The Role of Osteocalcin in Placenta Function in Gestational Diabetes Mellitus

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Research article

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Abstract

Background: Evidence for osteocalcin role in glucose and energy metabolism is increasing. However, little is known about osteocalcin function in gestational diabetes mellitus. The aim of this study is to examine the associations between osteocalcin and gestational diabetes mellitus.

Method: 36 patients with gestational diabetes mellitus and 40 normal glucose tolerance controls were recruited in the Maternal and Child Health Hospital Guangxi Zhuang Autonomous Region from May to August 2018. Total osteocalcin and biochemical indexes of maternal serum and umbilical vein serum were analyzed. Transcriptome of placenta were sequenced. Human trophoblast JAR cells were used for evaluated the affection of osteocalcin on trophoblast *In vitro*. There were no significant differences with maternal serum total osteocalcin levels between gestational diabetes mellitus and normal glucose tolerance groups. The gestational diabetes mellitus group has lower umbilical vein serum total osteocalcin ($51.46 \text{ ng/mL} \pm 24.29$ Vs $67.00 \text{ ng/mL} \pm 25.33$, $P = 0.008$), lower adiponectin ($1099.72 \text{ } \mu\text{g/L} \pm 102.65$ Vs $1235.85 \text{ } \mu\text{g/L} \pm 94.63$, $P < 0.001$), higher leptin ($7.41 \text{ } \mu\text{g/L} \pm 0.28$ Vs $6.02 \text{ } \mu\text{g/L} \pm 0.31$, $P = 0.001$). A significant relationship existed between umbilical vein serum total osteocalcin levels and leptin ($r = -0.456$, $P = 0.007$). Osteocalcin promote JAR trophoblast cells proliferation and HCG synthesis. 36 correlated gene modules of placental transcriptome were identified through weighted gene co-expression network analysis, 2 of them were associated with osteocalcin.

Conclusion: lower osteocalcin in umbilical vein serum is associated with gestational diabetes mellitus, osteocalcin may regulate placenta function via adiponectin.

Background

Gestational diabetes mellitus (GDM) is defined as glucose intolerance that is first recognized during pregnancy. With the development of living level and the alteration of life style, the morbidity of gestational diabetes is about 30% worldwide [1]. Hyperglycemia leads to complications, such as macrosomia, gestational hypertension, and preterm delivery. Moreover, postpartum patients and GDM offspring often carry a lifelong increased risk of glucose intolerance and obesity [2, 3].

Osteocalcin (OCN) is an osteoblast-specific protein, Osteocalcin is the the most abundant non-collagenous proteins which constitute bone, un carboxylation OCN (ucOCN) released into the circulation, evident shows that ucOCN involved in the regulation of glucose and fat metabolism[4, 5]. OCN could decrease serum glucose levels either directly by increases pancreas beta cells insulin secretion or indirectly by promoting glucose consume by liver, adipocyte, muscle and so on[6, 7]. Both of these functions are mediated by the G-protein-coupled receptor GPR C6A [8–10], inducing the synthesis of adiponectin (ADP) in adipocytes, muscle and other tissues, then facilitating insulin sensitivity [4, 5].

It has been confirmed that OCN serum levels are lower in individuals with type 2 diabetes than in normal controls [9, 11, 12]. Although GDM resembles type 2 diabetes in some ways, there is no consensus regarding the OCN levels in GDM. The results of some epidemiological investigations reveals that

maternal OCN, especially the biological activity un carboxylation OCN (ucOCN) levels are significantly higher in GDM patients [13–15], others reveals that the OCN levels has no significant relation with GDM morbidity [11]. It is interesting that some research suggest that ADP levels are significantly lower in women with GDM and may help predict the risk of diabetes after GDM [16, 17]. Since ADP is the down effect protein of OCN, and during pregnancy, the placenta is one of the major source organs of ADP, so we hypothesized that it couldn't exclude that OCN is involved in the occurrence of insulin resistance via the placenta. Therefore, the aim of this research is to examine the associations between OCN and GDM in GDM both in Maternal peripheral blood and umbilical cord blood.

