Twin Research and Human Genetics
Volume 20 ▌ Number 5 ▌ pp. 389–394 ▌ © The Author(s) 2017 ▌ doi:10.1017/thg.2017.41

Placental Expressions of *CDKN1C* and *KCNQ1OT1* in Monozygotic Twins with Selective Intrauterine Growth Restriction

Chenyu Gou,[1,2,*] Xiangzhen Liu,[3,*] Xiaomei Shi,[4] Hanjing Chai,[5] Zhi-ming He,[2] Xuan Huang,[2] and Qun Fang[2]

[1]*Department of Obstetrics and Gynecology, Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, China*
[2]*Fetal Medicine Center, Department of Obstetrics and Gynecology, First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China*
[3]*Department of Oral and Maxillofacial Surgery, First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China*
[4]*Genetic Medical Center, Guangdong Women and Children Hospital, Guangzhou, China*
[5]*Department of Obstetrics and Gynecology, Guangdong General Hospital, Guangzhou, China*

CDKN1C and *KCNQ1OT1* are imprinted genes that might be potential regulators of placental development. This study investigated placental expressions of *CDKN1C* and *KCNQ1OT1* in monozygotic twins with and without selective intrauterine growth restriction (sIUGR). Seventeen sIUGR and fifteen normal monozygotic(MZ) twin pairs were examined. Placental mRNA expressions of *CDKN1C* and *KCNQ1OT1* were detected by real-time fluorescent quantitative PCR. *CDKN1C* protein expression was detected by immunohistochemical assay and Western-blotting. In the sIUGR group, smaller fetuses had a smaller share of the placenta, and *CDKN1C* protein expression was significantly increased while *KCNQ1OT1* mRNA expression was significantly decreased. The *CDKN1C*/*KCNQ1OT1* mRNA ratio was lower in the larger fetus than in the smaller fetus ($p < .05$). In the control group, *CDKN1C* protein expression showed no difference between larger and smaller fetuses, while *KCNQ1OT1* mRNA expression was significantly lower in the larger fetus, and the *CDKN1C*/*KCNQ1OT1* mRNA ratio was higher in the larger fetus than in the smaller fetus ($p < .05$). Our findings showed that pathogenesis of sIUGR may be related to the co-effect of the up-regulated protein expression of *CDKN1C* and down-regulated mRNA expression of *KCNQ1OT1* in the placenta.

■ **Keywords:** monozygotic twins, placenta, selective intrauterine growth restriction, *CDKN1C*, *KCNQ1OT1*

Selective intrauterine growth restriction (sIUGR) occurs when the estimated fetal weight (EFW) of the smaller fetus of a pair of monozygotic (MZ) twins is less than the 10th percentile for the same gestational age. It represents an important contributor to perinatal mortality and morbidity in MZ twins, as well as being associated with a high risk of neurological damage in both fetuses (Gratacos et al., 2007; Valsky et al., 2010). Abnormal placental development and an unequal share of the placenta are considered to be the causes of sIUGR. The molecular mechanisms involved in discordant share of the placenta of MZ twins with sIUGR are unclear. Recent studies in singleton and animals suggest that *CDKN1C* and *KCNQ1OT1*, two imprinted genes, could affect placental development (Frost & Moore, 2010; Nelissen et al., 2011; Pateras et al., 2009; Shukla et al., 2011). However, these genes have been less studied in sIUGR. In this study, placental mRNA expressions of *CDKN1C*, *KCNQ1OT1*, and *CDKN1C* protein expression were investigated in MZ twin pairs with sIUGR to explore the pathogenesis of sIUGR.

Materials and Methods

The study was approved by the Hospital Ethics Committees of the First Affiliated Hospital of Sun Yat-Sen University and the Second Affiliated Hospital of Guangzhou Medical University, and informed consent from participants

RECEIVED 12 March 2017; ACCEPTED 1 June 2017. First published online 14 August 2017.

