined growth-discordant and concordant MCDA placentas (Table 1), we successfully applied next generation RNA-seq to elucidate differentially expressed placental genes involved in growth discordance. This was performed using human models of growth-discordant MCDA twin pregnancies in order to reduce the contribution of maternal factors and focus specifically on identifying novel placental factors. Detailed comparison of the poly A^+ transcriptomes derived from eight growth-discordant twin MCDA placentas identified two genes, *LEP* and *ARMS2*. The RNA-seq results were independently confirmed at the mRNA and protein level by qPCR and IHC analyses.

From the very large number of genes found to be expressed in placental tissue, remarkably, only *ARMS2* and *LEP* were significantly up-regulated in all of the smaller discordant MCDA twin placentas, leading us to speculate on their possible functional roles. The first gene that was found to be significantly up-regulated in the growth restricted twin placenta was *LEP*. In a previous study that examined placental angiogenic gene expression in MCDA growth-discordant twin placentas, three genes *LEP, Flt* (fms-like tyrosine kinase), and *Eng* (Endoglin) were consistently up-regulated in the smaller of the co-twin placentas, although leptin showed the strongest association (Schrey et al., 2013) and agrees with the findings in this study. Further, in other independent studies, higher placental levels of leptin gene expression has also been documented in cases of pre-eclampsia and insulin-dependent gestational diabetes, as well as in singleton pregnancies with small gestational age placentas and placentas with IUGR (Sagawa et al., 2002). Leptin is known to be expressed in a variety of tissues; in particular, the white and brown adipose tissue of the placenta (Allison & Myers, 2014). The most recognized function of leptin is regulation of the amount of stored fat in the body and thus is well-known as the 'satiety' hormone (Allison & Myers, 2014; Maffei et al., 1995).

Despite its important homeostatic role in regulating hunger, leptin has also been shown to have important autocrine effects on placental growth and function (Pérez-Pérez et al., 2015). Of further interest are two previous reports of leptin acting as a pro-angiogenic factor in the cornea of the eye (Sierra-Honigmann et al., 1998) and in developing embryos (Talavera-Adame et al., 2008). In this study, IHC analyses revealed that 10–20% of the blood vessels as well as the immediate surrounding chorionic villous cells had increased leptin expression, exclusively in the smaller placenta. This observation would support the notion that leptin may act as one of the key angiogenic factors to increase vascularization in specific regions of the placenta when under metabolic stress. Therefore, increased local levels of leptin expression, in concert with other angiogenic factors such as VEGF (Schrey et al., 2013), may be involved in modifying the vascular endothelium of the growth-restricted placenta. Thus, we postulate that during growth restriction, leptin signaling may form part of a critical survival or compensatory mechanism to increase blood supply and nutrient flow.

The second gene found to be up-regulated in growth restricted placenta was the *ARMS2* gene associated with

