Placenta 81 (2019) 46–53

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Placental expression of microRNAs in infants born small for gestational age

H. Östling[a], R. Kruse[b], G. Helenius[c], M. Lodefalk[d,e,*]

[a] Department of Obstetrics and Gynecology, School of Medical Sciences, Örebro University, SE-701 82, Örebro, Sweden
[b] Department of Clinical Research Laboratory, Faculty of Medicine and Health, Örebro University, SE-701 82, Örebro, Sweden
[c] Department of Laboratory Medicine, Faculty of Medicine and Health, SE-701 82, Örebro University, Örebro, Sweden
[d] Department of Pediatrics, School of Medical Sciences, Örebro University, SE-701 82, Örebro, Sweden
[e] University Health Care Research Center, Faculty of Medicine and Health, Örebro University, SE-701 82, Örebro, Sweden

ARTICLE INFO

Keywords:
Foetal growth
Inflammation
microRNA
Placenta
RNA-Sequencing
Small for gestational age

ABSTRACT

Introduction: The molecular mechanisms behind poor foetal growth are not fully known. The aim of this study was to explore global microRNA expression in placentas of infants born small for gestational age (SGA) compared to infants with a normal birth weight (NBW).

Methods: Placental biopsies from term infants were identified in a biobank and divided into four groups: infants born SGA with (n = 13) or without (n = 9) exposure to low maternal gestational weight gain (GWG) and infants born with NBWs with (n = 20) or without (n = 26) exposure to low GWG. All women and infants were healthy, and no woman smoked during pregnancy. Only vaginal deliveries were included. Next-generation sequencing was performed with single read sequencing of > 9 million reads per sample. Differential microRNA expression was analysed using ANOVA for unequal variances (Welch) with multiple testing corrections through the Benjamini-Hochberg method. A fold change > 2 and a corrected p value < 0.05 were considered significant. Adjustments for possible confounding factors were made using a linear regression model.

Results: A total of 1870 known, mature human microRNAs were detected in the sample. MiR-3679-5p and miR-193b-3p were significantly upregulated, and miR-379-3p, miR-335-3p, miR-4532, miR-519e-3p, miR-3065-5p, and miR-105-5p were significantly downregulated after adjustment for potential confounding factors in SGA infants with normal GWG compared to infants with NBWs and normal GWG.

Discussion: Infants born unexplained SGA show differential microRNA expression in their placenta. Important pathways for the differentially expressed microRNAs include inflammation and the insulin-IGF system.

1. Introduction

Foetal growth restriction (FGR) is defined as the failure of a foetus to attain its full growth potential. The prevalence of FGR varies globally, depending on the population and the country's state of development. It is one of the leading causes of perinatal morbidity and mortality and a well-known risk factor for adult diabetes, hypertension, and cardiovascular disease [1].

FGR is a heterogeneous condition resulting from a variety of causes and often leads to an infant born small for gestational age (SGA). However, not all infants born SGA are necessarily growth-restricted. Some infants might be constitutionally small but healthy since the normal size at birth varies by ethnicity, sex, parity, and maternal size [2]. As only SGA births due to FGR are associated with an increased risk of neonatal, childhood, and adult morbidity, attempts have been made to distinguish pathological SGA births from physiological ones [3,4].

Even though several of the conditions that lead to SGA births are known, as well as biological pathways that can be involved in foetal growth, i.e. immune response, cell adhesion, response to oxidative stress, and regulation of growth and cell differentiation [5], the molecular mechanisms behind poor foetal growth are not fully understood. This has led to a lack of appropriate diagnostic methods and effective treatments. In addition, it is necessary to identify early biomarkers indicating an increased risk for future disease in children born SGA. Increased knowledge about molecules involved in poor foetal growth could potentially assist in all these areas.

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate gene expression at the posttranscriptional level. They have been reported to be involved in many biological functions, as well as in several pathological processes [6].

MiRNAs are differentially expressed in various tissues, and their expression is influenced by a multitude of diseases and external factors

* Corresponding author. Department of Pediatrics, Örebro University Hospital, SE-701 85, Örebro, Sweden.
E-mail address: maria.lodefalk@regionorebrolan.se (M. Lodefalk).

https://doi.org/10.1016/j.placenta.2019.05.001
Received 1 August 2018; Received in revised form 22 March 2019; Accepted 1 May 2019

[7,8]. Additionally, miRNAs are expressed in the placenta, and differential miRNA expression in placenta has been reported in pregnancies complicated by preeclampsia (PE), FGR, or maternal smoking [7,9–16], indicating that miRNAs play a role in placental-associated diseases.

However, previous studies on miRNA expression in placentas of infants born SGA have shown some methodological problems. Some did not take into fully account possible confounding factors, such as abnormal maternal body mass index (BMI), premature delivery, maternal smoking, and differences in delivery mode, infant sex, or parity between SGA and normal weight infants. All these parameters might have influenced the results [11–14]. In addition, previous studies have only investigated a small number of miRNAs or miRNA clusters [11–14,17–21] or, when investigating a large number of miRNAs, have only included a very small number of subjects [15,22] or only twins [22]. To the best of our knowledge, no previous study has investigated the global miRNA expression in placenta using a sequencing technique in a larger sample set in relation to SGA births.

The aim of the present study was to explore the global expression of miRNAs in the placentas of term infants with birth weights < -2 standard deviations (SD) compared to normal birth weight (NBW) infants in order to improve our understanding of the pathogenesis underlying poor foetal growth. We aimed at excluding or correcting for possible confounding factors. We also aimed to describe pathways involved in SGA births according to bioinformatic analyses of the differentially expressed miRNAs that potentially could be found in the study.

2. Methods

2.1. Subjects

The placental biopsies were recruited from a sample collection at Örebro University Hospital, Sweden. The biopsies were collected during 2007–2012 at the Department of Obstetrics and Gynecology after written, informed consent was obtained from the woman. The study was approved by the Regional Board of Ethics in Uppsala, Sweden (2010/189).

The inclusion criteria were healthy women with a normal BMI (18.5–24.9) in early pregnancy, height > 150 cm, age 18–42 years at the time of delivery, and vaginal delivery of a healthy, singleton full term infant (gestational age 37 + 0 to 41 + 6).

The exclusion criteria were maternal smoking during pregnancy, any pregnancy complication (gestational hypertensive diseases, diabetes, or erythrocyte immunization), induction of the delivery with prostaglandins, asphyxia (defined as Apgar score < 7 at 5 min), chromosomal abnormality, or any anatomical malformation of the infant.

Placental biopsies of 22 infants born SGA (birth weights < -2 SD) and 46 infants with NBWs, who fulfilled all criteria, were identified. In order to try to make a subgroup of infants born SGA more homogeneous, the infants were sorted based on maternal gestational weight gain (GWG; low or normal) resulting in four groups totally. All SGA infants in the subgroup with poor maternal GWG shared an exposure that could have led to poor foetal growth, namely low GWG (LGWG) [23].