Methods

Participants and clinical measurement

Subgroup 1 including 36 pregnant women with GDM and 40 matched normal glucose tolerance (NGT) pregnant women, subgroup 2 including 13 NGT pregnant women, all of them were recruited from May to August 2018, at The Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region, Department of Obstetrics. Inclusion and exclusion criteria are shown in Tab. S1. Written informed consent was obtained from all participants, and the study was approved by The Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region ethics committee.

Pre-pregnancy body mass index (BMI) was calculated according to weight before pregnancy as weight (in kilograms)/height (in square meters). Birthweight, neonatal gender, delivery mode, placental size and weight, neonatal peripheral blood glucose levels were obtained from patient medical records.

Biochemical analysis

Maternal blood samples were taken from a cannulated vein before delivery, gestational diabetes mellitus umbilical vein (Uv) blood and umbilical artery (Ua) blood were taken from the umbilical cord venous and arterial storing in the vacuum blood collection tube during the third stage of labor. All blood samples were centrifuged (3000 rpm at room temperature for 5 minutes), and serum were collected in the 1.5 mL Eppendorf tubes. Placental tissue from the central cotyledon about 1 cm³ was obtained immediately after delivery. Serum aliquots and placental tissues were immediately stored at -80°C until analysis.

Measurement of serum indicators

The levels of total OCN (tOCN), 1,25-(OH)₂-VitD₃ (25(OH)D₃), parathormone, insulin-glucose, triglycerides (TG), total cholesterol (TC), high density lipoprotein total cholesterol (HDL-C), alkaline phosphatase (ALP), human chorionic gonadotropin (HCG), and estradiol (E₂) were analyzed by electrochemiluminescence immunoassay on COBAS 6000 system E601 (Elecsys module) immunoassay analyzers (Roche Diagnostics, GmbH, Mannheim, Germany). Leptin (LEP), ADP, and tartrate-resistant acid phosphatase 5b (TRACP-5b) were measured by ELISA kits according to the manufacturers' instructions (Shanghai Guangrui Biotechnology Co., Ltd).

serum cytokines analysis

8 serum cytokines, IL-2 IL-4 IL-6 IL-8 IL-10 IL-17A IFN- γ TNF- α , were analyzed on Bio-Plex liquid chip platform. Briefly, the biotinylated detection antibody was added into a 96-well plate, serum sample were mixed with the antigens coupled microspheres and added into each hole, then adding streptavidin phycoerythrin, detecting the fluorescence signal by a Bio-Plex liquid chip system, and judging the positive property/negative property of the sample according to the strength of the fluorescence signal.

RNA extraction and qRT-PCR

RNA extraction and purification were conducted using the E.Z.N.A.[™] Total RNA Kit I (Omega). cDNA synthesis was performed using the PrimeScript[™] RT Reagent Kit (Takara). SYBR Premix Ex Taq[®] (Tli RNaseH Plus) was used with the following PCR parameters, 1 cycle of 30 s at 95 °C and then 40 cycles of 95 °C for 5 s and 1 cycle of 30s at 60 °C. qRT-PCR was conducted using a LightCycler 96 (Roche). The primer sequences are presented in Tab. S2. The housekeeping gene β -actin was used as control. The relative levels of the mRNA of the genes of interest were normalized to the β -actin mRNA.

Immunohistochemistry

Placenta tissues were washed 3 times in PBS for 30 seconds and fixed in 4% formaldehyde for 24 hours. The placental tissues were then embedded in paraffin and sectioned at a thickness of 5 μ m. The tissue sections were deparaffinized, subjected to high-temperature antigen exposure, rehydrated in 3% H₂O₂, and blocked with 10% normal goat serum for 30 minutes. The sections were then incubated with antibodies to OCN (1:1000) (Santa cruz) or GPRC6A (1:1000) (Invitrogen). The secondary antibodies are provided with the DAB Substrate Kit for Peroxidase (Vector Laboratories). Olympus DP11 camera and Olympus Camedia software were used to produce the images.

