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Research article

FGR-associated placental insufficiency and capillary angiogenesis involves disruptions in human placental miRNAs and mRNAs

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A R T I C L E I N F O

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A B S T R A C T

Fetal growth restriction (FGR) is one of the most common pregnancy complications culminating in adverse fetal outcome, including preterm birth, neonatal mortality and stillbirth. Compromised placental development and function, especially disruption in angiogenesis and inadequate nutrient supply are contributing factors. Fetal sex also influences placental function. Knowledge of gene expression changes and epigenetic factors contributing to placental dysfunction in FGR pregnancies will help identify biomarkers and help target interventions. This study tested the hypothesis that FGR pregnancies are associated with disruptions in miRNA - an epigenetic factor and mRNAs involving key mediators of angiogenesis and microvessel development. Changes in expression of key genes/proteins involved in placental dysfunction by RT-PCR and immunohistochemistry and miRNA changes by RNA sequencing were undertaken with term placenta from 12 control and 20 FGR pregnancies. Findings showed changes in expression of genes involved in steroidogenesis, steroid action, IGF family members, inflammatory cytokines and angiogenic factors in FGR pregnancies. In addition, upregulation of *MIR451A* and downregulation of *MIR543* in placentas from FGR group with female newborns and upregulation of *MIR520G* in placentas from FGR group with male newborns were also noted. *MIR451A* and *MIR543* have been implicated in angiogenesis. Consistent with gene changes, CD34, the microvessel angiogenesis marker, also showed reduced staining only in female FGR group. These findings provide evidence that epigentically regulated gene expression changes in angiogenesis and capillary development influence placental impairment in FGR pregnancies. Our preliminary observations also support for these changes to be driven in a sex-specific manner.

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1. Introduction

Intrauterine growth restriction (IUGR) or fetal growth restriction (FGR) is one of the most common pregnancy complications. Approximately 10–15% of pregnancies are affected by FGR, defined as the failure of the fetus to reach its genetically determined growth potential with fetal weight less than the 10th percentile for gestation age [1]. FGR can lead to adverse fetal outcomes, including preterm birth, neonatal mortality and even stillbirth with 30% of stillbirth ascribed to FGR [2]. The incidence of preterm labor and fetal mortality of FGR offspring is significantly higher than the normal birth weight babies [3–5]. The surviving FGR offspring are also at increased risk of developing cardiometabolic diseases during childhood and adulthood [6–8]. This is supported by the evidence of multiple large population-based cohort studies that have demonstrated that lower birth weight is associated with higher risk of incident myocardial infarction and adverse left ventricular remodelling [9,10]. These observations and postulation of the developmental origin of health and disease (DOHaD) hypothesis has led to emphasis the need to understand the placental contribution on pre- and peri-natal infractions including FGR.

Placenta helps maintain a conducive intrauterine environment by undergoing structural and functional adaptations to accommodate fetal growth in response to changing environmental milieu [11]. While the majority of FGR cases are idiopathic causes, many involve compromised placental development and function, especially due to impairment of placental angiogenesis and insufficient oxygen and nutrient supply to the fetus [12,13]. Terminal villi in placenta is the basic functional unit for transfer of oxygen and nourishment between mother and the fetus. As the functional component in terminal villi [14], the efficiency of terminal villi and therefore of placenta is dependent on among other factors, the microvessel density within the villous core [15]. Since placental size, morphology, and angiogenesis dictates placental function [16], these changes are also associated with reduction in capillarization of the terminal villi [17], reduced cell proliferation along with increased apoptosis and necrosis [18].

Angiogenic factors, such as vascular endothelial growth factor A (VEGFA) [19], endocrine gland-derived vascular endothelial growth factor (EG-VEGF) [20], placenta growth factor (PlGF) [21], hypoxia inducible factor 1 subunit alpha (HIF1A) [22], Angiopoietin 1 (ANG1) and ANG2 [23] are among the factors reported to be involved in microvessel formation in terminal villi. Other mechanisms linking poor placental function in FGR pregnancies are the abnormal inflammatory response, both systemically at maternal and locally at the level of placenta [24], altered steroidogenesis [25], and impaired insulin-like growth factor (IGF) family signaling [26,27].

Aberrant expression of genes underlying placental disruptions in turn may be driven by epigenetic alterations mediated by, among others, the microRNA (miRNA). Placental miRNAs are being actively investigated not only because of their role in the growth and function of the placenta but also due to their evolving role in the pregnancy complications including FGR [28]. MiRNAs −10b and −363 are reported to be upregulated in the human term FGR placenta and these changes are associated with reduced expression of transcription factor KLF4 that promotes angiogenesis and genes associated with amino acid transport [29]. In addition, miRNAs −518B and -150-5p are demonstrated to cause inhibition of trophoblast migration and angiogenesis [30,31]. These findings establish a role for aberrant miRNA expression to dysregulate placental angiogenesis leading to development of FGR. Due to the heterogeneity of etiology in FGR, there were difference among previous findings that still requiring further study [32]. Though some miRNAs were reported to be regulated in a sex-specific manner [33], few evidence was shown on global miRNA expression.

Therefore, the goal of this project is to test the hypothesis that FGR pregnancies are associated with disruption in miRNA and mRNAs involving key regulators of angiogenesis and microvessel development. This hypothesis was tested in term placenta collected from primipara with normal and FGR pregnancies. While not the primary focus of the original study, inspite of the small sample size and uneven sex distribution, the impact of fetal sex on placental changes from this cohort were also examined to gain preliminary insight on sex-specific effects.

2. Methods

2.1. Human subjects

Human subjects were recruited following written informed consent and studies performed following the guidelines approved by the Institutional Ethics Committee at Hebei Medical University Affiliated Obstetrics and Gynecology Hospital (Approval ID: 20210030) and Chinese Clinical Trial Registry (Registration Number: ChiCTR2100043159). Primiparous pregnant women with singleton pregnancies who delivered between 37 and 41 weeks of gestation were recruited from the Fourth Hospital of Shijiazhuang affiliated with Hebei Medical University (from August 2020 to March 2021). Gestational age was determined by the ultrasound assessment. Placentae were collected from enrolled women at term immediately following delivery. Most of the enrolled subjects was underwent vaginal delivery with placenta collected from only 2 subjects in the FGR group underwent C-section without onset of labor. Based on offspring birth weight women were classed into two groups, control and FGR. Pregnancies were designated as FGR when birth weight was below the 10th percentile for the gestational age as defined by the American College of Obstetricians and Gynecologists [34]. Criteria for exclusion included pregnancy complications, malnutrition, velamentous placenta, severe placental calcification or infarction, congenital malformation, and umbilical cord compromise. The demographic details and birth outcomes such as maternal age, body mass index (BMI), maternal substance abuse or smoking, adverse pregnancy history, maternal weight, weight gain throughout pregnancy, offspring birth weight and gender information were collected from the medical record. Placental samples from 20 FGR (Male 7, female 13) and 12 controls (Male 9, female 3) were used in this study (Table 1).

2.2. Placenta collection

At term, whole placenta was collected immediately after the delivery and fetal membranes and the umbilical cord were removed and the weight and size were recorded. Placental volume (cm^3) was calculated by the formula: largest diameter x smallest diameter x thickness (cm). Considering the heterogeneity of the placenta tissue, placental tissue was harvested from 5 different points representing the 4 quadrants and center and sample from the same region were assigned for either RNA, histochemical or caspase activity analysis from all subjects. For RNA analysis, placenta was dissected and washed with normal saline to remove maternal blood and placed in a cryogenic vial containing RNAlater [35] frozen and stored in −80 °C until further analysis. For histomorphological and immunohistochemical analysis, a portion of placental fragments was also collected and fixed overnight at 4 °C in 4% paraformaldehyde and embedded in paraffin.