The subjects in the four groups were matched for sex of the infant, gestational age, parity, and age of the woman, as far as possible. However, since the woman-infant-pairs in the SGA groups were the only ones in the sample collection fulfilling all inclusion and none of the exclusion criteria, it was not possible to match these two groups to each other.

The characteristics of the subjects are presented in Table 1. There were no significant differences between the groups with respect to maternal age, weight, height, BMI, parity, gestational age, or sex of the infant. There were significant differences ($p < 0.001$) in GWG between the subjects with LGWG and the subjects with normal GWG (NGWG). There were also significant differences ($p < 0.001$) in birth weight, length, and head circumference between the SGA and the NBW infants. Despite all of the infants in groups 1 and 2 were SGA, the infants in Group 2 turned out to have lower birth weights than those in Group 1 ($p = 0.042$). There were no differences between the groups with regards to time of the year for the births.

2.2. Clinical definitions and study procedures

Data were retrieved from the maternal health records. Weight and height of the women were measured at the antenatal care clinics in the first trimester of pregnancy. Weight was also measured at the delivery ward at the time of delivery and GWG was calculated as the difference between late and early pregnancy weight.

Women with a height below 150 cm were excluded in an attempt to exclude infants born SGA physiologically. The cut off of 150 cm was chosen since it corresponds to a height 3 SD below the mean height of adult women in Sweden [24].

NGWG was defined as 11.5–16.0 kg based on current recommendations [25]. In the present study, LGWG was defined as ≤10 kg. To attain a significant difference between LGWG and NGWG, only women with at least 4 kg greater GWG than the corresponding woman in the LGWG groups were included in the NGWG groups.

Being SGA was defined as having a birth weight ≥2 SD below the population mean [26] using a Swedish reference population [27]. NBW was defined as a birth weight within ± 1 SD from the population mean. The z-scores for birth weight, length, and head circumference were calculated using Swedish reference data taking sex and gestational age into account [27].

2.3. Sample preparations

The placental biopsies were collected immediately after delivery. The amniotic membranes were removed and two biopsies, each the size of approximately 1 cm, were sampled from random locations on the maternal side of the placenta. The biopsies were rinsed in 30 mL of cold phosphate buffered saline (Gibco, Life Technologies, Stockholm, Sweden) and then stored at −80 °C in 3 mL of RNAlater (Ambion, Stockholm, Sweden) until further processing.

2.4. RNA isolation

The entire RNA was extracted from 10 to 30 mg of placental tissue using the Allprep DNA/RNA/miRNA Universal Kit (50), catalogue no. 80,224 (Qiagen, Nordic, Sollentuna, Sweden) per the manufacturer's instructions. RNA concentration and purity were determined with a Nano-Drop ND-1000 Spectrophotometer (Nano-Drop Technology Inc., Wilmington, DE, USA) and RNA quality was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) per the manufacturers' instructions. All samples passed the quality check, which included total extracted RNA optical density 260/280: 1.8–2.2 and RNA integrity number ≥8.

2.5. Next-generation sequencing

Next-generation sequencing (NGS) was performed at GATC Biotech AG (Konstanz, Germany). Illumina's Small RNA sample preparation protocol (TruSeq Small RNA Sample Prep Kits (Illumina, San Diego, CA, USA)) were used according to the manufacturer's instructions with minor adaptations taken for the generation of small RNA libraries. The libraries were sequenced on HiSeq 2500 (Illumina) according to the manufacturer's protocol, with single read sequencing of > 9 million reads per sample.

All data processing was performed using the Strand NGS software suit, version 3.3.1. Initial adaptor trimming was performed, followed by trimming of the bases with a base quality less than 20. Reads were then aligned and mapped to the reference genome (Genome build: *Homo*

Table 1
Clinical characteristics of the subjects.

	Group 1 SGA + LGWG (n = 13)	Group 2 SGA + NGWG (n = 9)	Group 3 NBW + LGWG (n = 20)	Group 4 NBW + NGWG (n = 26)	p-value
Maternal characteristics					
Age (years)	31.8 (22.6–40.7)	27.8 (21.9–35.6)	28.9 (23.4–35.5)	30.2 (22.5–35.9)	NS
Parity 0-para	9 (69%)	8 (89%)	13 (65%)	18 (69%)	NS
Weight (kg)	61 (51–72)	63 (52–68)	60.5 (48–79)	62.5 (53–74)	NS
Height (cm)	167 (158–174)	166 (158–178)	167.5 (158–178)	168.5 (158–178)	NS
BMI (kg/m^2)	22.3 (18.7–24.9)	22.6 (20.7–24.4)	21.6 (18.7–24.9)	22.1 (19.0–24.9)	NS
GWG (kg)	10 (6–10)	13 (12–15)	9 (7–10)	15 (12–16)	< 0.001[1]
Infant characteristics					
GA (weeks)	40.6 (37.7–41.9)	39.7 (38.6–41.7)	40.3 (38.3–41.6)	40.7 (37.0–41.7)	NS
Sex Females	3 (23.1%)	5 (55.6%)	6 (30%)	6 (23.1%)	NS
Birth weight (g)	2840 (2155–3160)	2620 (2290–3060)	3597 (3265–4510)	3735 (3080–4355)	< 0.001[2]
Birth length (cm)	48 (45–51)	49 (45–50)	50 (48–55)	51 (48–54)	< 0.001[2]
Head circum-ference (cm)	34.0 (30–35)	33.0 (32–35)	35.3 (33–37)	35.3 (33–38)	< 0.001[2]
Birth weight z-score	−2.24 (−3.36–−2.01)	−2.40 (−2.95–−2.11)	−0.07 (−0.92–0.96)	0.16 (−0.87–0.85)	< 0.001[2]
Birth length z-score	−2.05 (−3.52–−0.75)	−2.15 (−3.13–−0.78)	−0.54 (−2.28–1.52)	−0.28 (−1.40–1.37)	< 0.001[2]
Head circum-ference z-score	−1.63 (−3.35–−0.86)	−1.71 (−2.38–−1.20)	−0.46 (−1.65–1.11)	−0.22 (−1.82–2.01)	< 0.001[2]

Data are the medians (min-max) or numbers (%). BMI = body mass index. GWG = gestational weight gain. GA = gestational age. NBW = normal birth weight. NS = not significant. [1]Group 1 vs. Group 2, as well as Group 1 vs. Group 4. [2]Group 1 vs. Group 4, as well as Group 2 vs. Group 4.

sapiens, HG 19 (build 2009.06.14)) allowing up to one mismatch per uniquely aligned read. In total, 5002 different small RNAs were identified in the sample. Of these, 1870 were mapped to known sequences of miRBase-described mature human miRNAs. Data normalization was performed with DESeq normalization prior to statistical testing.