In vitro experiments

Cell culture: JAR trophoblast cells were giving by professor Longyu, JAR cells were maintained without antibiotics and RPMI-1640 medium (Gibco) containing 10% fetal bovine serum (FBS) (Gibco) at 37 °C under 5% CO₂. Osteogenic induction medium: α -MEM (Gibco) containing 10% FBS (fetal bovine serum), 100 nM dexamethasone (Sigma), 5 mM β -phosphoglyceride (Sigma) and 5 μ g/mL vitamin C (Sigma).

Alizarin red stain: Alizarin red stain positive indicate calcium. JAR cells were maintained in 6 well plate until 90% confluency. Osteogenic induction medium was added confluency to 6 well plate to maintain 7 days, and then JAR cells were fixed by 4% paraformaldehyde for 10 minutes. Adding alizarin red for 5 minutes. At each of steps, JAR cells were washed for 3 times by PBS.

Scanning electron microscope: JAR cells were maintained in 24 well plate until 80%. Osteogenic induction medium was added to 24 well plate to maintain 7 days. 3% glutaraldehyde was used to fixed JAR cells. The calcium nodules were observed by scanning electron microscope (Czech, TESCAN, VEGA 3 LMU).

Cell proliferation assay: Using the Cell Counting Kit-8 (CCK-8, Solarbio®) according to the manufacturer's instructions. This experiment was performed in triplicate.

shRNA knockdown: Human GPRC6A shRNA lentivirus plasmid pLV[shRNA]-EGFP: T2A: Puro-U6 > hGPRC6A purchases from Victor builder company (Saiye). Lipofectamine 2000 (Invitrogen) was used to package virus in 293T cells; a 5:3:2 ratio of target plasmid: psPAX2: PMD2G was used. Virus supernatant (100 µL) was added to JAR cells in 24-well plates for 24 hours. Green fluorescence could be observed after 72 h.

RNA sequencing and bioinformatics analysis

RNA sequencing: Total RNA was extracted from placental tissues using the E.Z.N.A.TM Total RNA Kit I (Omega). mRNA sequencing was performed on an Illumina HiSeq 4000 RNA-sequencing platform, and the results were uploaded to BaseSpace of the Illumina cloud server. The RNA-Seq Alignment and Cufflinks Assembly procedures were used in the sequencing analysis.

WGCNA: Co-expression networks were constructed using the WGCNA (v1.47) package in R. Without filtering the genes, gene expression values were imported into WGCNA and used to construct co-expression modules using the automatic network construction function block wise modules with default settings except that the power was set to 2. TOM Type was unsigned, and the minimum module size was 250. The genes were clustered into 36 correlated modules. Module eigengenes were used to calculate the correlation coefficients for samples or sample traits.

Gene ontology (GO) analysis: significantly enriched GO terms pathways were defined by the hypergeometric test using a threshold false discovery rate (FDR) ≤ 0.05 .

RNA-Seq data to this article can be found online at <https://www.ncbi.nlm.nih.gov/sra/>, SRR10812151-SRR10812189.

Statistical analyses

Statistical analyses were undertaken using SPSS 25(IBM, Chicago, IL, USA). The results are reported as the mean $\pm$ S.D. unless otherwise noted. Clinical characteristics that followed a normal distribution were compared between the two groups using Student's *t*-test. Categorical variables were analyzed using the χ^2 test. Spearman's correlation was used to examine associations between serum OCN and metabolic indices. Statistical analysis and graph plotting were performed using Prism 7 software and Adobe Illustrator. *P* < 0.05 was considered significant.

Results

No significant differences with maternal serum tOCN levels between GDM and NGT groups in third trimester

36 GDM patients and 40 NGT control participants were recruited. Compared with the NGT controls, GDM patients had higher maternal age, pre-pregnancy BMI, systolic blood pressure, diastolic blood pressure, and C-reactive protein. Overall weight gain was significantly lower in GDM patients than in the controls. However, there were no significant differences with maternal serum tOCN levels between GDM and NGT groups ($14.46 \text{ ng/mL} \pm 10.19$ Vs $16.01 \text{ ng/mL} \pm 8.54$, $P = 0.472$) (Tab. 1).