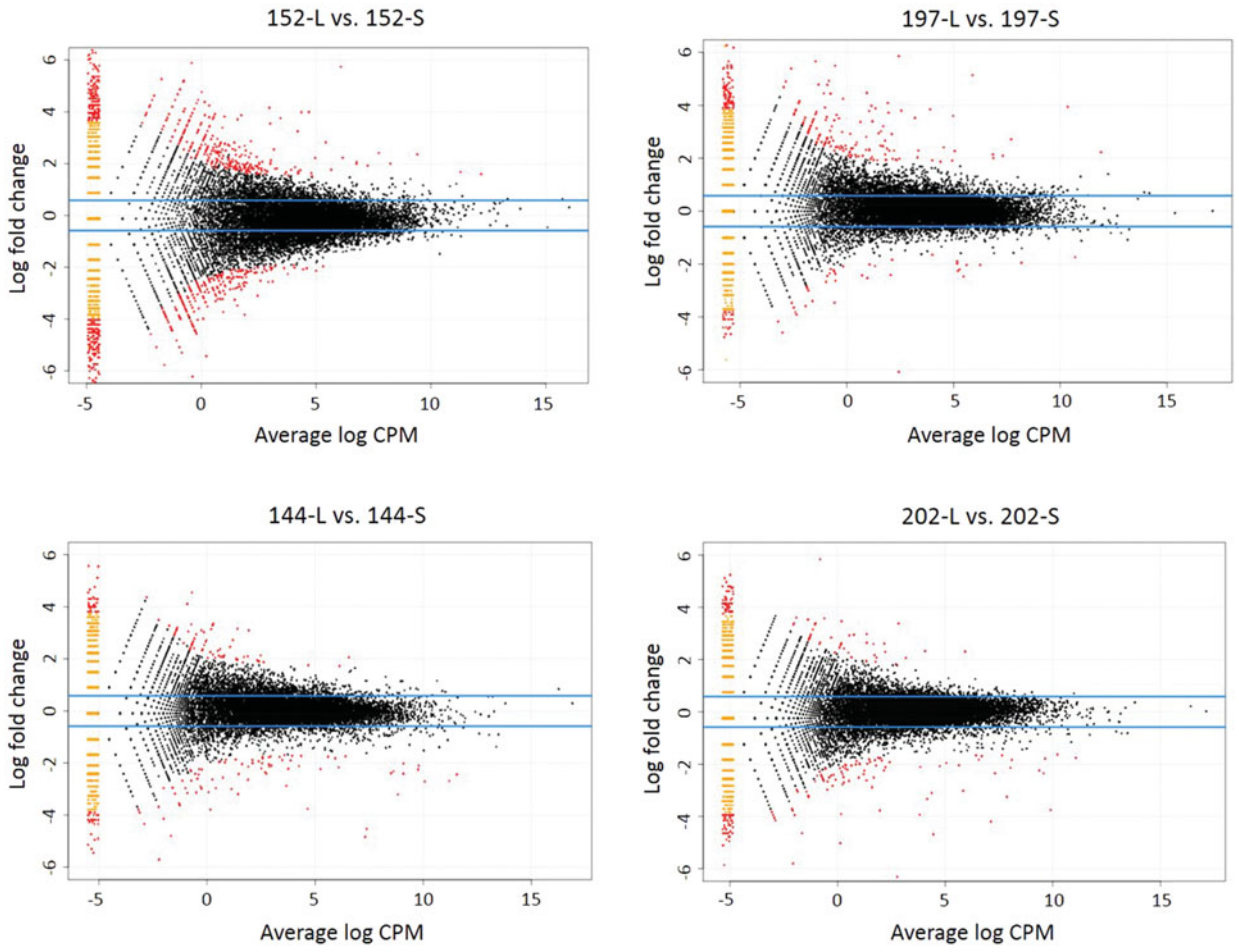

FIGURE 2.

(Colour online) Changes in mRNA gene expression between growth-discordant MCDA twin placentas. Note: Transcript distributions are plotted as log fold change (differential expression) versus average log CPM (abundance). The upper blue line marks 2-fold up-regulation and the lower blue line marks 2-fold down-regulation. The distribution of transcript expression change is shown by the cloud of black dots (non-significant change, $p > .05$) and red dots (significant change, $p < .05$). Representative plots for four of the eight growth-discordant MCDA placentas (152, 197, 144, and 202) are shown.