2.6. ddPCR analyses

Droplet digital polymerase chain reaction (ddPCR) analyses were performed in all samples of group 1, 2, and 4 (in total 48 samples) at the Clinical Research Laboratory, Örebro University Hospital, Sweden. Five miRNAs of interest and two reference genes were analysed for verification of the sequencing results. Complementary DNA (cDNA) was generated from 450 ng of RNA from each sample using the TaqMan®MicroRNA Reverse Transcription Kit (Thermo Fisher, Waltham, MA, USA) and TaqMan® Small RNA Assays (Primers: miR-379-3p, assayID 2133; miR-519e-3p, assayID 2370; miR-380-3p, assayID 569; miR-105-5p, assayID 2167; miR-193b, assayID 2367; snRNAU6, assayID 1973; and RNU58A, assayID 1207, from Thermo Fisher) in a C100 thermal cycler (BIO-RAD, Hercules, CA, USA). For each ddPCR assay, 5.5 µL cDNA was used in a total sample and master mix volume of 20 µL with 2 × ddPCR supermix for probes (BIO-RAD), 20 × TaqMan assay miRNA probe (Thermo Fisher) with droplet generation using the QX100 droplet generator (BIO-RAD). Droplet reactions were amplified by PCR for 40 cycles and droplets were read in the QX200 droplet reader and analysed using the Quantasoft™ version 1.7.4 software (BIO-RAD). Results were obtained as absolute numbers of cDNA copies/µL of 20 µL master mix. Target expression was normalized to the geometrical mean of the reference genes snRNAU6 and RNU58A and presented as target copies per 100,000 reference copies.

2.7. Statistic and bioinformatic analyses

Differences in clinical characteristics were assessed using SPSS Statistics, version 22. The Chi-square test was used for proportions and the Student's t-test or the Mann-Whitney *U* test was used for continuous variables, where appropriate. ANOVA testing for equal variances and Kruskal-Wallis testing were used when comparing more than two groups, where appropriate. A linear regression model was used to adjust for potential confounding factors. Data were presented as medians (min-max) or numbers (%). Statistical significance was set at a p value < 0.05.

Differential miRNA expression was analysed using ANOVA for unequal variances (Welch) with multiple testing corrections with the Benjamini-Hochberg correction. Statistical significance was set to a corrected p-value < 0.05 and biological significance was, after fold change analysis, set to a fold change > 2.0. Analysis of clustering was performed with hierarchical cluster analysis applied using Wards linkage rule and Euclidean similarity measure of distance (Strand NGS software suit).

Differentially expressed miRNAs were subjected to functional analyses through Ingenuity Pathway Analysis (IPA, QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis) Core analyses. Target prediction of miRNAs to potential mRNA targets was performed by Strand NGS through mappings from TargetScan, PicTar, TarBase, microRNA.org, and PITA target prediction databases.

The ddPCR data was analysed by Shapiro-Wilks test for normality followed by Kruskal-Wallis test. Correlations were analysed by Spearman's rank correlation test.

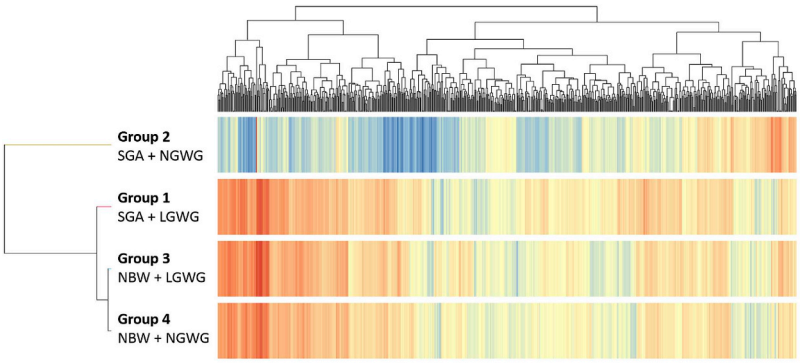
3. Results

3.1. Sequencing analyses

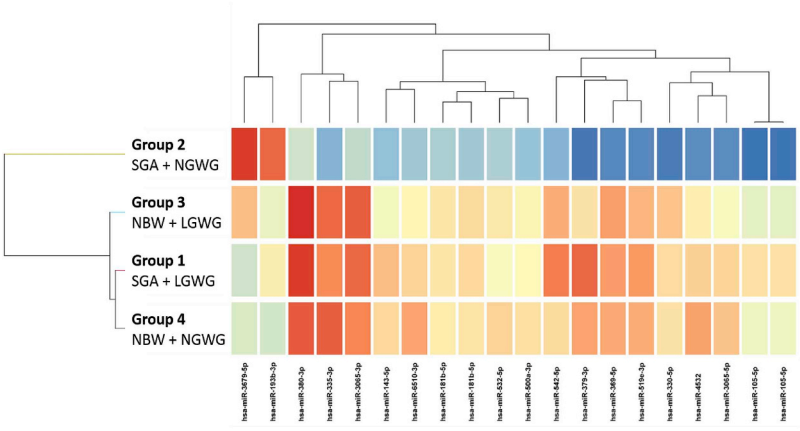
Hierarchical clustering analysis showed differential miRNA expression pattern in Group 2 (SGA + NGWG) compared to the other groups (Fig. 1). The infants in Group 2 showed biologically and statistically significant differential expression of eleven miRNAs in the placentas, compared to the infants in Group 4 (NBW + NGWG; Table 2). Two miRNAs were upregulated and nine were downregulated in Group 2. Group 2 also differed statistically and biologically significant in miRNA expression from Group 1 (SGA + LGWG), data not shown. No other significant differences in miRNA expression between the groups were found.

Since the sex distribution of infants in Group 2 appeared to differ from that in the other groups, although not statistically significant (see Table 1), a secondary analysis was performed, where the miRNA expression was compared between sexes (48 boys vs. 20 girls) regardless of birth weight or GWG. This analysis showed no differential miRNA expression in relation to sex.

Furthermore, the SGA infants in Group 2 had significantly lower birth weights than the SGA infants in Group 1 (see Table 1). Therefore, another secondary analysis was performed comparing the twelve infants with the lowest birth weights (2575 g (2155–2790)) regardless of GWG with twelve NBW infants matched for sex, parity, and GWG. This analysis showed no differential miRNA expression between these two



a) Hierarchical clustering of miRNAs expressed in a minimum of 75% of the samples in at least one group (n= 661).



b) Hierarchical clustering of miRNAs with statistically significant differential expression between groups (n= 20).

Fig. 1. Hierarchical clustering of miRNAs between groups according to NGS Strand. SGA = small for gestational age. NBW = normal birth weight. NGWG = normal gestational weight gain. LGWG = low gestational weight gain. A stronger heat-map color denotes higher (red) or lower (blue) expression.