The Uv serum tOCN levels is lower in GDM patient

Birth length and head circumference were significantly higher in the GDM group than in the NGT control group, but there was no difference in birth weight (Tab. S3). Interestingly, The Uv serum tOCN levels is significantly lower in GDM patient than in the NGT controls ($51.46 \pm 24.29 \text{ ng/mL}$ Vs $67.00 \text{ ng/mL} \pm 25.33$, $P = 0.008$). Meanwhile ADP is lower in the GDM group ($1099.72 \text{ } \mu\text{g/L} \pm 102.65$ Vs $1235.85 \text{ } \mu\text{g/L} \pm 94.63$, $P = 0.001$), while LEP is higher in GDM group ($7.41 \text{ } \mu\text{g/L} \pm 0.28$ Vs $6.02 \text{ } \mu\text{g/L} \pm 0.31$, $P < 0.001$, respectively) (Tab. 1). blood glucose 24-28 weeks OGTT, fasting, 1, 2-hour glucose were higher in the GDM group, all $P < 0.001$. the Fasting Glucose before delivery show rising trend in the GDM group, but has no significant different, $P = 0.074$

Spearman's correlation analysis is shown that Uv serum tOCN levels were negatively correlated with LEP and neonatal peripheral blood glucose ($R = -0.456$, $P = 0.007$ and $R = -0.278$, $P = 0.026$, respectively) and positively correlated with TNF- α ($R = 0.395$, $P = 0.016$). It is not significantly correlated with birth weight, birth length and chest circumference, or bone metabolism indexes $25(\text{OH})\text{D}_3$ and TRACP-5b (Tab. 2).

OCN exerts autocrine loop in trophoblast

Since there is no obvious correlation between Uv serum tOCN levels and fetal bone mass, we suspected that some of the OCN present in Uv blood may be produced by trophoblast cells. To test this prediction, firstly, the concentrations of total OCN in maternal peripheral blood and in Ua and Uv were measured in 13 healthy pregnant women, the serum sample was collected during pre-delivery or third stage of labor. The tOCN concentration in Uv serum was significantly higher than that in maternal serum and Ua serum (Fig. 1 a). Secondly, OCN and its receptor GPRC6A expression were verified by placenta immunohistochemistry staining, which were positive expression in syncytiotrophoblasts ($N = 3$) (Fig. 1b). Thirdly, the ability of trophoblasts to synthesis OCN was further investigated by JAR trophoblast cells [human]. 7 days after osteoblast induction, qRT-PCR analysis revealed that the bone formation-related genes *OCN*, *Dmp1*, *Col1A1* were highly expressed in osteogenesis induces differentiated JAR cells (Fig. 1c). JAR cells showed the presence of mineralized nodules within the cells, indicated by Alizarin Red stained or transmission electron microscopic images staining (Fig. 1d).

OCN affected JAR trophoblast cells function

To determine the effects of OCN on JAR trophoblasts cells, 100 ng/ml OCN was added to the culture medium. OCN markedly promoted JAR cells proliferation and HCG synthesis. qRT-PCR analysis is shown that ADP expression is increased while LEP expression is decreased, meanwhile enzymes associated with

steroid hormone metabolism such as *CYP-11A1*, *CYP-19A1*, *CYP-27B1*, *HSD-17B1*, and inflammatory factors IL-6, TNF- α were significantly increased than without AND (Fig. 1e-g).

GPRC6A is the receptor of OCN, and GPRC6A was knockdown in JAR trophoblast cells which was confirmed by qRT-PCR (Fig. S1). As expected, shRNAs targeting GPRC6A efficiently attenuated JAR trophoblast cells proliferation and HCG synthesis. LEP, ADP, enzymes associated with steroid hormone metabolism and inflammatory factors were also affected by GPRC6A shRNA (Fig. 1h-j). $P < 0.001$

Placenta mRNA sequence analysis found 6 modules correlated with ADP and LEP

mRNA sequence analysis was performed on 39 (GDM=18, NGT=21) placental samples. A total of 18329 genes were detected and selected for subsequent analysis. Co-expression modules were constructed using the WGCNA (v1.47) package in R. Scale-free networks were constructed in which the power value was equal to 2 (Fig. S2a). The grey module, which consisted of genes not belonging to any other module, was excluded, then 36 distinct gene co-expression modules were separated (Fig. S2b).