ADDRESS FOR CORRESPONDENCE: Professor Qun Fang, Fetal Medicine Center, Department of Obstetrics and Gynecology, the First Affiliated Hospital of Sun Yat-Sen University, 58, 2nd Zhongshan Road, Guangzhou 510080, China. E-mail: fang_qun@163.com

* These authors contributed equally to this study.

was obtained. All twin pairs included were delivered in these two hospitals from July 2013 to July 2015, and they were confirmed as monochorionic twin pairs by ultrasound examination at first trimester and monozygotic by postpartum short tandem repeat (STR) assay. Pregnancies complicated by twin-to-twin transfusion syndrome (TTTS), miscarriage, fetal death, severe congenital anomalies, and maternal complications were excluded. Placental tissues around the umbilical cord insertion were collected immediately after cesarean section, and maternal blood was washed off by phosphate buffered saline and immediately frozen in liquid nitrogen and stored at −80°C for DNA, RNA, and protein extraction. Some of the placental tissue was fixed with formalin for immunohistochemistry.

Each umbilical cord was labeled at delivery to identify the twin. The arteries and veins from each umbilical cord were catheterized with 1.5 mm plastic catheters and flushed with heparin saline solution at room temperature. Barium sulfate dyed with eosin was injected in arteries, while barium sulfate dyed with methylene blue was injected in veins. Digital photographs of placentas were taken perpendicularly to the chorionic surface. The placental area of each fetus was measured by following the margins demarcated by inter-twin anastomoses. The placental ratio of each twin was calculated as area of each placental portion/area of whole MC placenta × 100%.

DNA was extracted with MiniBEST Universal Genomic DNA Extraction Kit (TAKALA, Japan) and amplified with GeneMapper ID-X (Version 1.2) (Applied Biosystems, USA) to detect 19 autosomal STR loci and gender determination marker. If all the loci were identical, the probability of MZ would be up to 99.99%.

Placental total RNA was extracted with RNAiso Plus (TAKARA, Japan). First-strand cDNA was synthesized from 500 ng of DNase-treated total RNA using PrimeScript RT Master (TAKARA, Japan). *CDKN1C* and *KCNQ1OT1* mRNA expressions were examined by quantitative real-time PCR (Q-PCR) analysis (SYBR Premix Ex TaqTM Kit, TAKARA, Japan and an ABI 7500 Real-Time PCR system, Applied Biosystems, Foster City, CA).

Primers were as follows: *GAPDH*: F-GCACCGTCAA GGCTGAGAAC, R- GGTGAAGACGCCAGTGGA. *CDKN1C*: F- CCCATCTAGCTTGCAGTCTCTT, R- CAGACGGCTCAGGAACCATT. *KCNQ1OT1*: F-GGGAGCTGTTGTCCCTTACC, R- TTCGGAGTGGT AACTGTGCC.

Expression values were calculated with the delta Ct method using the housekeeping gene *GAPDH* as the control.

Indirect immunohistochemical staining for all cases of formalin-fixed placental tissue was performed using the streptavidin-biotin-peroxidase complex method with an SP Rabbit HRP Kit (Cwbiotech, China). A rabbit polyclonal antibody (Abcam, UK) of CDKN1C was used at a 1:200 dilution as the primary antibody.

TABLE 1

Clinical Information of MC Twins With and Without sIUGR

	sIUGR ($n = 17$)	Normal control ($n = 15$)
Maternal age (years)	27.9 ± 3.5	30.2 ± 4.7
Gestational age at delivery(weeks) (range)	34.1* (31–36.7)	36.1 (33.8–37.7)
Birth weight of larger fetus (g)	2085 ± 396*	2574 ± 337
Birth weight of smaller fetus (g)	1424 ± 419*	2366 ± 229
Birth weight discordance (%)	30.3 ± 16.1*	8.4 ± 6.0
Placental ratio of larger fetus (%)	70.9 ± 9.5	55.8 ± 2.0
Placental ratio of smaller fetus (%)	29.1 ± 9.5	44.2 ± 3.3

Note: *$p < .05$ compared with normal control.