Table 2
miRNAs with differential expression, both biologically (defined as fold change > 2) and statistically significant, in placentas from SGA infants compared to infants with normal birth weights. All mothers had normal GWG.

miRNA	Fold change (Group 2 vs. Group 4)
miR-3679-5p	2.48
miR-193b-3p	2.09
miR-379-3p	−3.27
miR-335-3p	−3.04
miR-369-5p	−2.90
miR-4532	−2.87
miR-519e-3p	−2.85
miR-3065-5p	−2.57
miR-330-5p	−2.52
miR-105-5p	−2.28
miR-380-3p	−2.25

SGA = small for gestational age. GWG = gestational weight gain.

Table 3
Associations between placental gene expression of miRNAs and birth weight class (SGA vs. NBW), unadjusted and adjusted for possible confounding factors. All infants' mothers had normal GWG during the pregnancy.

Gene expression of miRNAs	Unadjusted	p-value	Adjusted	p-value
	Coefficient		Coefficient	
miR-3679-5p	−0.66	< 0.001	−0.74	< 0.001
miR-193b-3p	−0.53	0.003	−0.57	0.003
miR-379-3p	0.86	0.006	0.83	0.012
miR-335-3p	0.80	0.004	0.82	0.009
miR-369-5p	0.77	0.016	0.69	0.051
miR-4532	0.76	0.005	0.76	0.009
miR-519e-3p	0.76	0.002	0.68	0.012
miR-3065-5p	0.68	0.012	0.58	0.044
miR-330-5p	0.67	0.008	0.45	0.078
miR-105-5p	0.59	0.002	0.71	0.001
miR-380-3p	0.58	0.032	0.58	0.063

The coefficients adjusted for maternal age, parity, and infant's sex. SGA = small for gestational age. NBW = normal birth weight. GWG = gestational weight gain.

new groups.

The correlations between the differentially expressed miRNAs and the group destination (Group 2 vs. Group 4) were adjusted for maternal age, parity, and infant sex in a linear regression model. Only minor changes of the coefficients were found after the adjustment. However, for three of the miRNAs the statistical significance of the correlations decreased to a tendency toward significance (Table 3).

3.2. ddPCR analyses

The ddPCR analyses showed the following fold changes (Group 2 vs. Group 4) and mean concentrations (95% confidence intervals): For miR-379-3p −1.90 (5.36 copies per 100,000 reference copies (−2.73–13.46)

vs. 10.90 (6.43–15.37), p = 0.025); for miR-519e-3p −3.06 (799.85 copies per 100,000 reference copies (−85.23–1684.94) vs. 2690.00 (1402.51–3977.49), p = 0.016); for miR-380-3p −1.22 (1.41 copies per 100,000 reference copies (0.64–2.17) vs. 1.81 (1.29–2.33), p = 0.675); for miR-105-5p −1.95 (4.28 copies per 100,000 reference copies (2.06–6.50) vs. 8.93 (6.55–11.30), p = 0.036); and for miR-193b 2.21 (44,806.91 copies per 100,000 reference copies (935.21–88,678.60) vs. 20,694.83 (16,559.28–24,830.37), p = 0.319).

The correlation coefficient between sequencing and PCR data was for miR-379-3p 0.79 ($p < 0.001$), for miR-519e-3p 0.67 ($p < 0.001$), for miR-380-3p 0.57 ($p < 0.001$), for miR-105-5p 0.62 ($p < 0.001$), and for miR-193b 0.74 ($p < 0.001$).

3.3. Bioinformatic analyses

The IPA of the eleven miRNAs with differential expression between Group 2 and Group 4 showed no common upstream regulator, but downstream effects targeted the Disease and Function Analysis group "Cancer Drug Resistance". The Disease and Function analysis of them based on IPA found 101 statistically significant annotations, of which involved the biology of different tumours. Other important annotations involved cell viability, cell migration, and inflammation. One involved severe late-onset PE. The network between the differentially expressed miRNAs, other miRNAs involved, and their associated proteins in relation to the function "Inflammation" is shown in Fig. 2. The corresponding network in relation to "Cell viability" was very similar (data not shown).

The 20 most statistically significantly predicted targeted mRNAs of the eleven differentially expressed miRNAs are presented in Table 4.

4. Discussion

In the present study, significant differences in miRNA expression in the placentas of term infants born SGA compared to matched infants with NBWs were found. The results support previous evidence that miRNA expression in the placenta seems to be involved in the pathogenesis of or the compensatory mechanisms for poor foetal growth [12,13,15]. The bioinformatic analyses showed that the differentially expressed miRNAs in this study are involved in cell viability, cell migration, and inflammation, i.e. processes of importance to placental function or dysfunction and, consequently, foetal growth. The analyses also showed connections to tumours, which may be due to the large number of miRNA studies performed in relation to tumours [28]. However, the placenta shows similarities with tumours with regards to invasive and rapid growth [29], indicating that miRNAs that are significant in tumours may also be relevant to placental dysfunction.

It is interesting that differential miRNA expression was evident in Group 2, clearly shown by the hierarchical clustering analyses (see Fig. 1), and not in the smallest children, as indicated by one of the secondary analyses performed. A common feature of the infants in Group 2 is that their low birth weights have no obvious explanation since no factor known to be involved in FGR are applicable to them (maternal smoking during pregnancy, poor maternal health, low GWG, abnormal maternal body size, pregnancy complications, or malformations of the infant). Our findings may point at pathways of importance for unexplained SGA births.

To the best of our knowledge, only one of the differentially expressed miRNAs found in this study has been reported before as differentially expressed in placentas in relation to SGA births. That miRNA is miR-519, which was downregulated both in the present and in previous reports [12,13]. In contrast, Wang et al. [18] reported upregulation of miR-519a in placentas from FGR pregnancies. In disagreement with the findings on SGA births by others [12,13,18], downregulation of miR-518 was not demonstrated in this study. These differences may be due to differences in study design.

The miR-519 gene belongs to the Chromosome 19 miRNA cluster (C19MC) found at 19q13.41. This cluster is paternally imprinted and primarily expressed in placenta and other organs of the reproductive system, suggesting its importance in embryonic development [30]. Downregulation of miR-519e has been found together with upregulation of miR-193b and miR-4532 in placentas from pregnancies complicated with PE superimposed on chronic hypertension [31]. Upregulation of miR-193b alone or together with downregulation of miR-379 have been found in preeclamptic placentas [32,33], a finding consistent with our findings, indicating that the combination of downregulated miR-519 and miR-379 and upregulated miR-193b seems to be important in placentas complicated by either PE or FGR.

MiR-193b-3p decreased the migration and invasion of trophoblast cells [33], indicating that upregulation of miR-193b may be part of the pathogenesis of poor foetal growth. However, miR-193b has also been found to improve insulin signalling [34], which would promote growth. This inconsistency may be due to the diverse effects of miRNAs in different cells and circumstances and needs further investigations.

MiR-379 has been found to target insulin-like growth factor I (IGF-I) [35], which belongs to the insulin-IGF system, which is crucial to and regulates placental and foetal growth and development [36,37]. It is also important for energy homeostasis [38]. The downregulation of miR-379-3p found in this study is supposed to increase the expression of IGF-I [35]. Therefore, downregulation of miR-379-3p would constitute a compensatory mechanism for poor foetal growth. This needs to be further elucidated in future studies.