There are 2 modules show the highest correlations with osteocalcin (Fig. 2). The *darkturquoise* (n = 355 genes), *darkmagenta* (n = 269 genes), showed positive correlations with osteocalcin (Fig. 2).

The enriched GO biological processes—molecular function and cellular component were used to show the gene function of two correlations *darkmagenta* and *darkturquoise* module. The genes in the *darkturquoise* module appear to be enriched in glucagon receptor activity, brown fat cell differentiation. *darkmagenta* module corresponded to muscle system process, cAMP-dependent protein kinase regulator activity (Fig. S3).

Hub genes for the modules of interest were chosen according to the criteria MM (Module Membership) > 0.8, GS(Gene Significance) > 0.4 and significantly ($P < 0.05$) correlated with osteocalcin. (Fig. S4, Tab. S4).

Discussion

We found no differences in maternal serum OCN in GDM and NGT groups, in fact, the levels of OCN Uv serum were lower in the GDM group than those in NGT group, these result was consisted with previous research, which demonstrated that in the third trimester or predelivery OCN has no significant difference between GDM and control group [11, 13]. What we go further than these reports are that we analyze the relationship between OCN and biochemical indexes in Uv serum. Our result suggest that the OCN levels in Uv serum were lower in GDM patient. Uv OCN was negatively correlated with Uv LEP and fetal blood glucose. Based on these founding we guess that maybe the placenta could synthesis OCN by itself, further research reveal that Uv serum concentrations of OCN are higher than that in Ua serum and maternal peripheral blood plasma concentrations, suggesting that the steroidal environment of the placenta differs significantly from that in maternal peripheral blood.

By placenta immunohistochemical staining and in vitro JAR trophoblast cells experiment, we identified a potential OCN autocrine feed-forward loop in the placenta. OCN is the most abundant non-collagenous protein found in the skeleton and was previously thought to be expressed only in mature osteoblasts. However, there is increasing evidence regarding the ectopic production OCN by other organs. This is not the first report about that OCN expression was found in placenta tissue. Telejko had reported that OCN mRNA were detected in placenta tissue [18]. Moreover, it has been reported that OCN is expressed in adipose tissue [18–20], tendon sheath tissues [21], endothelial progenitor cells [22], and prostate cancer [23] and so on. The function of ectopic production OCN in placenta is worth further investigation.

It is believed that OCN facilitates insulin sensitivity by inducing the expression of ADP through the GPRC6A receptor [4, 24]. ADP is a well-known gene involved in glucose metabolism regulation, which promotes sugar uptake and reduces gluconeogenesis in liver and muscle, resulting in reduced insulin resistance and promoting insulin sensitivity [25, 26]. The level of insulin resistance during pregnancy is closely associated with proteins secreted by the placenta, such as ADP, inflammatory cytokines and so on [27]. A consensus has been reached that lower ADP levels are significantly correlated with insulin secretion and IR indices in GDM [28, 29]. However, it is unclear that OCN plays a role in glucose homeostasis through placenta or mediated through ADP during pregnancy.

In this research we reported that OCN synthesis by trophoblast, in vitro JAR trophoblast cells experiment proved that OCN favorable ADP, sex hormone, adipocyte cytokine production. mRNA sequencing of placenta and subsequent WGCNA analysis was performed for further investigate the OCN function in the placenta. It is interesting that 2 of 36 module show correlations with osteocalcin, including *darkmagenta* and *darkturquoise*. Hub genes were identified in these two modules. Some of these genes have been reported to be associated with diabetes or lipid metabolism, such as MAGI1 [30], ACTC1[31], ACTA1 [32]. Overall, the result suggested that repress OCN in placenta regulated trophoblast function through ADP. Although some of these genes have been reported to be associated with glucolipid metabolism, their importance in placenta metabolic programming and the relationship with OCN and ADP require further investigation.