Stained sections were scanned on a digital pathology system (Olympus, Japan) both at 20× and 40× magnification. Integrated optic density (IOD) and total area of tissue stained with DAB were measured with the Image-Pro Plus 6.0 software (Media Cybernetics, Europe). Mean light density, obtained by dividing the IOD for the total area, was used to evaluate the intensity of staining.

Western blot analysis was performed on whole-tissue extracts. A total of 50 μg of protein was loaded on 10% SDS-PAGE gel, transferred to polyvinylidene difluoride (PVDF) membrane and blocked with 3% bovine serum albumi (BSA) in Tween tris-buffered saline (TTBS). *CDKN1C* was detected using a rabbit polyclonal anti-*CDKN1C* antibody (Abcam, UK), 1:1,000 and then incubated with a secondary antibody labeled with peroxidase. The blots were visualized by an enhanced chemiluminescence system and were measured with the ImageJ 1.43 software (National Institutes of Health, USA).

Statistical Analyses

Gestational age of each group is presented as the median; the other data are presented as the means ± *SD*. The results of continuous and categorical variables were analyzed with one-way ANOVA. Statistical analysis was performed with SPSS 17.0.

Results

Seventeen sIUGR and fifteen normal MZ twin pairs were included in this study. Clinical information is shown in Table 1. Placental ratios of three types of sIUGR are shown in Figure 1.

In the sIUGR group, placental *CDKN1C* mRNA expression showed no difference between larger and smaller fetuses while *KCNQ1OT1* mRNA expression was significantly higher in the larger fetus (0.72 ± 0.43) than in the smaller fetus (0.36 ± 0.19; $p < .05$). The

https://doi.org/10.1017/thg.2017.41 Published online by Cambridge University Press