MiR-379 has also been found to inhibit cell proliferation and induce cell cycle arrest [39], indicating that low miR-379 expression would promote growth.

The genes encoding miR-379-3p, miR-369-5p, and miR-380-3p belong to the Chromosome 14 miRNA cluster (C14MC) found at the region 14p32 [30]. Decreased expression of them were found in this study in SGA infants. C14MC is maternally imprinted and may be important to human embryonic development [30]. Mouse pups with a heterozygote maternal deletion of C14MC displayed partially penetrant neonatal lethality with defects in the maintenance of energy homeostasis [40].

There is compelling evidence to support the idea that C14MC is a tumour suppressor locus involved in multiple cancers [41]. MiR-105, miR-193b, miR-330-5p, miR-335, and miR-519 are also reported to be tumour suppressors in different forms of cancer [42–46]. These miRNAs showed differential expression in this study, but they do not belong to C14MC. Besides that, miR-105 also seems to have a tumour promoting effect [47].

The expression of miR-335 has been found to be regulated by hypoxia in human trophoblasts [48]. MiR-335 has also been found to negatively regulate the migration of trophoblasts [49]. In this study, miR-335-3p was significantly downregulated in placentas from infants born SGA without a known reason. This is in contrast to findings of upregulation of miR-335 in placentas of women with PE [49,50], indicating that even though PE often cause FGR, the placental molecular pathways may differ between these two pregnancy complications.

In this study, miR-3679-5p was significantly upregulated, and miR-3065-5p was significantly downregulated in placentas from infants born SGA compared to infants with NBWs. To the best of our knowledge, none of them has been described before in relation to human pregnancy, embryology, placenta, or foetal growth. The significance of their expression in placenta to human foetal growth needs to be further investigated.

The network analysis performed in the present study showed that four of the eleven differentially expressed miRNAs, namely miR-379-3p, miR-335-3p, miR-330-5p, and miR-193b-3p, were connected to the insulin-IGF system (see Fig. 2). This indicates that miRNAs in the placenta may influence, at least to some extent, foetal growth through this important growth-promoting system. The network analysis was based on the function "inflammation", which was one of the important annotations for the differentially expressed miRNAs in this study.

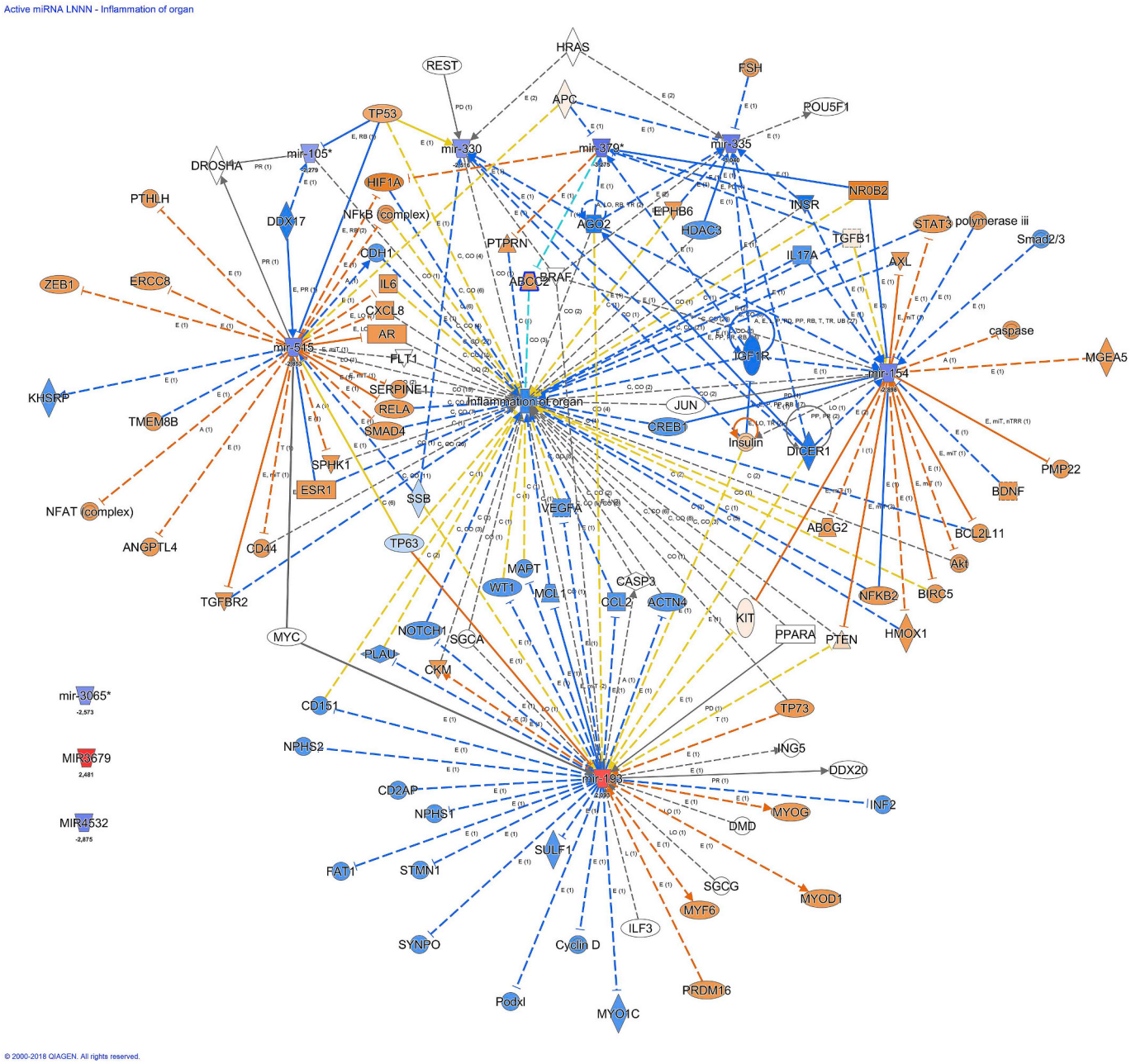


Fig. 2. Network of differentially expressed miRNAs with associated gene products and their connections to the function "Inflammation of organ" according to the NGS Strand. The color of associated gene products and their nodes denotes a predicted activation (orange) or inhibition (blue) of their function with regards to the differential expression of the miRNAs.

Inflammation inhibits postnatal growth, probably by inhibition of inflammatory cytokines on the insulin-IGF system [51]. Placental inflammation has also been postulated to be involved in the development of FGR [51].