2.3. Histological examination

Paraffin-embedded tissues were sectioned (4 μm) and stained with hematoxylin and eosin (H&E) following the traditional H&E staining protocol. For examination of CD34 expression, paraffin embedded placenta was sectioned (4 μm) and immunostained with a mouse monoclonal anti-human CD34 antibody (QBEnd 10, Gene Tech (Shanghai) Company Limited, Ch). Immunostaining was performed on VENTANA BenchMark GX@ (Roche, Tucson, AZ USA) automated staining instrument following manufacturer recommended protocol. Briefly, the sections were stained with hematoxylin-eosin and immunostained with an ultraView Universal DAB Detection Kit using the BenchMark Ultra immunohistochemistry/in situ hybridization staining module. The sections were incubated with primary antibodies for 32 min at 37 °C. The primary antibodies, which were diluted as per the specific suppliers' instructions of 1:100 dilution of the 1 mg/mL stock. High-resolution, whole-slide digital scan of both H&E and immuno stained slides was performed at 40 × magnification using Aperio CS2 Digital Pathology Scanner and Aperio Imagescope software (Aperio Technologies Inc. San Diego USA). Acquired images were saved as Tagged Image Format File (tiff) for further analysis. From each slide, five equal size regions of interest (ROI; 0.2645 μm2 each) were selected by experienced pathologist. Regions of interest was restricted to the microvessel rich area from different locations. Color deconvolution and microvessel analysis algorithms were employed to qualify the CD34 staining intensity and microvessel-associated parameters, respectively. With color deconvolution algorithm, the parameter settings for the DAB channel were tailored to efficiently identify CD34 staining in placenta villi and the parameter settings were saved as a Macro for the repeat use. Using the macro, average CD34 staining intensity, percentage of stained area, and total stained region was obtained. The procedures for Ki67 staining using the human Ki67 antibody (NO.GM027, Gene Tech (Shanghai) Company Limited, Ch) was similar to that of CD34 immunostaining with 1:100 dilution as recommended by the manufacturer followed by analysis with Aperio CS2

Table 1
Demographics of the pregnant women (n = 32).

Mother		N (%) or Median (range min-max)			N (%) or Median (range min-max)		
		FGR	Male	Female	Control	Male	Female
Infant sex		20	7	13	12	9	3
Age (years)		28.45(23–34)	26.71(23–31)	29.38(26–34)	27.67(21–32)	27.89(22–32)	27(21–32)
Race	Asian	20	7	13	12	9	3
Pre-pregnancy BMI		20.31	20.36	20.38	20.70	20.73	19.73
Pre-pregnancy weight (kg)		53.38(40–70)	52.36(46–62)	53.92(40–70)	54.67(46–65)	55.22(48–65)	53(46–63)
Weight near term (kg)		67.90(55–88)	69.29(60–76)	67.15(55–88)	71.50(60–88)	71.33(65–85)	72.00(60–88)
Gestational weight gain (kg)		12.63(-4-23)	16.93(8–23)	13.23(-4-21)	16.83(10–25)	16.11(10–23)	19.00(10–25)
Parity	0	100%	7	13	100%	9	3
	1	–	–	–	–	–	–
	2	–	–	–	–	–	–
	3	–	–	–	–	–	–
	4	–	–	–	–	–	–
Smoking status	Never	100%	7	13	100%	9	3
	Past smoker	–	–	–	–	–	–
Education (n = 32)	High school	5	3	2	5	3	2
	Some college	1	1	0	1	1	0
	≥ Bachelor's	14	3	11	6	5	1
Marital status	Married	100%	7	13	100%	9	3
	Single	–	–	–	–	–	–
Infant							
Route of delivery	Cesarean	2	1	1	0	0	0
	Vaginal	18	6	12	12	9	3
Gestational age (days)		39.49 (38.00–40.43)	39.20 (38.00–40.43)	39.65 (38.14–40.57)	39.32 (38.29–40.43)	39.33 (38.57–40.43)	39.29 (38.29–40.14)
Birth weight (g)		2650.50 (2130–2900)	2655.71 (2500–2790)	2647.69 (2130–2900)	3250.83 (3000–3620)	3240.00 (3040–3620)	3283.33 (3000–3500)

software.

2.4. Assessment of caspase-3 activity

Caspase 3 activity was assessed in cyro-preserved tissues utilizing a colorimetric kit (No.C1116, Beyotime Biotechnology, Shanghai, Ch) following manufacturer's recommendations. Briefly, 100 μl lysis buffer was added to about 10 mg of cryo-preserved placental tissue from each subject, homogenized using a cryogenic homogenizer and incubated on ice for 5min. Following this the homogenate was centrifuged at 16,000–20,000r/min for 10–15min and the placental lysate was collected and used to assess the Caspase 3 activity. To normalize between samples, total protein was also estimated in homogenate using Bradford Protein Assay Kit (NO.P0006, Beyotime Biotechnology, Shanghai, CH) and caspase-3 activity was expressed as ratio to mg amount of total protein.

2.5. Real-time reverse transcriptase–polymerase chain reaction (RT-PCR)

Frozen placental tissue was lysed using tissue cryogrinder (KZ–III–FP, Wuhan Servicebio Technology Company Limited, CH) and total RNA was isolated using Eastep@Supper Total RNA Extraction kit (Shanghai Promega Biological Products Ltd., Shanghai, CH) following manufacture's protocol. The concentration and 260/280 ratios were determined using Nanodrop. In addition, the RNA integrity was tested by using RNA Agarose gel electrophoresis where the ratio of 28s RNA to 18s RNA was ~2:1 in all samples that are part of this analysis. Subsequently, 1000 ng of total RNA was reverse transcribed to complementary DNA (cDNA) with first-strand complementary synthesis system (Invitrogen, Life Technologies). Relative mRNA concentrations was assessed using SYBRgreen based real time RT-PCR using PowerUpTMSYBRTM Green Master Mix (ThermoFisher). Sequences for the oligonucleotide primers for the genes under study designed using Primer3 are shown in Table 2. The relative amount of each transcript was calculated using the ΔΔCT method and normalized to the endogenous reference gene beta-actin. The reference gene was chosen based on literature review [36–39].

2.6. RNAseq analysis

2.6.1. Sequencing

The total RNA was extracted by using Eastep@Supper Total RNA Extraction kit (Shanghai Promega Biological Products Ltd., Shanghai, CH) following manufacturer's protocol, then purified by using the Total RNA Purification Kit (LC Sciences, Houston, USA), according to the manufacturer's protocol. The total RNA quantity and purity were analyzed using Agilent Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, Santa Clara, CA USA) and RNA from subjects (control group: n = 6, 3 male and 3 female; FGR group: n = 7, 4 male and 3 female) with RIN number >7.0 was used for sequencing. Approximately 1,000 ng of total RNA were used to prepare small RNA library according to protocol of TruSeq Small RNA Sample Prep Kits (Illumina, San Diego, CA USA). The general procedure was as follows: the RNAs were ligated to 3' adapters and the 5' adapters were ligated to the other end of the RNA molecules. Then the

Table 2
Sequence of primers used.

Gene ID	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Accession Number
β-Actin	GGCACCCAGCACAATGAAG	GCCGATCCACACGGAGTACT	NM_001101.5
IGF1	GCCCAAGACCCAGAAGTATCAGC	TCCAATCTCCCTCCTCTGCTCT	NM_001111285.3
IGF2	TTCTCACCTTCTTGGCCTTCG	GCGGAAACAGCACTCCTCAA	NM_001291862.2
IGFBP1	AGGAGCCCTGCCGAATAGAA	CCATGGATGTCTCACACTGTCT	NM_000596.4
IGFBP2	CAAAAGCACGCGCTCTTCTCC	TCATCGCCATTGTCTCCGC	NM_001313992.2
IGFBP3	CATCAAGAAAGGGCATGCTAAA	GAGGAGAAGTTCTGGGTATCTG	XM_047420325.1
IGFBP4	CCCACGAGGACCTCTACATC	ATCCAGAGCTGGGTGACACT	NM_001552.3
IGF1R	AGTGCTGTATGCCTCTGTGAACC	ATAGACCATCCCAAACGACCC	XM_011521517.3
IGF2R	GAGGGAAGAGGCAGGAAAG	TGTGGCAGGCATACTCAG	NM_000876.4
VEGF	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA	NM_001025366.3
VEGFR1	CAGTGTGAGCGGCTCCCTTATG	CACAGTCCGGCACGTAGGTGAT	NM_002019.4
VEGFR2	CCAGCAAAAGCAGGGAGTCTGT	TGTCTGTGTCATCGGAGTGATATCC	NM_002253.4
PIGF	CAGAGGTGGAAGTGGTACCCTTCC	CGGATCTTTAGGAGCTGCATGGTGAC	NM_002632.6
HIF1A	GAACGTCGAAAAGAAAAGTCTCG	CCTTATCAAGATGCGAACTCACA	NM_001243084.2
EGVEGF	AGGTCCCCTTCTTCAGGAAACG	TCCAGGCTGTGCTCAGGAAAAG	NM_032414.3
ANGPT1	CAGACTGCAGAGCAGACCAGAA	CTCTAGCTTGTAGGTGGATAATGAATTC	NM_001199859.3
ANGPT2	AGGAGGCGGGTGGACAATT	CTCCTGAAGGGTTACCAAATCC	NM_001118887.2
CCL2	CTCTGCCGCCCTTCTGT	CTTCTTTGGGACACTTGCTG	NM_002982.4
TNF	CCCAGAGGGAAGAGTTCCCCA	GGCTTGTCACTCGGGGTTCG	NM_000594.4
CD68	GCTACATGGCGGTGGAGTACAA	ATGATGAGAGGCAGCAAGATGG	NM_001040059.2
ESR1	CAGGAACCAGGGAAAATGTG	AACCGAGATGATGTAGCCAGC	XM_017010383.2
PGR	GGCAGCACAACTACTTATGTGC	TCATTTGGAACGCCCACT	XM_006718858.4
AR	CCTGGCTTCCGCAACTTACAC	GGACTTGTGCATGCGGTACTCA	NM_000044.6
CYP19	GGCAAGCTCTCCTCATCAAA	CAACTCAGTGGCAAAGTCCA	NM_001347252.2
HSD3B	AGAGGCCTGTGTCCAAGCTA	TTTTGCTGTGTGGGTATGGA	NM_000862.3
HSD17B	ATCCAGAGCCTCATCCATTG	AACGCCTTGGAAGCTGAGTA	XM_047423304.1