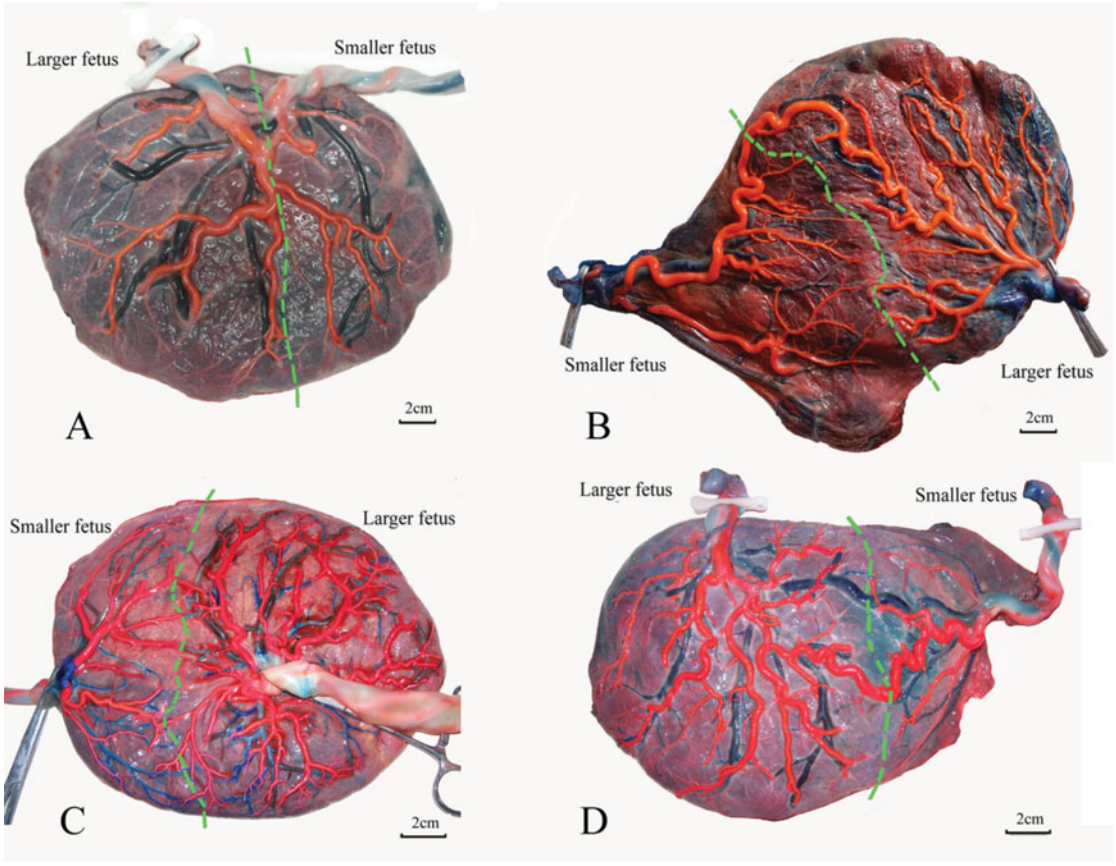

FIGURE 1

(Colour online) Placental ratio of normal and sIUGR MZ twins. Note: A = normal MZ twins, B = Type I sIUGR, C = Type II sIUGR, D = Type III sIUGR. Dash lines showed the margins demarcated by inter-twin anastomoses. The birth weights and share of placenta were similar in normal control group. The share of placenta was less in the smaller fetus of the sIUGR group. Scale bar = 2 cm.

CDKN1C/*KCNQ1OT1* mRNA ratio was lower in the larger fetus (2.92 ± 1.97) than in the smaller fetus (6.01 ± 3.54; $p < .05$). In the normal control group, placental *CDKN1C* mRNA showed no difference between larger and smaller fetuses either. But *KCNQ1OT1* mRNA expression was significantly lower in the larger fetus (0.63 ± 0.32) than in the smaller fetus (1.08 ± 0.67), and *CDKN1C*/ *KCNQ1OT1* mRNA ratio was higher (3.07 ± 1.95) in the larger fetus than in the smaller fetus (1.73 ± 1.07; $p < .05$) (Figure 2). Immunohistochemistry and western blotting showed that the mean light density of *CDKN1C* was higher in the smaller fetus than in the larger fetus in sIUGR group ($p <$ .05), while no difference was shown in the control group (Figures 3 and 4).

Discussion

The etiology of sIUGR in MZ twins is not clear, yet the placenta is indispensable in the pathogenic mechanism of sIUGR. Placental dysplasia of the smaller fetus might be critical in MZ sIUGR (De Paepe et al., 2010; Gou et al., 2017; Lopriore et al., 2007). Q-PCR, immunohistochemistry, and western blotting showed that *CDKN1C* and *KCNQ1OT1* were expressed in MZ placentas, which indicated that *CDKN1C* and *KCNQ1OT1* might be important during placental development.

CDKN1C is a paternally imprinted gene that encodes a potent inhibitor of several cyclin/Cdk complexes. *CDKN1C* is primarily expressed in terminally differentiated cells; it associates with G1 Cdks, and its overexpression causes a complete cell cycle arrest in the G1 phase. *KCNQ1OT1* is a maternally imprinted gene, and its transcript is an unspliced long non-coding RNA. It interacts with chromatin and silences *CDKN1C* transcription through epigenetic modifications.

According to parental conflict theory, paternally imprinted genes suppress fetal growth, while maternally imprinted genes promote fetal growth (Diplas et al., 2009). Expressions of paternally and maternally imprinted genes should be kept in balance to ensure the normal development of both placenta and embryo. Although *CDKN1C* mRNA expression showed no difference between the larger

https://doi.org/10.1017/thg.2017.41 Published online by Cambridge University Press