Some of the predicted targeted genes in the present study (see Table 4) may be more relevant to foetal growth than other based on current knowledge on them. The possibly more relevant predicted targeted genes were grouped according to their functions: the ubiquitin (or ubiquitin-like) system (UFL, UBE3D, COPS3, COMMD3); inflammation (IFNW1, NLRC4, MPO); angiogenesis (CLEC14A, EFNA4); metabolism of toxins (CYP2E1, KLHL1); and transcription factors (TFAP2A). Exploring these genes in placental tissue and their relationship with foetal growth in future studies may reveal new, important mechanisms for FGR.

Previous studies on miRNAs in placenta have used microarrays or RT-PCR depending on the number of molecules of interest. Only a few studies have used a sequencing technique to profile the placental transcriptome or global miRNA expression [12,31,52,53], even though sequencing has the potential to find new miRNAs [54]. Most of the previous sequencing studies in human placenta have had small sample sizes.

Koh et al. [55] found a strong correlation between sequencing and microarray results in serum, indicating that either method can be used.

The strengths of this study include the strictly selected subjects. Mother-infant pairs with maternal hypertensive disease, abnormal BMI, diabetes, other chronic diseases, maternal smoking during pregnancy, prematurity, caesarean section, or asphyxia were excluded as these conditions may influence gene expression in the placenta. In addition, infants born SGA were carefully matched to the NBW infants. Cox et al. [5] emphasized the importance of closely matching the control subjects,

Table 4
Top 10 targeted mRNAs as predicted by NGS Strand for the up and downregulated miRNAs found in Group 2 in comparison to Group 4.

mRNA	Upregulated miRNAs found in Group 2	p-value
NLRC4	miR-193b-3p	0.005
MPO	miR-3679-5p, miR-193b-3p	0.006
COPS3	miR-3679-5p, miR-193b-3p	0.006
ASPRV1	miR-3679-5p, miR-193b-3p	0.007
ATP5C1	miR-3679-5p, miR-193b-3p	0.007
EFNA4	miR-3679-5p, miR-193b-3p	0.008
CAMK2N2	miR-3679-5p, miR-193b-3p	0.009
AAED1	miR-3679-5p, miR-193b-3p	0.009
UBE2L6	miR-3679-5p, miR-193b-3p	0.010
ATF5	miR-3679-5p, miR-193b-3p	0.011
mRNA	**Downregulated miRNAs found in Group 2**	p-value
UFL1	miR-519e-3p, miR-3065-5p, miR-330-5p, miR-105-5p, miR-379-3p, miR-380-3p, miR-335-3p	0.001
DKFZP586I1420	miR-379-3p, miR-330-5p, miR-380-3p, miR-105-5p, miR-335-3p	0.001
FAM74A1	miR-3065-5p, miR-519e-3p, miR-330-5p, miR-105-5p, miR-335-3p	0.001
FMO6P	miR-105-5p, miR-335-3p, miR-380-3p	0.001
IFNW1	miR-379-3p, miR-380-3p, miR-3065-5p, miR-105-5p	0.001
PCNP	miR-519e-3p, miR-3065-5p, miR-330-5p, miR-105-5p, miR-379-3p, miR-380-3p,miR-335-3p	0.001
TFAP2A	miR-519e-3p, miR-3065-5p, miR-330-5p, miR-105-5p, miR-369-5p, miR-379-3p, miR-380-3p, miR-335-3p	0.001
KCNG1	miR-3065-5p, miR-519e-3p, miR-379-3p, miR-4532, miR-380-3p	0.001
CLEC14A	miR-379-3p, miR-330-5p, miR-380-3p, miR-335-3p	0.001
UBE3D	miR-519e-3p, miR-3065-5p, miR-330-5p, miR-105-5p, miR-379-3p, miR-380-3p, miR-335-3p	0.001

Group 2 = SGA + NGWG. Group 4 = NBW + NGWG. SGA = small for gestational age. NBW = normal birth weight. NGWG = normal gestational weight gain.

since factors such as gestational age, infant sex, and mode of delivery influence miRNA expression in the placenta. Furthermore, we adjusted for potential confounding factors in a regression model.

Another strength was the inclusion of only the smallest infants and the exclusion of very short women, which probably led to exclusion of infants born physiologically small. The definition of SGA used in this study (birth weight < - 2 SD [26]) corresponds to the smallest approximately 2% of infants, while the other commonly used definition of SGA (birth weight < 10th percentile [56]) corresponds to the smallest 10% of infants, which increases the risk of including physiologically small infants. However, FGR was not verified by ultrasonography during pregnancy in this study.

The results from the ddPCR analyses were in good agreement with the results from the sequencing analyses. This technical validation shows the robustness of the findings, but biological validation in another cohort is needed in the future.

In conclusion, term infants born SGA without any known cause show significant differential expression of miRNAs in the placenta compared to infants born with NBWs. A common link for the differentially expressed miRNAs are inflammation and the insulin-IGF system. We propose that term infants born unexplained SGA may have been subjected to subclinical placental inflammation, leading to disturbances in the insulin-IGF system. This needs to be investigated further in future research.

Declaration of interest

We declare that there are no conflicts of interest associated with this work and that there has been no financial support for this study that could have influenced the results. We confirm that the manuscript has been read and approved by all authors and that there are no other people who satisfied the criteria for authorship that are not listed.

Acknowledgements

We would like to thank Dr Helena Fadl for critical reading and commenting on the manuscript. This project was supported by grants from the Research Committee of Region Örebro County and ALF funding Region Örebro County.