RNAs which were ligated with 3' and 5' adapters were reverse transcribed to create single stranded cDNA. The cDNAs were amplified, gel purified and used to generate libraries. The small RNA library quality was assessed by using Bioanalyzer 2100 (Agilent) with High Sensitivity DNA Chip Kit (Agilent) and single-end sequencing (1 × 50bp) on an Illumina Hiseq2500 at the LC-BIO (Hangzhou, China) following the vendor's recommended protocol was performed.

2.6.2. Dimensionality reduction

Dimensionality reduction modeling was performed using SIMCA 17 (Sartorius Stedim Data Analytics AB, Sweden). Normalized counts for miRNA from placentae from both male and female offspring tissues were imported to SIMCA software for the analysis. Multivariate modeling was performed using unit variance (UV) scaling (mean centered and divided by the standard deviation). To get an overview of the data and identify patterns/groupings unsupervised principal component analysis (PCA) was performed. Specifically two-dimensional and three dimensional (3D) PCA clustering were employed, and the respective plots were explored. The scatter score plot of components 1 and 2 were explored to visualize differences in the two groups (classes). Observations that are close to each other have more similar miRNA expression profiles compared to the observations that are distant from each other.

2.6.3. miRNA trimming

Raw reads from miRNA sequencing were trimmed using cutadapt (v3.2) and specifically trimmed from the 5′ end using the sequence 'TGGAATTCTCGGGTGCCAAGG'. Sequences were then sub-selected for reads that were less than 17bp. Finally, low-quality reads that did not match the default quality control scores were removed.

2.6.4. Quality control metrics

Fastqc was used to evaluate and multiqc was used to summarize the quality control metrics for both raw and trimmed files.

2.6.5. Alignment and counts

Trimmed reads were aligned to Genome Reference Consortium Human Build 38 patch release 14 (GRCh38.p14) (GRCh38.p14) using Spliced Transcripts Alignment to a Reference (STAR) aligner (v2.6.0c). FeatureCounts (v1.6.1) was used to count aligned fragments and then differential expression performed.

2.6.6. Differential gene expression testing

DESEq2 (1.24.0) utilizing negative binomial distribution on counts was used for determining the differential expression of miRNA. Sex-specific and treatment (control vs. FGR) effects in miRNA expression in placental tissue were also determined by comparing 1) control male with control female, 2) FGR male vs. FGR female, 3) FGR male vs. control male and 4) FGR female vs. control female. For miRNA differentially expressed transcripts that met the FDR <0.1 and absolute log2Fold Change >0.5 were considered significant. Finally, differentially expressed transcripts were visualized using volcano plots, and heatmaps were generated using the heatmap.2 package. All differential expression testing and plots were processed using R statistical software (v3.5.1).

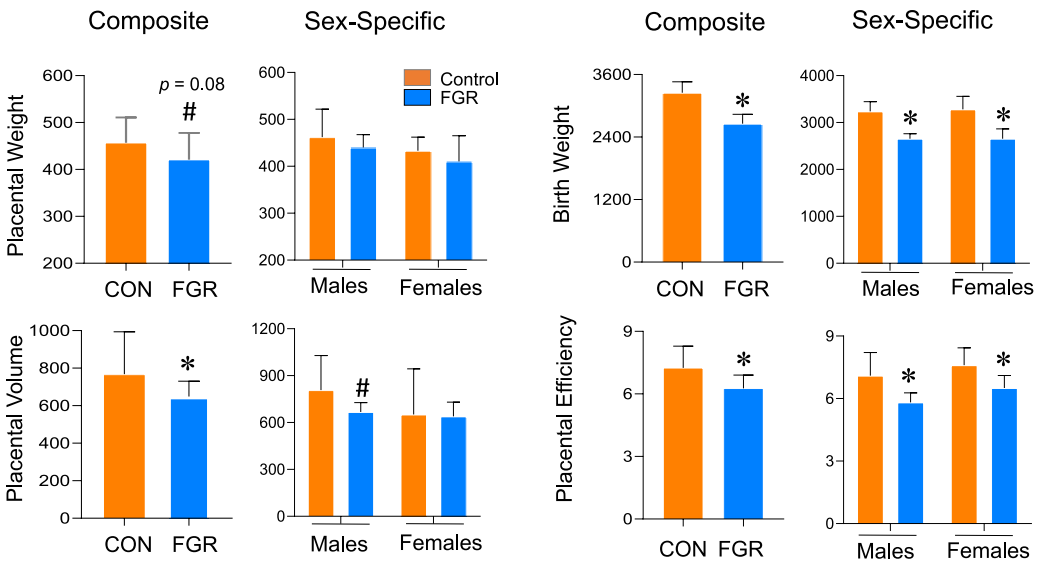

Fig. 1. Placental characteristics – weight, volume and efficiency and newborn birth weight from the entire cohort (composite) and segregated by newborn sex are shown. Data are presented as mean ± SD with open bars representing the controls and solid bars representing the FGR groups (control group: n = 13; FGR group: n = 20). Asterisk (*) represents $p < 0.05$ by Student's t-test and # represents large magnitude change by Cohen's effect size analysis with Cohen's d values ≥ 0.8 comparing control and FGR groups.

2.7. Statistical analysis

Demographic, immunohistological and RT-PCR data were examined for homogeneity of variance using Fisher's test and Student's *t*-test was employed to compare the quantitative difference of assessed parameters between Control and FGR groups. Differences among the placentas from male and female newborn between control and FGR groups were also assessed by Student's *t*-test. A p-value lower than 0.05 was considered significant. Additionally, data were also analyzed using Cohen's effect size analysis [40,41] as smaller sample size and the lack of statistical significance may not be sufficient to reject the null hypothesis [42]. The computed Cohen's d value of 0.8 and above indicates large effect size differences between the control and FGR groups.