age-related maculopathy, which is a degenerative condition of the retina that leads to progressive visual loss (Horie-Inoue & Inoue, 2014; Ratnapriya & Chew, 2013). To date, the function of ARMS2 has remained elusive due to a lack of a non-human homologue for manipulation in an animal model. ARMS2 mRNA is known to be highly expressed in the both the retina (Wang et al., 2011) and in the placenta (Fritsche et al., 2008), and the mature form of the protein is secreted into the cytosol (Kortvely et al., 2010). In the retina, ARMS2 binds to a number of extracellular matrix proteins implicated in macular dystrophies (Kortvely et al., 2010). Further, it is localized mainly to regions of choroid pillars in the retina where pathological drusen deposits occur in patients with age-related maculopathy (Ratnapriya & Chew, 2013). Thus, based on the available evidence by association with age-related maculopathy, we speculate that elevated levels of ARMS2 protein detected in the growth-restricted twin placenta may reflect an underlying pathology originating from dysfunction of the normal extracellular matrix. Furthermore, as *ARMS2* mRNA is almost exclusively expressed in the placenta (Fritsche et al., 2008) and is secreted (Kortvely et al., 2010), it may represent a useful measurable biomarker of growth discordance in MCDA twins.

One limitation of the study was that the growth-discordant MCDA placentas consented for comparative analyses were obtained after deliveries during the late third trimester of pregnancy; thus, we were only able to focus on genes exerting an effect at the end stage of growth discordance. Obtaining access to placental mRNA, prospectively during the course of a MCDA twin pregnancy would allow the identification of placental genes more relevant to the initiation and perpetuation of growth discordance. One emerging approach is the analysis of the circulating RNA