References

[1] T. Saleem, N. Sajjad, S. Fatima, N. Habib, S.R. Ali, M. Qadir, Intrauterine growth retardation–small events, big consequences, Ital. J. Pediatr. 37 (2011) 41.
[2] J. Zhang, M. Merialdi, L.D. Platt, M.S. Kramer, Defining normal and abnormal fetal growth: promises and challenges, Am. J. Obstet. Gynecol. 202 (6) (2010) 522–528.
[3] A. Ego, Definitions: small for gestational age and intrauterine growth retardation, J. Gynecol. Obstet. Biol. Reprod. 42 (8) (2013) 872–894.
[4] J. Gardosi, New definition of small for gestational age based on fetal growth potential, Horm. Res. 65 (Suppl 3) (2006) 15–18.
[5] B. Cox, K. Leavey, U. Nosi, F. Wong, J. Kingdom, Placental transcriptome in development and pathology: expression, function, and methods of analysis, Am. J. Obstet. Gynecol. 213 (4 Suppl) (2015) S138–S151.
[6] S.M. Hammond, An overview of microRNAs, Adv. Drug Deliv. Rev. 87 (2015) 3–14.
[7] M.A. Maccani, M. Avissar-Whiting, C.E. Banister, B. McGonnigal, J.F. Padbury, C.J. Marsit, Maternal cigarette smoking during pregnancy is associated with downregulation of miR-16, miR-21, and miR-146a in the placenta, Epigenetics 5 (7) (2010) 583–589.
[8] Z. Xu, D. Nayak, W. Yang, G. Baskaran, S. Ramachandran, N. Sarma, A. Aloush, E. Trulock, R. Hachem, G.A. Patterson, T. Mohanakumar, Dysregulated MicroRNA expression and chronic lung allograft rejection in recipients with antibodies to donor HLA, Am. J. Transplant. 15 (7) (2015) 1933–1947.
[9] K.R. Bounds, V.L. Chiasson, L.J. Pan, S. Gupta, P. Chatterjee, MicroRNAs: new players in the pathobiology of preeclampsia, Frontiers in cardiovascular medicine 4 (2017) 60.
[10] D.B. Chen, W. Wang, Human placental microRNAs and preeclampsia, Biol. Reprod. 88 (5) (2013) 130.
[11] L. Guo, S.Q. Tsai, N.E. Hardison, A.H. James, A.A. Motsinger-Reif, B. Thames, E.A. Stone, C. Deng, J.A. Piedrahita, Differentially expressed microRNAs and affected biological pathways revealed by modulated modularity clustering (MMC) analysis of human preeclamptic and IUGR placentas, Placenta 34 (7) (2013) 599–605.
[12] A. Higashijima, K. Miura, H. Mishima, A. Kinoshita, O. Jo, S. Abe, Y. Hasegawa, S. Miura, K. Yamasaki, A. Yoshida, K. Yoshiura, H. Masuzaki, Characterization of placenta-specific microRNAs in fetal growth restriction pregnancy, Prenat. Diagn. 33 (3) (2013) 214–222.
[13] I. Hromadnikova, K. Kotlabova, M. Ondrackova, P. Pirkova, A. Kestlerova, V. Novotna, L. Hympanova, L. Krofta, Expression profile of C19MC microRNAs in placental tissue in pregnancy-related complications, DNA Cell Biol. 34 (6) (2015) 437–457.
[14] M.A. Maccani, J.F. Padbury, C.J. Marsit, miR-16 and miR-21 expression in the placenta is associated with fetal growth, PLoS One 6 (6) (2011) e21210.
[15] S. Thamotharan, A. Chu, K. Kempf, C. Janzen, T. Grogan, D.A. Elashoff, S.U. Devaskar, Differential microRNA expression in human placentas of term intrauterine growth restriction that regulates target genes mediating angiogenesis and amino acid transport, PLoS One 12 (5) (2017) e0176493.
[16] A. Lykoudi, A. Kolialexi, G.I. Lambrou, M. Braoudaki, C. Siristatidis, G.K. Papaioanou, M. Tzetis, A. Mavrou, N. Papantoniou, Dysregulated placental microRNAs in early and late onset preeclampsia, Placenta 61 (2018) 24–32.
[17] L. Huang, Z. Shen, Q. Xu, X. Huang, Q. Chen, D. Li, Increased levels of microRNA-424 are associated with the pathogenesis of fetal growth restriction, Placenta 34 (7) (2013) 624–627.