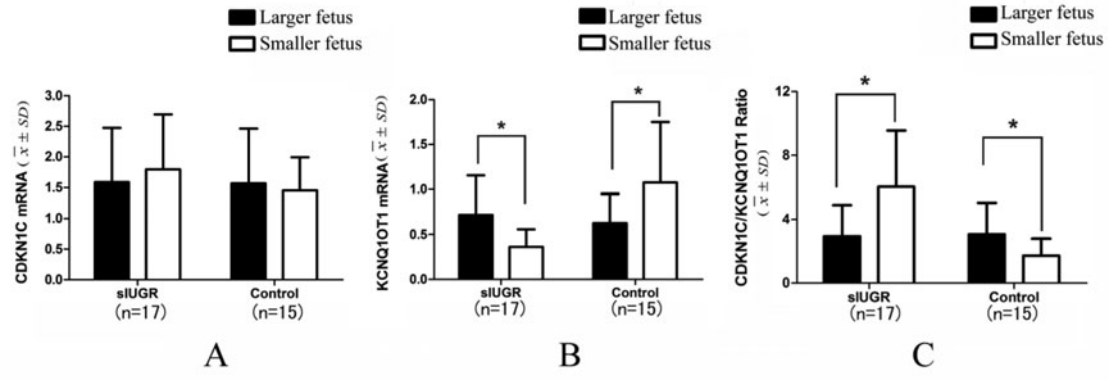

FIGURE 2

mRNA expressions of *CDKN1C* and *KCNQ1OT1* (*$p < .05$). Note: A = *CDKN1C*, B = *KCNQ1OT1*, C = *CDKN1C/KCNQ1OT1* mRNA ratio.

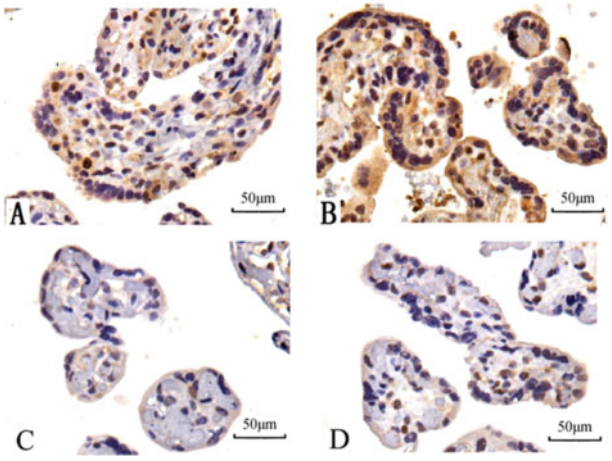

FIGURE 3

(Colour online) Immunohistochemistry *of CDKN1C* in placenta (×400). Note: A = larger fetus of the sIUGR group, B = smaller fetus of the sIUGR group, C = larger fetus of the control group, D = smaller fetus of the control group. Scale bar = 50 μm.

and smaller fetuses in the sIUGR group, CDKN1C protein expression was higher in the smaller fetus than the larger fetus. CDKN1C was reported to increase in IUGR placentas of human singletons (McMinn et al., 2006; Unek et al., 2014). Animal studies have shown that CDKN1C protein staining was stronger in placentas of IUGR rats induced with dexamethasone (Unek et al., 2012), and *CDKN1C* over-expressing animals have significantly smaller placental weight and fetal growth restriction (Andrews et al., 2007; Serman et al., 2007). These results showed some similarity with our study. Our results showed that placental *KCNQ1OT1* expression decreased in smaller fetuses of the sIUGR group but increased in smaller fetuses of the control group. There were some similar discoveries in animal studies.

CDKN1C-knockdown rats and *KCNQ1OT1*-imprinting-loss rats showed the same pathologic changes, such as placental hypertrophy, trophoblast hyperplasia, and mesenchymal dysplasia (Armes et al., 2012; Du et al., 2004; Shukla et al., 2011). The previous studies and our study showed that sIUGR smaller fetuses had a smaller share of the placenta (De Paepe et al., 2010; Gou et al., 2017; Lopriore et al., 2007). Increased CDKN1C protein and/or insufficient *KCNQ1OT1* expression might play an important role in the placental dysplasia of the sIUGR smaller fetus.

The *CDKN1C*/*KCNQ1OT1* mRNA ratio in this study could intuitively reflect the expression balance between paternally and maternally imprinted genes. In the sIUGR group, the *CDKN1C*/*KCNQ1OT1* mRNA ratio was almost twice as high in the smaller fetus than in the larger fetus, but in the control group, it was only 50% in the smaller fetus. It indicated that *CDKN1C* expression was dominant in sIUGR smaller fetuses, and this might be closely related to a poorly developed placenta. *CDKN1C* expression was found to increase more than *IGF-2* in placentas of IUGR rats induced by alcohol: *CDKN1C*/*IGF-2* mRNA ratio was 1.5 in IUGR rats and 0.9 in the control group (Shukla et al., 2011).