The relationship between Uv OCN and neonatal growth index, 25(OH)D₃ were not found out. Uv OCN is negative associated with neonatal peripheral blood glucose. Importantly, LEP is negative correlated with Uv OCN. LEP is mainly secreted by placenta regulating the insulin sensitivity, insulin secretion as well as glucose and lipid metabolism [33]. The level of LEP has an important role in bone metabolism. Previous study report that LEP deficient ob / ob and LEP resistant db / db can increase osteocalcin levels [34]. However, other research shows that LEP increase osteoblast-specific osteocalcin [35]. One explanation may be the different sources of LEP. Uv LEP may mainly secreted by the placenta to satisfy maternal and fetal growth during pregnancy.

In this research, the Uv OCN is positively correlated with TNF- α , and GPRC6A knockdown JAR cells model was stimulated by OCN increased secreting IL-6 and TNF- α . The role of OCN in inflammation in human beings is still inconclusive. The previous study suggest that OCN improves whole - body insulin

resistance by decreasing inflammation, and increasing insulin signaling and the expression of *Slc2a4/GLUT4* [36]. Moreover, OCN could be a therapeutic target for protecting against chronic inflammation in T2D [37].

Limitations Of The Study

The limitations of this study are as follows. 1) This is a study of a small sample of women in southern China. Although is neonate birth weight and Maternal peripheral blood glucose higher in GDM group, but there was no statistic difference, which may be the sample size is too small, still need a large number of high quality. 2) OCN has two molecular forms, carboxylated or ucOCN, it is ucOCN appears linked endocrine function. Although tOCN may reflect indirectly ucOCN levels, it is still very regret that only tOCN, not specifically ucOCN was measured in this research. 3) This is a cross-sectional study, the findings reported here do not necessarily reflect causal associations. 4) OCN were strongly expressed in syncytiotrophoblasts by immunohistochemistry, however, for verification the trophoblast OCN synthesis ability, we use Jar cells, which are cytotrophoblasts.

Conclusion

In the third trimester, lower osteocalcin concentration of umbilical venous serum is associated with gestational diabetes mellitus. Osteocalcin exerts autocrine loop in trophoblast may regulate placenta function via adiponectin.

Abbreviations

GDM, gestational diabetes mellitus; NGT, normal glucose tolerance; BMI, Body Mass Index; SBP, systolic blood pressure; DBP, diastolic blood pressure; OGTT, oral glucose tolerance test; OCN, osteocalcin; 25(OH)D3, 1,25-(OH)2-VitD3; CRP, C-reactive protein; ALP, alkaline phosphatase; HOMA-IR, homeostasis model assessment of insulin resistance; HDL-C, high density lipoprotein total cholesterol; TC, total cholesterol; TG, triglyceride; HCG, human chorionic gonadotropin; E2, estradiol; TRACP-5b, tartrate-resistant acid phosphatase 5b; LEP, leptin; ADP, adiponectin; IL, interleukin; IFY γ , interferon γ ; TNF- α , tumor necrosis factor α . Uv, umbilical vein;

Declarations

Availability of data and materials

RNA-Seq data to this article can be found online at <https://www.ncbi.nlm.nih.gov/sra/>, SRR10812151-SRR10812189.

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Authors' contributions

Y.J., and H.X. conceived and designed the experiments. L.S., C.H., Z.P., F.F., J. L., and Y.L. performed the epidemiologic study. H.Y. performed the cell biology experiments. H.Y., N.Q., and Y.G. performed the pathology and immunohistochemistry experiments. Y.H. performed the bioinformatic analysis experiments. Y.J. and Y.H. wrote the manuscript.

Ethics declaration

The study was approved by The Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region ethics committee.

Consent for publication

This manuscript contains no individual person's data in any form and consent for publication is not applicable for this section.

Competing interests

All authors declared no competing interests.

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Tables

Table 1. Clinical and biochemical characteristics of the GDM and NGT groups.