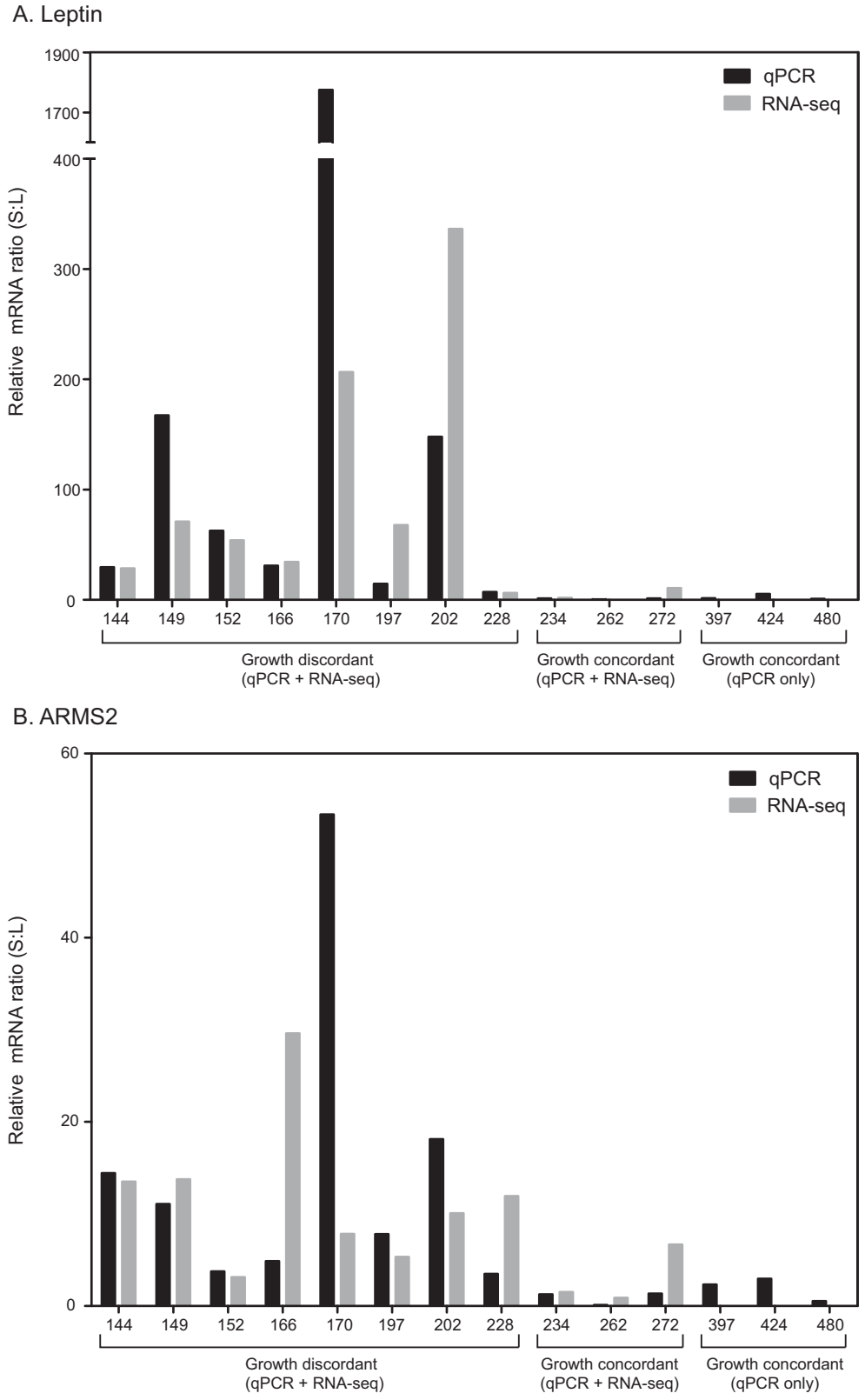

FIGURE 3.

Differential expression of *LEP* and *ARMS2* mRNA. Note: The relative expression levels of *LEP* mRNA (A) and *ARMS2* mRNA (B) were determined by the ratio of transcript abundance between the small (S) and large (L) placenta of the growth-discordant and concordant MCDA placentas. qPCR and RNA sequencing results are shown by black and grey bars, respectively.