According to our results, we speculate that CDKN1C protein expression of the smaller fetus in sIUGR increases secondarily to the decreased expression of *KCNQ1OT1*, and CDKN1C over-expression causes early termination of trophoblast proliferation, which in turn might cause placental dysplasia. Contrarily, in the normal MZ group, the expression of *KCNQ1OT1* in the smaller fetus increases; this might compensate placental development; therefore, the birth weight discordance is small.

CDKN1C protein and *KCNQ1OT1* mRNA are expressed differentially in larger and smaller fetuses in the placenta of MZ with sIUGR. The pathogenesis of sIUGR may be related to the co-effect of the up-regulated protein expression of *CDKN1C* and down-regulated expression of *KCNQ1OT1* mRNA in the placenta. The imbalanced expression between *CDKN1C* and *KCNQ1OT1* may be involved in the unequal placental development in sIUGR.

https://doi.org/10.1017/thg.2017.41 Published online by Cambridge University Press

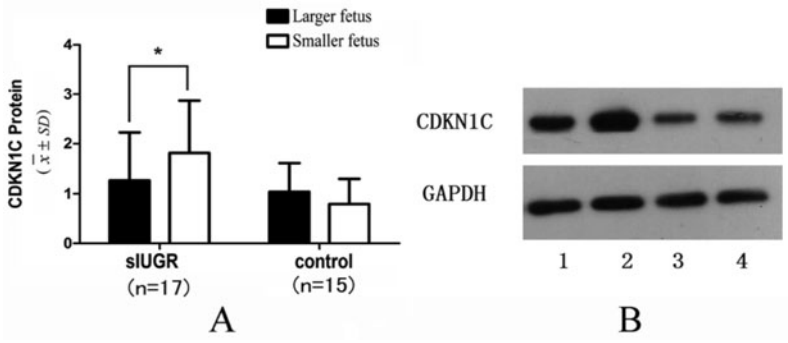

FIGURE 4

Western blot of *CDKN1C* in placenta (*$p < .05$). Note: 1 = larger fetus of the sIUGR group, 2 = smaller fetus of the sIUGR group, 3 = larger fetus of the control group, 4 = smaller fetus of the control group.

Acknowledgments

We thank Professor Lanzhen Zhang, Professor Zhiqiong Liao, and Professor Yu Gao for placenta collection, and Ms Junhong Chen for clinical data collection. National Nature Science Foundation of China (Grant no.8127075); Science and Technology Planning Project of Guangzhou, China (1563000549); Research Fund for the Doctoral Program of Guangzhou Medical University (2015C16) have provided financial support.

Ethical Approval

The study was approved by the Hospital Ethics Committees of the First Affiliated Hospital of Sun Yat-Sen University and the Second Affiliated Hospital of Guangzhou Medical University.

References

Andrews, S. C., Wood, M. D., Tunster, S. J., Barton, S. C., Surani, M. A., & John, R. M. (2007). Cdkn1c (p57Kip2) is the major regulator of embryonic growth within its imprinted domain on mouse distal chromosome 7. *BMC Developmental Biology, 7*, 53–66.

Armes, J. E., McGown, I., Williams, M., Broomfield, A., Gough, K., Lehane, F., & Lourie, R. (2012). The placenta in Beckwith–Wiedemann syndrome: Genotype-phenotype associations, excessive extravillous trophoblast and placental mesenchymal dysplasia. *Pathology, 44*, 519–527.

De Paepe, M. E., Shapiro, S., Young, L., & Luks, F. I. (2010). Placental characteristics of selective birth weight discordance in diamniotic-monochorionic twin gestations. *Placenta, 31*, 380–386.