3. Results

3.1. Subjects and placental characteristics

The demographics of the 32 subjects from this study are summarized in Table 1. This cohort comprised of Asian women with a mean age of 28.18 (range 21–34 years). This homogeneous group consists of only married, non-smoking, primiparous women of similar pre-pregnancy body mass index (BMI) and comparable gestational weight gain. Most women reported to have a college degree and had mostly vaginal birth (with two undergoing elective c-section without onset of labor) and were of full term and showed no difference among mothers from control and FGR group. Deliveries were determined as FGR at birth based on the birth weight with those below the 10th percentile for the gestational age as defined by the American College of Obstetricians and Gynecologists was designated as FGR pregnancies. The placental and fetal characteristics comparing the control and FGR subjects are shown in Fig. 1. The fetal birth weight was significantly lower in FGR group compared with control group (3249.23 ± 210.61 g vs 2656.09 ± 181.08 g, $p < 0.01$). This was evident even when the newborns were segregated based on their sex. The placental weight tended ($p = 0.08$) to be lower among FGR patients however this was not evident when FGR mothers were segregated based on the sex of the newborn. The placental volume showed significant decrease ($p = 0.04$) among FGR mothers with this trend evident by large magnitude effect among the mothers with male newborns. Likewise, the placental efficiency calculated as the ratio of birth weight to the placental weight [43] was significantly

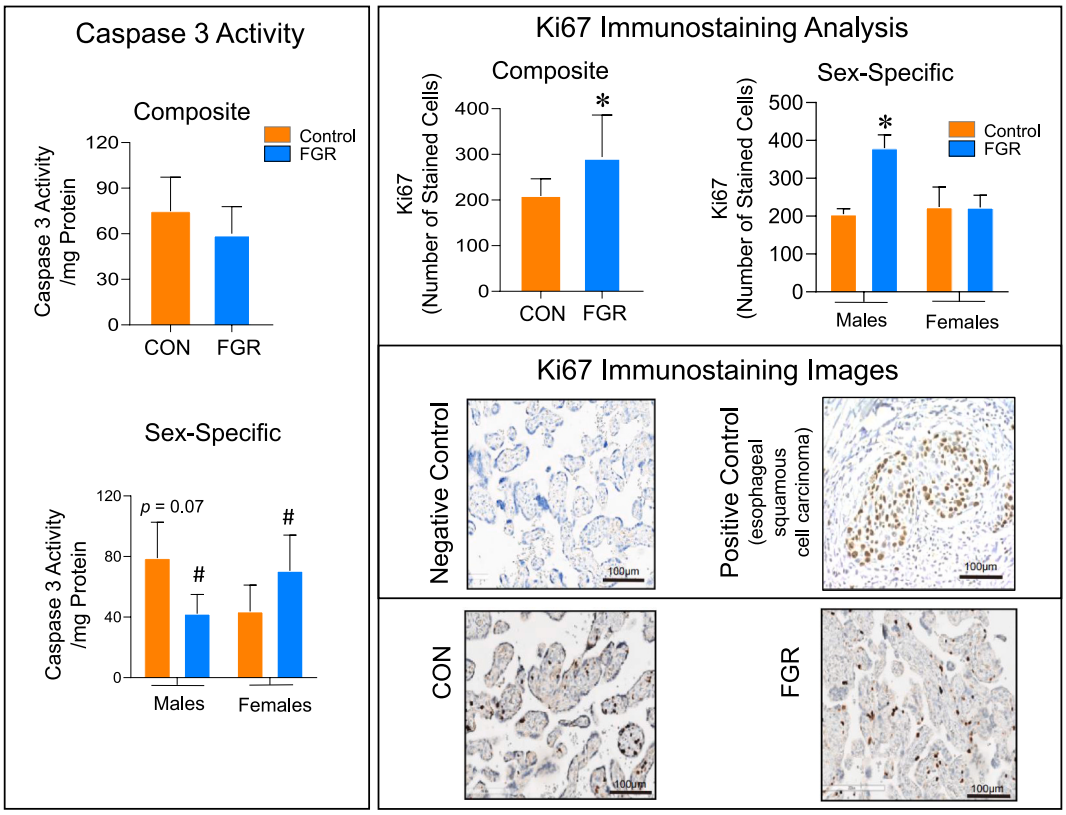

Fig. 2. Placental caspase 3 activity and Ki67 immunostaining. Data for caspase activity and number of Ki67 positive/stained cells are presented as mean ± SD with open bars representing the controls and solid bars representing the FGR groups (control group: n = 13; FGR group: n = 20). Asterisk (*) represents $p < 0.05$ by Student's t-test and # represents large magnitude change by Cohen's effect size analysis with Cohen's d values ≥ 0.8 comparing control and FGR groups. Photomicrographs of the immunostaining from negative control generated by omitting primary antibody; squamous cell carcinoma as positive controls along with control and FGR placentae are shown.

lower ($p < 0.01$) among FGR subjects and this was also evident among both sexes of the newborn.

3.2. Placental apoptosis and proliferation state

Placental apoptosis measured via caspase 3 activity did not show any changes between control and FGR groups. However, when segregated by the sex of the offspring born the caspase 3 activity leaned to be lower in placentas from male newborn ($p = 0.07$) while in those with female newborns there was a large magnitude increase in caspase 3 activity ($p = 0.06$) (Fig. 2). Cellular proliferation marker Ki67 immunostaining showed a significant increase ($p = 0.03$) in placentas from the FGR group. This increase was only evident among the placentas with male newborns when segregated by sex of the newborn (Fig. 2).

3.3. Placental relative mRNA concentrations of steroidogenic genes

Placental relative mRNA concentrations of 17 hydroxy steroid dehydrogenase B (*HSD17B*) and aromatase (*CYP19*) were significantly lower ($p < 0.05$) in the FGR group (Fig. 3). When segregated by the sex of the newborn, *HSD17B* leaned ($p = 0.07$) to be lower in placentas with male newborn only. In contrast, *CYP19* expression was significantly lower in placentas with female newborns while in the males there was a large magnitude decrease (Fig. 3). While no change in androgen (AR) and progesterone (PR) receptor were evident when examined compositely, when segregated by sex of the newborn *AR* and *PR* expression showed a large magnitude increase in placentas with female and male newborns respectively (Fig. 3). No change in expression was observed for *HSD3B* and *ESR1* expression.

3.4. Placental relative mRNA concentrations of insulin like growth factor (IGF) family members

The placental expression of *IGF1* and IGF binding protein 3 (*IGFBP3*) was significantly lower in FGR placentas (Fig. 4). When segregated by sex of the newborn, both *IGF1* and *IGFBP3* was significantly lower ($p < 0.05$) in females (Fig. 4). The expression of *IGF1, IGFBP1* and *IGF2* receptor (*IGF2R*) did not show any change between control and FGR. However, when segregated by sex of the newborn, the expression of *IGF2* mRNA appear to be lower with a large magnitude decrease in placentas from female newborns while