	GDM	NGT	<i>p</i>
	N (%) or mean ± SD	N (%) or mean ± SD	
N	36	40	
Maternal age (years)	32.86±4.20	30.33±3.09	0.004
Ethnicity			0.222
Han	17(47.2)	26(65.0)	
Zhuang	18(50.0)	13(32.5)	
Others	1(2.8)	1(2.5)	
Pre-pregnancy BMI (kg/m²)	24.39±3.39	21.08±3.17	0.001
Overall weight gain (kg)	9.91±5.08	13.77±2.69	0.001
Blood pressure (mmHg)			
SBP	117.94±11.18	113.05±8.52	0.044
DBP	76.19±8.51	70.58±10.01	0.011
Parity			0.365
Primiparous	31(86.1)	37(92.5)	
Multiparous	5(13.9)	3(7.5)	
Mode of delivery			0.812
Vaginal	28(77.8)	32(80.0)	
Caesarean section	8(22.2)	8(20.0)	
24-28 weeks OGTT (mg/dL)			
Fasting glucose	5.05±1.11	4.3±0.44	0.001
1-hour glucose	10.81±1.30	6.99±1.49	0.001
2-hour glucose	9.03±1.78	6.28±1.34	0.001
Maternal serum before delivery:			
OCN (ng/mL)	14.46±10.19	16.01±8.54	0.472
25(OH)D ₃ (ng/mL)	22.74±7.75	19.54±6.80	0.059
CRP (mg/dL)	71.09±42.75	44.44±31.92	0.015
ALP (U/L)	162.97±50.11	158.38±52.26	0.701
Fasting Insulin (μIU/mL)	42.67±79.74	47.03±36.88	0.742

Fasting Glucose (mg/dL)	5.31±2.64	6.35±2.40	0.074
HOMA-IR	16.01±39.70	15.68±16.53	0.962
Umbilical vein serum:			
Parathormone (pg/mL)	6.15±6.74	5.99±2.61	0.891
Insulin (μU/mL)	19.79±32.39	8.14±5.92	0.028
Glucose (mmol/L)	4.60±2.56	6.35±2.40	0.574
OCN (ng/mL)	51.46±24.29	67.00±25.33	0.008
25(OH)D ₃ (ng/mL)	28.79±8.86	25.62±10.11	0.152
ALP (U/L)	156.12±38.89	179.66±52.70	0.031
HDL-C (mmol/L)	0.97±0.24	1.05±0.28	0.200
TC (mmol/L)	1.83±0.61	1.86±0.49	0.836
TG (mmol/L)	0.33±0.14	0.25±0.14	0.095
HCG (mIU/mL)	80.59±58.75	62.21±55.16	0.167
E ₂ (pg/mL)	12863.80±13743.04	10825.14±10183.10	0.462
TRACP-5b (ng/L)	210.85±15.71	240.91±13.30	0.001
LEP (μg/L)	7.41±0.28	6.02±0.31	0.001
ADP (μg/L)	1099.72±102.65	1235.85±94.63	0.001
IL-2 (pg/mL)	0.56±0.52	0.72±0.78	0.601
IL-4 (pg/mL)	4.20±3.19	4.51±3.17	0.601
IL-6 (pg/mL)	7.22±13.34	7.04±16.88	0.960
IL-8 (pg/mL)	10.36±21.78	8.45±12.09	0.634
IL-10 (pg/mL)	6.78±13.41	22.82±66.13	0.157
IL-17 (pg/mL)	1.16±0.71	1.16±0.48	0.997
IFYG (pg/mL)	1.26±1.20	1.55±1.08	0.408
TNF-α (pg/mL)	8.19±2.07	9.29±2.91	0.065

GDM, gestational diabetes mellitus; NGT, normal glucose tolerance; BMI, Body Mass Index; SBP, systolic blood pressure; DBP, diastolic blood pressure; OGTT, oral glucose tolerance test; OCN, osteocalcin; 25(OH)D₃, 1,25-(OH)₂-VitD₃; CRP, C-reactive protein; ALP, alkaline phosphatase; HOMA-IR, homeostasis model assessment of insulin resistance; HDL-C, high density lipoprotein total cholesterol; TC, total cholesterol; TG, triglyceride; HCG, human chorionic gonadotropin; E₂, estradiol; TRACP-5b, tartrate-

resistant acid phosphatase 5b; LEP, leptin; ADP, adiponectin; IL, interleukin; IFY γ , interferon γ ; TNF- α , tumor necrosis factor α .