Diplas, A. I., Lambertini, L., Lee, M. J., Sperling, R., Lee, Y. L., Wetmur, J., & Chen, J. (2009). Differential expression of imprinted genes in normal and IUGR human placentas. *Epigenetics*, *4*, 235–240.

Du, M., Zhou, W., Beatty, L. G., Weksberg, R., & Sadowski, P. D. (2004). The KCNQ1OT1 promoter, a key regulator of genomic imprinting in human chromosome 11p15.5. *Genomics, 84*, 288–300.

Frost, J. M., & Moore, G. E. (2010). The importance of imprinting in the human placenta. *PLoS Genetics, 6*, e1001015.

Gou, C., Li, M., Zhang, X., Liu, X., Huang, X., Zhou, Y., & Fang, Q. (2017). Placental characteristics in monochorionic twins with selective intrauterine growth restriction assessed by gradient angiography and three-dimensional reconstruction. *Journal of Maternal-Fetal & Neonatal Medicine, 21*, 1–6.

Gratacos, E., Lewi, L., Munoz, B., Acosta-Rojas, R., Hernandez-Andrade, E., Martinez, J. M., ... Deprest, J. (2007). A classification system for selective intrauterine growth restriction in monochorionic pregnancies according to umbilical artery Doppler flow in the smaller twin. *Ultrasound in Obstetrics and Gynecology, 30*, 28–34.

Lopriore, E., Sueters, M., Middeldorp, J. M., Oepkes, D., Walther, F. J., & Vandenbussche, F. P. (2007). Velamentous cord insertion and unequal placental territories in monochorionic twins with and without twin-to-twin-transfusion syndrome. *American Journal of Obstetrics and Gynecology, 196*, 151–159.

McMinn, J., Wei, M., Schupf, N., Cusmai, J., Johnson, E. B., Smith, A. C., & Tycko, B. (2006). Unbalanced placental expression of imprinted genes in human intrauterine growth restriction. *Placenta, 27*, 540–549.

Nelissen, E. C., van Montfoort, A. P., Dumoulin, J. C., & Evers, J. L. (2011). Epigenetics and the placenta. *Human Reproduction Update, 17*, 397–417.

Pateras, I. S., Apostolopoulou, K., Niforou, K., Kotsinas, A., & Gorgoulis, V. G. (2009). p57KIP2: 'Kip'ing the cell under control. *Molecular Cancer Research, 7*, 1902–1919.

Serman, L., Vlahovic, M., Sijan, M., Bulic-Jakus, F., Serman, A., Sincic, N., & Katusic, A. (2007). The impact of 5-azacytidine on placental weight, glycoprotein pattern and proliferating cell nuclear antigen expression in rat placenta. *Placenta, 28*, 803–811.

Shukla, P. K., Sittig, L. J., Ullmann, T. M., & Redei, E. E. (2011). Candidate placental biomarkers for intrauterine

https://doi.org/10.1017/thg.2017.41 Published online by Cambridge University Press

alcohol exposure. *Alcoholism Clinical and Experimental Research, 35*, 559–565.

Unek, G., Ozmen, A., Kipmen-Korgun, D., & Korgun, E. (2012). Immunolocalization of PCNA, Ki67, p27 and p57 in normal and dexamethasone-induced intrauterine growth restriction placental development in rat. *Acta Histochemica, 114*, 31–40.

Unek, G., Ozmen, A., Ozekinci, M., Sakinci, M., & Korgun, E. T. (2014). Immunolocalization of cell cycle proteins (p57, p27, cyclin D3, PCNA and Ki67) in intrauterine growth retardation (IUGR) and normal human term placentas. *Acta Histochemica*, *116*, 493–502.

Valsky, D. V., Eixarch, E., Martinez, J. M., Crispi, F., & Gratacos, E. (2010). Selective intrauterine growth restriction in monochorionic twins: Pathophysiology, diagnostic approach and management dilemmas. *Seminars in Fetal and Neonatal Medicine, 15*, 342–348.

https://doi.org/10.1017/thg.2017.41 Published online by Cambridge University Press