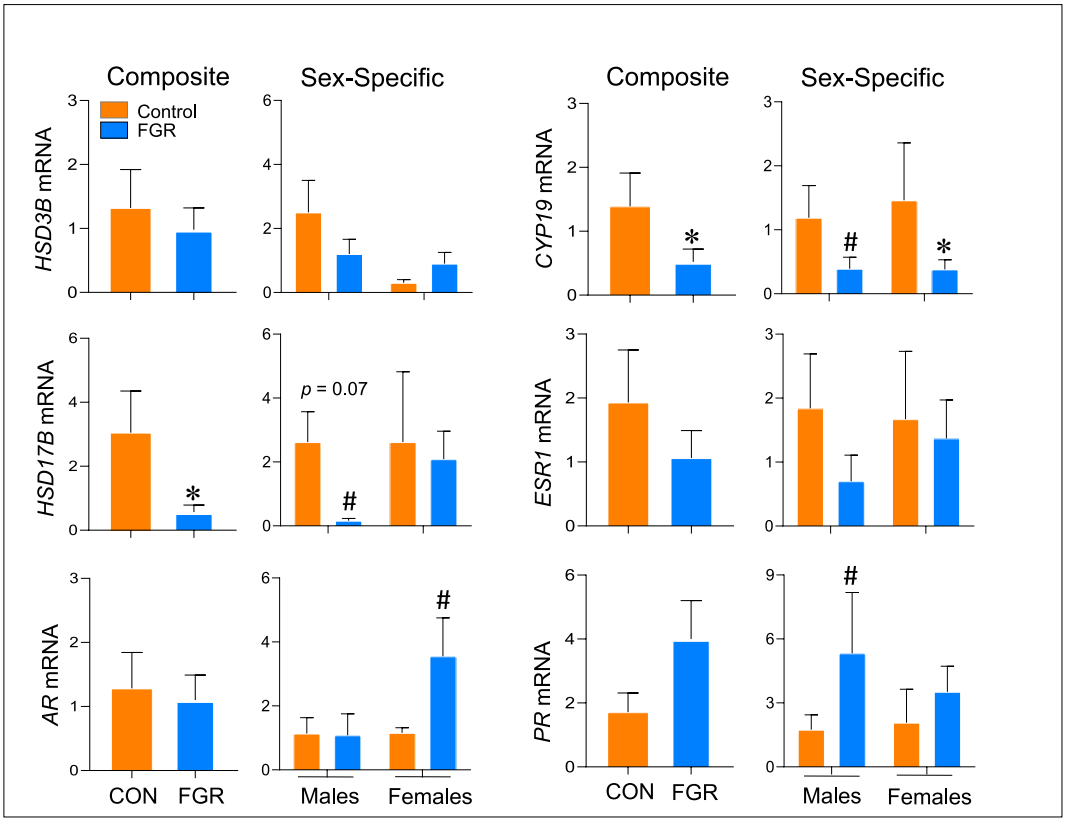

Fig. 3. The Relative mRNA concentrations of steroidogenic genes *HSD3B*, *HSD17B* and *CYP19* and steroid receptors *ESR1*, *AR* and *PR* in placentas from entire cohort (composite) and segregated by newborn sex are shown. Data are presented as mean ± SD with open bars representing the controls and solid bars representing the FGR R groups (control group: n = 13; FGR group: n = 20). Asterisk (*) represents $p < 0.05$ by Student's t-test and # represents large magnitude change by Cohen's effect size analysis with Cohen's d values ≥ 0.8 comparing control and FGR groups.

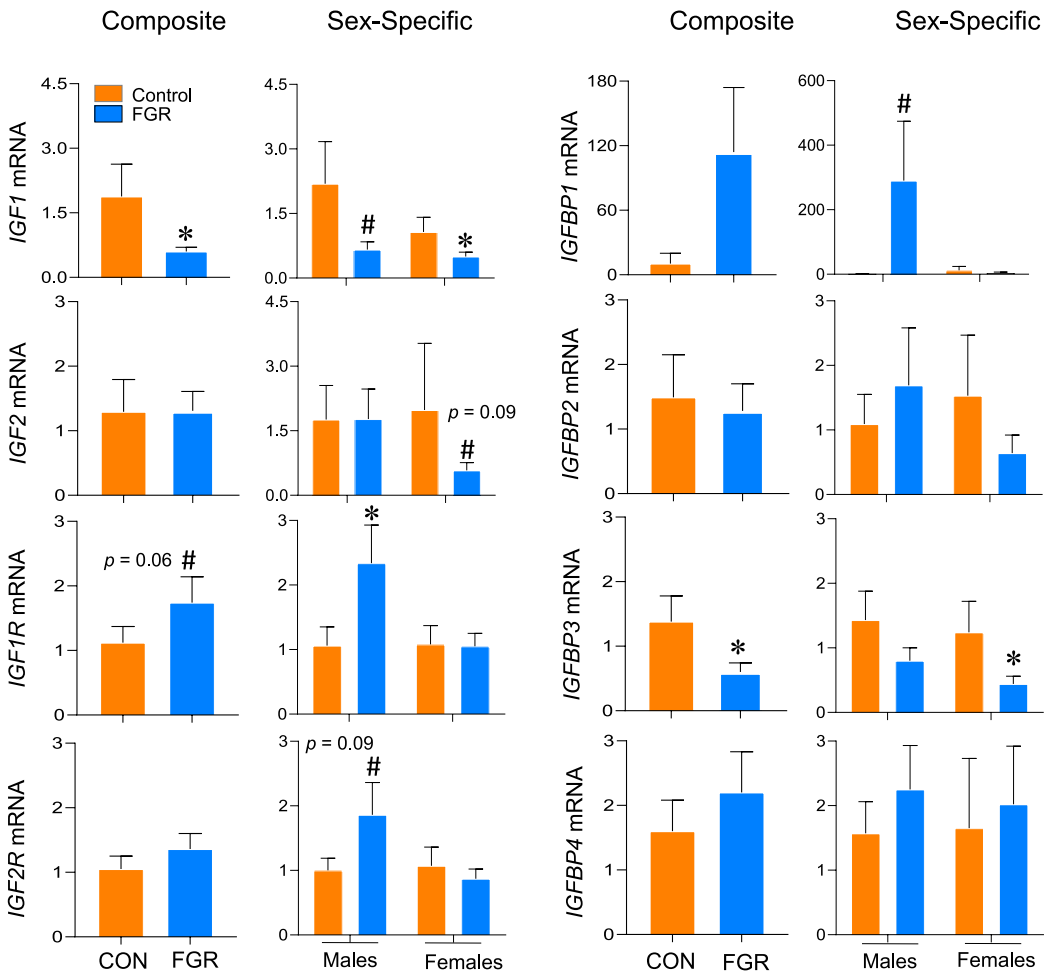

Fig. 4. The Relative mRNA concentrations of IGF family members *IGF1*, *IGF2, IGF1R, IGF2R, IGFBP1, IGFFBP2, IGFBP3* and *IGFBP4* in placentas from entire cohort (composite) and segregated by newborn sex are shown. Data are presented as mean ± SD with open bars representing the controls and solid bars representing the FGR groups (control group: n = 13; FGR group: n = 20). Asterisk (*) represents $p < 0.05$ by Student's t-test and # represents large magnitude change by Cohen's effect size analysis with Cohen's d values ≥ 0.8 comparing control and FGR groups.

IGF2R appeared to be higher in placentas from male newborns (Fig. 4). The relative levels of *IGFBP1* mRNA appeared to show a large magnitude increase in FGR placentas with male newborns but no change in expression of *IGFBP2* and *IGFBP4* expression was observed.

3.5. Placental relative mRNA concentrations of inflammatory markers

The qPCR analysis of the genes associated with inflammation such as cytokine TNF, chemokine CCL2 and macrophage infiltration marker CD68 in the placenta from control and FGR subjects are shown in Fig. 5. No change in the expression of these markers was evident between the control and FGR groups. However, when placental relative mRNA concentrations when assessed by segregating the newborn sex, the expression of the chemokine *CCL2* and macrophage marker *CD68* were significantly lower ($p < 0.05$) in placentas from pregnancies with female newborn (Fig. 5).

3.6. Placental relative mRNA concentrations of angiogenic markers

The RT-PCR analysis of the genes associated with angiogenesis in the placenta from control and FGR subjects are shown in Fig. 6. Relative mRNA concentration analysis showed that the mRNA expression of *VEGFA* leaned to be lower by large magnitude in placenta from FGR group while the *VEGF* receptors *VEGFR1* and *VEGFR2* showed no changes. Similarly, no change in the expression of *EGVEGF* was observed between the two groups. The expression of *HIF1A* and *PIGF* were significantly lower ($p < 0.05$) among the placentas from FGR group. While this trend was not observed among placentas when segregated by newborn sex for *HIF1A*, placentas with male pregnancies had a significantly lower ($p = 0.02$) *PIGF* mRNA. While no change in the relative mRNA levels of *ANG1* and *ANG2* was evident among the placentas from entire group, large magnitude increase in among placentas with female newborn was observed for

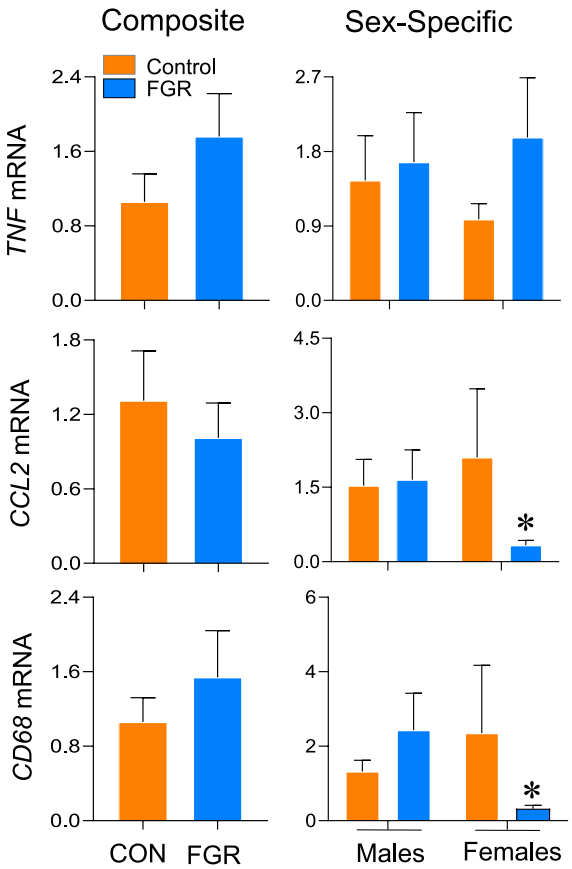

Fig. 5. The Relative mRNA concentrations of inflammatory cytokine TNF, chemokine CCL2 and macrophage marker CD68 in placentas from entire cohort (composite) and segregated by newborn sex are shown. Data are presented as mean ± SD with open bars representing the controls and solid bars representing the FGR groups (control group: n = 13; FGR group: n = 20). Asterisk (*) represents $p < 0.05$ by Student's t-test comparing control and FGR groups.