Table 2. Correlations between osteocalcin and parameters of biochemical index.

	Uv OCN (ng/mL)	
	R	P
Neonatal		
Birth weight (kg)	-0.108	0.382
Birth length (cm)	-0.159	0.195
Chest circumference (cm)	-0.072	0.557
Head circumference (cm)	-0.069	0.574
Umbilical vein serum		
Insulin (μU/mL)	-0.164	0.180
Glucose (mmol/L)	-0.132	0.288
25(OH)D ₃ (ng/mL)	-0.030	0.809
ALP(U/L)	-0.275	0.023
HDL-C (mmol/L)	-0.109	0.377
TC (mmol/L)	-0.126	0.304
TG (mmol/L)	-0.295	0.014
HCG (mIU/mL)	-0.127	0.307
E ₂ (pg/mL)	-0.166	0.177
TRACP-5b (ng/L)	0.168	0.343
LEP (μg/L)	-0.456	0.007
ADP(μg/L)	0.194	0.272
Neonatal peripheral blood glucose (mg/dL)	-0.278	0.026
Inflammatory cytokines		
IL-2 (pg/mL)	-0.186	0.270
IL-4 (pg/mL)	-0.161	0.340
IL-6 (pg/mL)		-0.260
IL-8 (pg/mL)	-0.193	0.253
IL-10 (pg/mL)	-0.173	0.307
IL-17A (pg/mL)	-0.079	0.642
IFY γ (pg/mL)	-0.233	0.165

TNF- α (pg/mL)	0.395	0.016
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Uv, umbilical vein; OCN, osteocalcin; BMI, body mass index; OGTT, oral glucose tolerance test; ALP, alkaline phosphatase; HDL-C, high density lipoprotein total cholesterol; TC, total cholesterol; TG, triglyceride; HCG, human chorionic gonadotropin; E₂, estrogen; TRACP-5b, tartrate-resistant acid phosphatase 5b; LEP, leptin; ADP, adiponectin; CRP, C-reactive protein.

Adjusted for the BMI and age.

Figures

Figure 1

Osteocalcin exerts an autocrine loop in trophoblast. a: OCN umbilical vein serum levels were significantly higher than those in umbilical arterial serum and maternal serum. b: Immunohistochemical staining of placental tissue for OCN and GPRC6A. Seven days after JAR trophoblast cells osteogenic differentiation. c: The expression of OCN, Dmp1, Col1A1 was significantly elevated. d: Alizarin Red S stained. E: Transmission electron microscopic images. OCN improved JAR trophoblast cells (e) proliferation, (f) HCG secretion, (g) ADP, aromatase-related genes, genes encoding inflammatory factors synthesis. GPRC6A gene knockdown by shRNA in JAR trophoblast cells, decrease (h) proliferation, (i) HCG secretion, (j) aromatase-related genes and genes encoding inflammatory factors synthesis. The data are presented

as the mean ± SE of the values obtained in 3 independent experiments. * P < 0.05, ** P < 0.01, ***P < 0.001). OCN, osteocalcin; HCG: Human Chorionic Gonadotropin; ADP, adiponectin.

Figure 2

Module-trait associations. Each row corresponds to a module eigengene, and the columns correspond to clinical indexes. Red indicates a positive correlation, and green indicates a negative correlation. Each cell contains the corresponding correlation and P-value.

Figure 3

Schematic diagram of OCN autocrine of trophoblast in GDM. Lower trophoblasts synthesis OCN contributes to placenta dysfunction in GDM, Lower OCN resulted in a decrease in HCG, In the same way, IL-6, TNF- α , and ADP levels were reduced, but LEP increased.

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