[18] D. Wang, Q. Na, W.W. Song, G.Y. Song, Altered Expression of miR-518b and miR-519a in the placenta is associated with low fetal birth weight, Am. J. Perinatol. 31 (9) (2014) 729–734.
[19] I. Hromadnikova, K. Kotlabova, L. Hympanova, L. Krofta, Cardiovascular and cerebrovascular disease associated microRNAs are dysregulated in placental tissues affected with gestational hypertension, preeclampsia and intrauterine growth restriction, PLoS One 10 (9) (2015) e0138383.
[20] J.F. Mouillet, T. Chu, C.A. Hubel, D.M. Nelson, W.T. Parks, Y. Sadovsky, The levels of hypoxia-regulated microRNAs in plasma of pregnant women with fetal growth restriction, Placenta 31 (9) (2010) 781–784.
[21] Q. Tang, W. Wu, X. Xu, L. Huang, Q. Gao, H. Chen, H. Sun, Y. Xia, J. Sha, X. Wang, D. Chen, Q. Xu, miR-141 contributes to fetal growth restriction by regulating PLAG1 expression, PLoS One 8 (3) (2013) e58737.
[22] H. Wen, L. Chen, J. He, J. Lin, MicroRNA expression profiles and networks in placentas complicated with selective intrauterine growth restriction, Mol. Med. Rep. 16 (5) (2017) 6650–6673.
[23] M. Viswanathan, A.M. Siega-Riz, M.K. Moos, A. Deierlein, S. Mumford, J. Knaack, P. Thieda, L.J. Lux, K.N. Lohr, Outcomes of maternal weight gain, Evid. Rep. Technol. Assess. 168 (2008) 1–223.
[24] K.A. Wikland, Z.C. Luo, A. Niklasson, J. Karlberg, Swedish population-based longitudinal reference values from birth to 18 years of age for height, weight and head circumference, Acta Paediatr. 91 (7) (2002) 739–754.
[25] K.M. Rasmussen, A.L. Yaktine, Weight Gain during Pregnancy: Reexamining the Guidelines, National Academies Press (US), Washington (DC), 2009.
[26] P.A. Lee, S.D. Chernausek, A.C. Hokken-Koelega, P. Czernichow, International Small for Gestational Age Advisory Board consensus development conference statement: management of short children born small for gestational age, April 24-October 1, 2001, Pediatrics 111 (6 Pt 1) (2003) 1253–1261.
[27] A. Niklasson, K. Albertsson-Wikland, Continuous growth reference from 24th week of gestation to 24 months by gender, BMC Pediatr. 8 (2008) 8.
[28] M. Acunzo, G. Romano, D. Wernicke, C.M. Croce, MicroRNA and cancer–a brief overview, Adv Biol Regul 57 (2015) 1–9.
[29] G.J. Burton, E. Jauniaux, What is the placenta? Am. J. Obstet. Gynecol. 213 (4 Suppl) (2015) S6–S8 S6.e1.
[30] D.M. Morales-Prieto, S. Ospina-Prieto, W. Chaiwangyen, M. Schoenleben, U.R. Markert, Pregnancy-associated miRNA-clusters, J. Reprod. Immunol. 97 (1) (2013) 51–61.
[31] E.S. Vashukova, A.S. Glotov, P.V. Fedotov, O.A. Efimova, V.S. Pakin, E.V. Mozgovaya, A.A. Pendina, A.V. Tikhonov, A.S. Koltsova, V.S. Baranov, Placental microRNA expression in pregnancies complicated by superimposed pre-eclampsia on chronic hypertension, Mol. Med. Rep. 14 (1) (2016) 22–32.
[32] P. Xu, Y. Zhao, M. Liu, Y. Wang, H. Wang, Y.X. Li, X. Zhu, Y. Yao, H. Wang, J. Qiao, L. Ji, Y.L. Wang, Variations of microRNAs in human placentas and plasma from preeclamptic pregnancy, Hypertension 63 (6) (2014) 1276–1284.
[33] X. Zhou, Q. Li, J. Xu, X. Zhang, H. Zhang, Y. Xiang, C. Fang, T. Wang, S. Xia, Q. Zhang, Q. Xing, L. He, L. Wang, M. Xu, X. Zhao, The aberrantly expressed miR-193b-3p contributes to preeclampsia through regulating transforming growth factor-beta signaling, Sci. Rep. 6 (2016) 19910.
[34] B. Siddeek, N. Li, C. Mauduit, H. Chehade, E. Rigal, J.F. Tolsa, J.B. Armengaud, C. Yzydorczyk, M. Benahmed, C. Vergely, U. Simeoni, Transient postnatal over nutrition induces long-term alterations in cardiac NLRP3-inflammasome pathway, Nutr. Metabol. Cardiovasc. Dis. 28 (9) (2018) 944–951, https://doi.org/10.1016/j.numecd.2018.03.013.
[35] K. Li, Y. Wang, A. Zhang, B. Liu, L. Jia, miR-379 inhibits cell proliferation, invasion, and migration of vascular smooth muscle cells by targeting insulin-like factor-1, Yonsei Med. J. 58 (1) (2017) 234–240.
[36] U. Hiden, E. Glitzner, M. Hartmann, G. Desoye, Insulin and the IGF system in the human placenta of normal and diabetic pregnancies, J. Anat. 215 (1) (2009) 60–68.
[37] S. Setia, M.G. Sridhar, Changes in GH/IGF-1 axis in intrauterine growth retardation: consequences of fetal programming? Horm. Metab. Res. 41 (11) (2009) 791–798.
[38] A. Lancha, G. Fruhbeck, J. Gomez-Ambrosi, Peripheral signalling involved in energy homeostasis control, Nutr. Res. Rev. 25 (2) (2012) 223–248.
[39] J. Yi, Y. An, Circulating miR-379 as a potential novel biomarker for diagnosis of acute myocardial infarction, Eur. Rev. Med. Pharmacol. Sci. 22 (2) (2018) 540–546.
[40] S. Labialle, V. Marty, M.L. Bortolin-Cavaille, M. Hoareau-Osman, J.P. Pradere, P. Valet, P.G. Martin, J. Cavaille, The miR-379/miR-410 cluster at the imprinted Dlk1-Dio3 domain controls neonatal metabolic adaptation, EMBO J. 33 (19) (2014) 2216–2230.
[41] S.V. Laddha, S. Nayak, D. Paul, R. Reddy, C. Sharma, P. Jha, M. Hariharan, A. Agrawal, S. Chowdhury, C. Sarkar, A. Mukhopadhyay, Genome-wide analysis reveals downregulation of miR-379/miR-656 cluster in human cancers, Biol. Direct 8 (2013) 10.
[42] X. Liu, H. Wang, Z. Zhu, Y. Ye, H. Mao, S. Zhang, MicroRNA-105 targets SOX9 and inhibits human glioma cell progression, FEBS Lett. 590 (23) (2016) 4329–4342.
[43] L. Wang, Y. Zhang, L. Zhao, S. Liu, S. Yu, Y. Ma, G. Sun, MicroRNA-193b inhibits the proliferation, migration and invasion of gastric cancer cells via targeting cyclin D1, Acta Histochem. 118 (4) (2016) 323–330.
[44] B.B. Su, S.W. Zhou, C.B. Gan, X.N. Zhang, MiR-330-5p regulates tyrosinase and PDIA3 expression and suppresses cell proliferation and invasion in cutaneous malignant melanoma, J. Surg. Res. 203 (2) (2016) 434–440.
[45] K. Wang, X. Chen, Y. Zhan, W. Jiang, X. Liu, X. Wang, B. Wu, miR-335 inhibits the proliferation and invasion of clear cell renal cell carcinoma cells through direct suppression of BCL-W, Tumour Biol 36 (9) (2015) 6875–6882.
[46] G. Yu, T. Zhang, Y. Jing, Q. Bao, Q. Tang, Y. Zhang, miR-519 suppresses nasopharyngeal carcinoma cell proliferation by targeting oncogene URG4/URGCP, Life Sci. 175 (2017) 47–51.
[47] W. Zhou, M.Y. Fong, Y. Min, G. Somlo, L. Liu, M.R. Palomares, Y. Yu, A. Chow, S.T. O'Connor, A.R. Chin, Y. Yen, Y. Wang, E.G. Marcusson, P. Chu, J. Wu, X. Wu, A.X. Li, Z. Li, H. Gao, X. Ren, M.P. Boldin, P.C. Lin, S.E. Wang, Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis, Cancer Cell 25 (4) (2014) 501–515.
[48] J.F. Mouillet, T. Chu, D.M. Nelson, T. Mishima, Y. Sadovsky, MiR-205 silences MED1 in hypoxic primary human trophoblasts, Oxid. Med. Cell. Longev. 24 (6) (2010) 2030–2039.
[49] F. Jiang, J. Li, G. Wu, Z. Miao, L. Lu, G. Ren, X. Wang, Upregulation of microRNA335 and microRNA584 contributes to the pathogenesis of severe pre-eclampsia through downregulation of endothelial nitric oxide synthase, Mol. Med. Rep. 12 (4) (2015) 5383–5390.
[50] Y. Hu, P. Li, S. Hao, L. Liu, J. Zhao, Y. Hou, Differential expression of microRNAs in the placentae of Chinese patients with severe pre-eclampsia, Clin. Chem. Lab. Med. 47 (8) (2009) 923–929.
[51] F. Cirillo, P. Lazzeroni, C. Sartori, M.E. Street, Inflammatory diseases and growth: effects on the GH-IGF Axis and on growth plate, Int. J. Mol. Sci. 18 (9) (2017).
[52] J. Kim, K. Zhao, P. Jiang, Z.X. Lu, J. Wang, J.C. Murray, Y. Xing, Transcriptome landscape of the human placenta, BMC Genomics 13 (2012) 115.
[53] M. Majewska, A. Lipka, L. Paukszto, J.P. Jastrzebski, K. Myszczynski, M. Gowkielewicz, M. Jozwik, M.K. Majewski, Transcriptome profile of the human placenta, Funct. Integr. Genom. 17 (5) (2017) 551–563.
[54] K.R. Kukurba, S.B. Montgomery, RNA sequencing and analysis, Cold Spring Harb. Protoc. 11 (2015) (2015) 951–969.
[55] W. Koh, W. Pan, C. Gawad, H.C. Fan, G.A. Kerchner, T. Wyss-Coray, Y.J. Blumenfeld, Y.Y. El-Sayed, S.R. Quake, Noninvasive in vivo monitoring of tissue-specific global gene expression in humans, Proc. Natl. Acad. Sci. U. S. A 111 (20) (2014) 7361–7366.
[56] I.M. Beune, F.H. Bloomfield, W. Ganzevoort, N.D. Embleton, P.J. Rozance, A.G. van Wassenaer-Leemhuis, K. Wynia, S.J. Gordijn, Consensus based definition of growth restriction in the newborn, J. Pediatr. 196 (2018) 71–76 e1.