ANG1 as opposed to a large magnitude decrease *ANG2* among the placentas with male newborn.

3.7. miRNA differential expression in FGR placenta

3.7.1. Descriptive statistics

Post-trimming, miRNA reads exhibited high mean read quality scores, per sequence GC content ranging from 20 to 80% and sequence duplication levels up to 30%

3.7.2. miRNA expression in control group

A partial separation in miRNA expression profiles was observed in 2D and 3D PCA plots generated using unsupervised model (Fig. 7). Comparison of miRNA expression (FDR <0.1 and absolute log2 fold change (abs log2FC > 0.5) between female and male controls demonstrated 7 miRNA specific in placentas with female newborns and 3 genes specific in the placentas with male newborns (Supplemental Table 1). In the control group, placentas from female newborns miRNAs *MIR490* (log2FC 1.827; p = 0.097), *MIR193B* (log2FC −1.104; p = 0.00137), and 5 of the *MIR941* family *MIR941-1* (log2FC −1.5899; p = 0.009), *MIR941-2* (log2FC −1.602; p = 0.009), *MIR941-3* (log2FC −1.602; p = 0.009), *MIR941-4* (log2FC −1.602; p = 0.009), and *MIR941-5* (log2FC −1.602; p = 0.009) were upregulated compared to placentas from male newborns (Fig. 7). On the other hand, *MIR451A* (log2FC 1.635; p = 0.037), *MIR372* (log2FC 1.656; p = 0.097) and *MIR136* (log2FC 0.995; p = 0.097) were upregulated in the placentas with male newborns compared to the placentas from female newborns (Fig. 7).

3.7.3. miRNA expression in FGR group

In the FGR groups comparing the placenta from male and female newborns, only one miRNA *MIR518B* (log2FC 1.589; p = 0.001) was upregulated in the placentas from female newborns compared to the male newborns (Fig. 7; Supplemental Table 2).

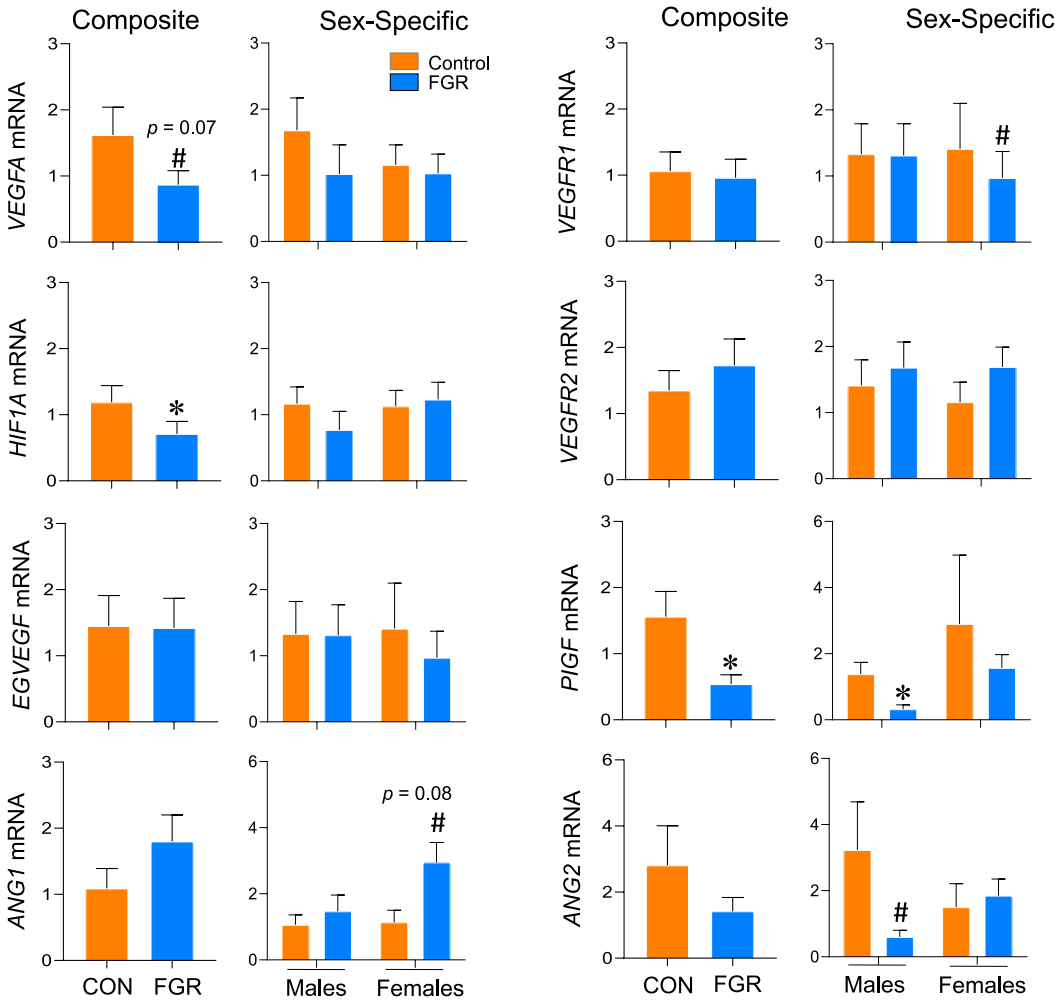

Fig. 6. The Relative mRNA concentrations of angiogenic markers *VEGFA* and its receptors *VEGFR1* and *VEGFR2*, *EBVEGF*, *HIF1A*, *PIGF*, *ANG1* and *ANG2* in placentas from entire cohort (composite) and segregated by newborn sex are shown. Data are presented as mean ± SD with open bars representing the controls and solid bars representing the FGR groups (control group: n = 13; FGR group: n = 20). Asterisk (*) represents $p < 0.05$ by Student's t-test and # represents large magnitude change by Cohen's effect size analysis with Cohen's d values ≥ 0.8 comparing control and FGR groups.

3.7.4. miRNA differential expression in control versus FGR group

In mixed gender analysis, no change in miRNA was evident, however, distinct changes were observed when segregated by fetal sex. Among placentas from female newborns, 2D and 3D PCA plots generated by unsupervised clustering miRNA showed overlap between control and FGR groups (Fig. 8). Analysis by DESeq2 for miRNA found 2 dysregulated miRNAs (FDR<0.1 and absolute log2FC > 0.5). *MIR451A* was upregulated (log2FC 1.962; p = 0.049) whereas *MIR543* was downregulated (log2FC −1.331; p = 0.054) in placentas from FGR group with female newborns (Fig. 8, Supplemental Table 3). Among placentas from male newborns, 2D and 3D PCA plots generated by unsupervised clustering showed an overlap between control and FGR groups. FGR group modulated one miRNA at FDR <0.1 and absolute log2FC > 0.5. The sole miRNA observed to be regulated was *MIR520G* which is upregulated (log2FC 0.820; p = 0.049) in the FGR group placentas with male newborns (Supplemental Table 4).