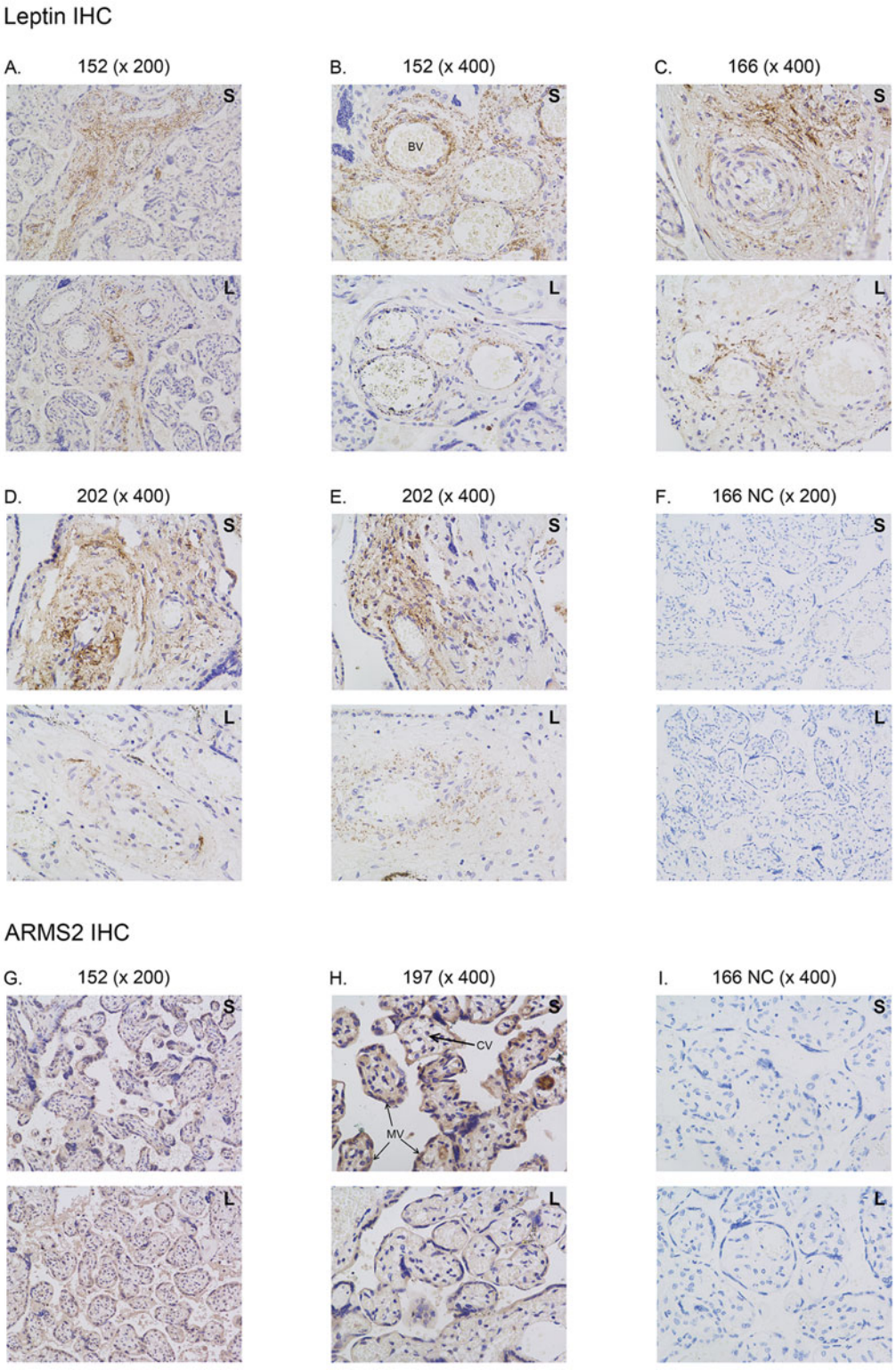

FIGURE 4.

(Colour online) Differential leptin and ARMS2 protein expression. Note: IHC using leptin and ARMS2 specific antibodies was performed on selected tissue sections of growth-discordant placentas (magnification either x200 or x400). IHC results for smaller (S) and larger (L) placenta are shown in the top and bottom panels, respectively. H&E blue staining indicates cellular nuclei and brown cellular staining represents positive leptin (panels A, B, C, D, and E) and ARMS2 (panels G and H) staining. There was no staining of the negative control sections (panels F and I). BV = blood vessel (panel B), CV = chorionic villous, and MV = microvillous (panel H).

fraction in the maternal plasma during pregnancy (Koh et al., 2014). Robust techniques for isolating the total RNA fraction (Spornraft et al., 2014) and identifying key elements of the entire transcriptome, including mRNA and miRNA (Huang et al., 2013), are already well-advanced. In addition, proof of concept for detection of placental-specific mRNAs such as H19 that was found to be highly expressed in our study, as well as pregnancy-specific RNAs, has now been recently demonstrated (Tsui et al., 2014). Therefore, multiple blood sampling to obtain a liquid plasma RNA biopsy would be an ethically acceptable research study for women with MCDA twin pregnancies. This type of minimally non-invasive approach may provide novel insights into the key genes and biological pathways involved in the development of growth discordance in MCDA twins. Furthermore, such an approach would also have potential to reveal valuable measurable biomarkers for identifying and monitoring MCDA twin pregnancies that are at high risk for growth discordance.

In conclusion, we identified two placental proteins, leptin and ARMS2, strongly associated with growth discordance in MCDA twins. We postulate that leptin and ARMS2 may play a key role to drive angiogenesis as a survival mechanism to compensate for the decreased blood and nutrient supply in the growth-restricted MCDA twin. Further, since both leptin and ARMS2 are produced as soluble proteins, they may also have potential as secreted biomarkers for predicting growth discordance in MCDA twins. Continued studies are required to understand the key epigenetic changes in growth-discordant MCDA twin pregnancies, so that medical interventions or potential therapies can be developed to alleviate the adverse perinatal outcomes associated with this pregnancy complication.

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Conflict of Interest

LS, JZ, and DSC are employees of Berry Genomics Corporation, Beijing.

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