3.8. Placental microvessel characteristics

Endothelial cell marker CD34 expression in placental microvessels immunohistochemically visualized and assessed is summarized in Fig. 9. The relative staining intensity, total stained area and percent total intensity of CD34 immunostaining was not different between control and FGR placentas. When segregated with sex, the relative placental staining intensity for CD34 showed a large magnitude increase in placentas from FGR pregnancies with male newborn while the percentage of total intensity of the stain was significantly lower ($p = 0.004$) in placentas from FGR pregnancies with female newborn. Assessment of the microvessel density and the microvessel area did not show any difference between control and FGR subjects. When segregated by sex, microvessel density was

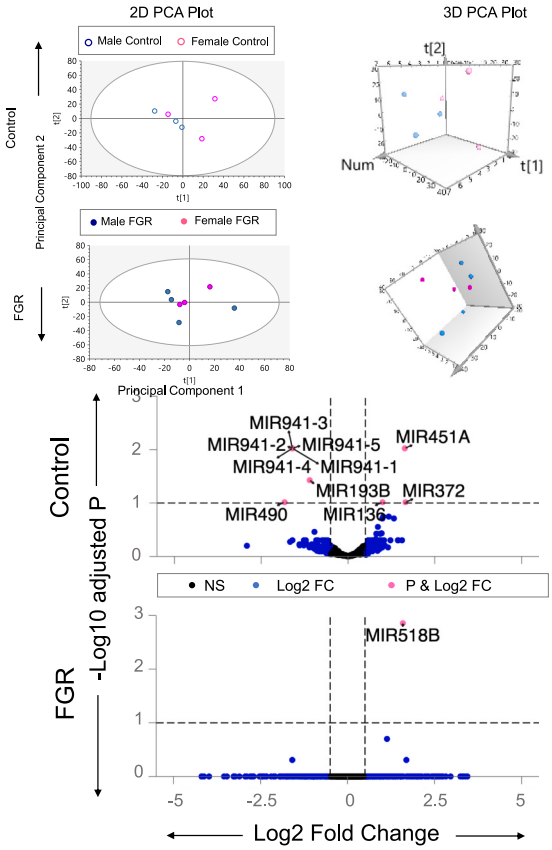

Fig. 7. Sex-specific miRNA sample clustering and differential expression in control or FGR placentas. Principal Component Analysis (PCA) 2D among placentas from controls or FGR group with male or female newborns are shown on the left column (control group: n = 6, 3 male and 3 female; FGR group: n = 7, 4 male and 3 female). Similarly, the 3D PCA plots are shown in the right for similar samples. For the PCA the 2D plots, 3D PCA plots are plotted with principal component 1 on X-axis and principal component 2 on Y-axis showing separation. Each point represents one sample. Volcano plot showing differences in miRNA expression comparing control female vs control male samples (bottom-top) and FGR female vs FGR male samples (bottom). miRNA are plotted by log2 fold change and -log10 adjusted p-values. The pink points represent miRNA that have absolute log2 fold change>0.05 and FDR <0.1. Blue dots represent miRNA that met absolute log2 fold change >0.5 but did not meet FDR cut off 0.1. Black dots represent those miRNAs that did not meet either the log2 fold change or FDR cut off. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

significantly lower ($p = 0.015$) in placentas from FGR pregnancies with female newborns.

4. Discussion

Utilizing term placenta from a homogeneous cohort of Asian primiparous pregnant women with and without FGR, expression levels of coding genes, proteins and miRNA that influence placental angiogenesis and function were assessed. Our findings showed changes in expression of genes involved in steroidogenesis, steroid action, IGF family members, inflammatory cytokines and angiogenic factors in FGR pregnancies. Most of significant changes were manifested in female placenta. A preliminary assessment also showed these changes were impacted by the offspring sex. At miRNA level, although no comprehensive changes were observed, significant changes were only evident in sexual dimorphic manner with upregulation of *MIR451A* and downregulation of *MIR543* in FGR placentas with female newborns and upregulation of *MIR520G* in FGR placentas with male newborns. In keeping with these findings, the microvessel angiogenesis marker CD34 also showed reduced staining in female FGR placental. Although the placenta function was impaired both in male and female placenta, the change of microvessel density was only observed in female placenta but not in male, possibly due to the less susceptible on gene and protein expression in males associated with placental environment [44]. The significance of these sex-specific findings and their role in development of FGR are discussed below.

4.1. Compromised placental function in FGR pregnancies

Changes in placental morphology and angiogenesis have been shown to underlie placental dysfunction in FGR pregnancies [45]. Recruitment of primiparous women without pregnancy complication allowed exclusion of maternal parameters and fetal

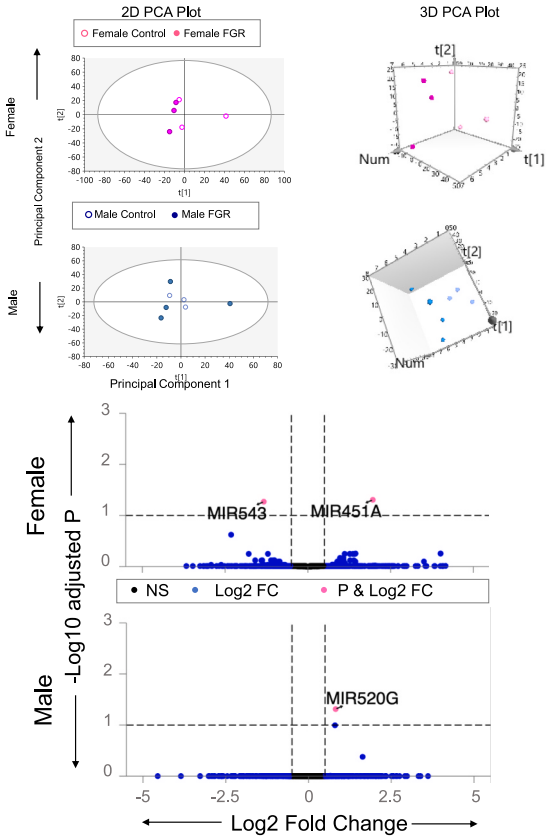

Fig. 8. Sex-specific miRNA sample clustering and differential expression between control and FGR placentas. Principal Component Analysis (PCA) 2D among placentas from male or female newborns between control and FGR are shown on the left column (control group: n = 6, 3 male and 3 female; FGR group: n = 7, 4 male and 3 female). Similarly, the 3D PCA plots are shown in the right for similar samples. For the PCA the 2D plots, 3D PCA plots are plotted with principal component 1 on X-axis and principal component 2 on Y-axis showing separation. Each point represents one sample. Volcano plot showing differences in miRNA expression comparing control vs FGR among placentas from female newborns (bottom-top) and male newborns (bottom). The pink points represent miRNA that have absolute log2 fold change>0.05 and FDR <0.1. Blue dots represent miRNA that met absolute log2 fold change >0.5 but did not meet FDR cut off 0.1. Black dots represent those miRNAs that did not meet either the log2 fold change or FDR cut off. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

malformation thus enforcing the focus on the main etiology resulting from placenta. Consistent with previous findings, lowered placental efficiency were evident in FGR pregnancies [46]. Meanwhile, the impaired placental function was evident in both male and female fetal placenta.

4.2. Relative mRNA concentration changes in FGR placenta function and angiogenesis

Placenta being a dynamic organ that undergoes significant anatomical and functional changes to keep up the demands of fetal growth requires to be constantly regulated by various processes. In line with this premise, genes that encode steroid synthesis and action, inflammatory process, IGF family members and angiogenic genes were altered in FGR group. The lower expression of *CYP19,* key gene in estradiol biosynthesis, in the placentas is in line with lower level of maternal estradiol observed in mothers with FGR pregnancies [47] and estrogen-suppressed placenta manifesting an impaired placental angiogenesis in animal study [48]. The lower placental expression of *IGF1* and *IGFBP3* in FGR group are also supportive of the role of IGF family members in development of FGR. IGFs are anabolic hormones that promote proliferation, mitochondrial protection, cell survival, tissue growth and development, anti-inflammatory, antioxidant, anti-fibrogenic and anti-aging activities in the developing fetus [49]. With placenta being a major source of IGFs [50], the lower expression of IGF is in line with low birth weight and impaired placental efficiency and capillary growth [51]. Preliminary analysis also indicated that the lower expression of *IGF1* and *IGFBP3* in the female placenta was observed along with the decreased capillary density in terminal villi and expression of pro-inflammatory chemokine *CCL2* and macrophage marker *CD68*.

Significant downregulation of angiogenic genes *HIF1A* and *PIGF* were evident among the placentas from FGR group, while placental expression of *VEGF, ANG1*, *ANG2*, and angiopoietin receptor (*TIE-2*) were found to be higher [52–54]. Since HIF1A regulates the expression of VEGF and angiopoietins, it is possible that lower expression of *HIF1A* in our cohort may have contributed to the lack of change in the expression of other angiogenic markers, consistent with the findings in a preeclamptic placentas where no significant

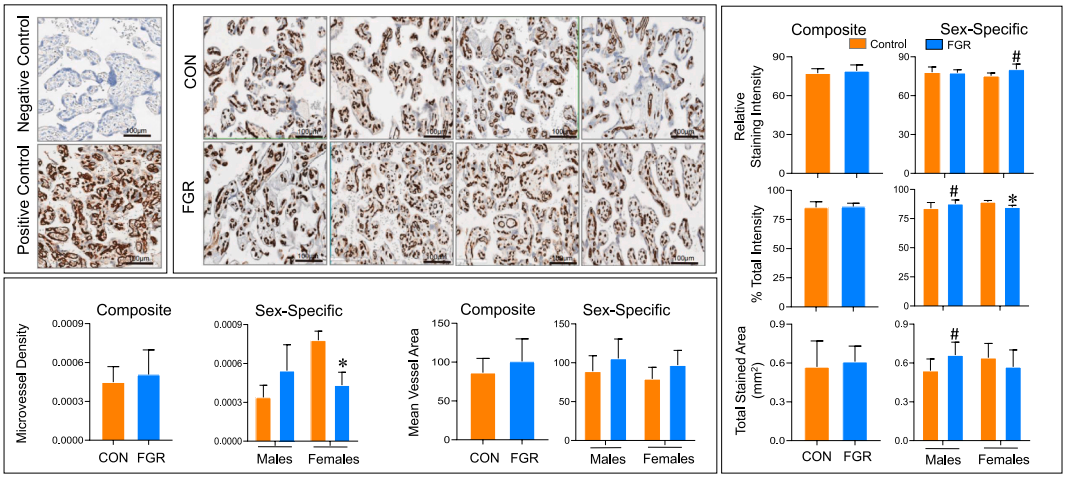

Fig. 9. Photomicrographs of endothelial cell marker CD34 immunostaining in the control and FGR placentas along with analysis of the staining intensity, percentage of total stain intensity and area of the stain, and microvessel density and area in placentas from the entire cohort (composite) and segregated by newborn sex are shown. Data for staining and microvessel characteristics are presented as mean ± SD with open bars representing the controls and solid bars representing the FGR groups (control group: n = 13; FGR group: n = 20). Asterisk (*) represents $p < 0.05$ by Student's t-test and # represents large magnitude change by Cohen's effect size analysis with Cohen's d values ≥ 0.8 comparing control and FGR groups.

changes in angiogenic factors were observed [55]. Though the role of fetal sex plays in the placental perturbations are widely studied [29,56], our preliminary observations did not indicate any sex-dependent change on angiogenesis associated relative mRNA concentrations.

Consistent with the compromised placental phenotype there was an increase in caspase activity in the placenta with female pregnancies. In contrast, in spite of the decreased placental efficiency and a trend for decreased placental volume, paradoxically the placenta from male pregnancies showed an increase in proliferative activity as evidenced by the Ki67 staining and decrease in caspase activity suggestive of an adaptive response to overcome compromise. In keeping with this premise of adaptive response, a trend for an increase in capillary density was evident in placenta of male pregnancies. Adaptive changes in angiogenesis reflective of increased efficiency have been reported with smaller size placenta [57]. Our preliminary observation of a trend for an increase in capillary density in male FGR placenta as opposed to downregulation in female FGR placenta is supportive of sexually-dimorphic compensatory responses. Previous studies [58,59] have also shown a trend for increase in capillary in male FGR placenta.

4.3. Placental miRNA expression changes

miRNAs are noncoding RNAs that regulate gene expression through micro-ribonucleoprotein effector complexes and sequence-specific recognition of target sites. As has been reported before [60] dimorphic expression of miRNA was observed between pregnancies with male and female newborns in both control and FGR groups. The significance of these sex-specific findings needs to be further explored. Interestingly, decreased expression of *MIR451A* expression in hepatocellular carcinoma was reported to play a role in increasing angiogenesis and VEGF expression [61,62]. This increase of *MIR451A* in FGR pregnancies in the present study was not associated with a change in VEGF expression. However, our finding of reduced staining for CD34, a microvessel endothelial marker is suggestive of reduced angiogenesis in FGR placentas with female newborns. This is in line with the negative regulation of angiogenesis associated with *MIR451A* elevation. In contrast to upregulation of *MIR451A*, downregulation of *MIR543* in placentas from FGR group with female newborns was observed. However, while *MIR451A* negatively regulates angiogenesis, *MIR543* expression positively regulates angiogenesis as demonstrated in cardiac endothelial cells [63] and non-small cell lung cancer [64]. Due to the opposing roles of *MIR451A* and *MIR543* in regulating angiogenesis, the balance of the two may dictate the final impact on angiogenesis. These changes in miRNA in the FGR associated pregnancies with female fetuses are however indicative that dysregulation of angiogenesis may have epigenetic basis.

In contrast in FGR associated pregnancies with male fetuses upregulation of *MIR520G* was observed. While a role for angiogenesis for this miRNA is not yet known, elevated expression of *MIR520G* have been observed in serum from preeclampsia pregnancies [65]. Additionally, this miRNA has been shown to negatively regulate trophoblast migration and invasion [65] processes essential for placental function. These findings support for the possibility that compromised placental trophoblast migration and invasion may be the basis for the development of FGR in pregnancies carrying male fetuses.

4.4. Changes in placental angiogenesis

CD34 is a cell surface marker that is expressed by a broad range of cells including hematopoietic, stromal, epithelial, and endothelial cells and is now widely regarded as a marker of vascular endothelial progenitor cells [66]. CD34 positive endothelial cells under

angiogenic stimuli are shown to migrate and from sprouting tip cells which are present in the leading edge of angiogenesis [67]. Because of this role of CD34 in active angiogenesis the evidence that reduced immunostaining intensity for CD34 in FGR associated placentas with female pregnancies suggests impaired angiogenesis as a contributory factor for development of FGR. Evidence that VEGF and CD34 expression correlates with angiogenesis in tumors [68] is supportive for the evidence that the large magnitude decrease in expression of VEGF receptor VEGFR1 may also contribute to this impeded angiogenesis in FGR placenta with female offspring. However, the upregulation of CD34 total staining intensity and stained area pointing to the proliferation of endothelial cell in male FGR placenta may again be reflective of a compensatory response to maintain non-branching angiogenesis in terminal villi of male FGR placenta.

5. Limitations and conclusions

This study provides strong evidence for impaired placental angiogenesis as a contributory factor for development of FGR and our preliminary observations supports that this occurs in a sex dimorphic manner. However, these findings should be viewed considering some limitations. The limitations are that this study was carried out in a small set of mothers from a non-diverse cohort. However, the fact that this study is from a non-diverse cohort of Asian women can be viewed as a strength as confounds arising from race can be avoided. As only primipara and term-pregnancies were chosen for recruitment, it lead to difference in distribution of fetal sex from both control and FGR groups. Nonetheless, this preliminary investigation showing statistical differences in sex-specific outcomes achieved in the face of low numbers emphasize the need for large scale investigations to validate the potential miRNA biomarkers identified. This comprehensive analysis of gene and key protein expression coupled with use of next generation sequencing to determine differentially expressed miRNA is the strengths of this study. However, our preliminary findings of sex-specific changes observed here should be viewed as providing proof of concept on the influence of fetal sex on FGR outcomes via impaired placental angiogenesis and function and needs to be explored further in a larger and more diverse cohort. Nonetheless this study points to the role for changes in key genes modulated through epigenetic regulation to have a contributory role in impaired angiogenesis underlying the development of FGR.

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Data availability statement

Data will be available from corresponding author upon request.

Author contributions

Song W performed conceptualization, formal analysis, writing the original draft.
Guo Q contributed to the project design, funding Application.
Puttabyatappa M analyzed and interpreted the data, writing-original draft.
Venkateswaran R Elangovan performed the miRNA expression analysis and related draft.
Wu XH, project administrator, supervised the whole process of experiment, funding acquisition, writing-reviewing & editing.
Wang JP, Li F, Liu F, Bi X, Li H, Fu G collected data and samples, performed the experiment.
Padmanabhan V contributed to revised the original draft and gave suggestions for experiment.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28007.

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