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Serum levels of miR-628-3p and miR-628-5p during the early pregnancy are increased in women who subsequently develop preeclampsia

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ABSTRACT

Objective: Preeclampsia pathogenesis involves imbalances of oxidative stress networks including the heat shock protein (HSP) pathway. Micro-RNAs regulate gene networks associated with preeclampsia. Hsp90 and Runx2 are transcriptional targets of miR-628-3p. Considering that potential participation of hsa-miR-628-3p in PE development is still not elucidated, the aim of this study was to evaluate serum microRNA expression of hsa-miR-628-3p and hsa-miR-628-5p and their association with the preeclampsia development.
Study design: A retrospective nested cohort case-control study was conducted. Serum samples from 16 pregnant women who developed preeclampsia (WWD-PE) during the follow-up period were selected and individually matched to that from 18 women in the cohort who had healthy pregnancies without complications (controls).
Main outcome measures: The levels of hsa-miR-628-3p and hsa-miR-628-5p were measured in serum samples from study groups at 12, 16, and 20 weeks of gestation (WG) using TaqMan probes. Additionally serum levels were measured at the moment of diagnosis, in women with preeclampsia.
Results: Serum levels of hsa-miR-628-3p were higher than controls in WWD-PE at 12 WG (RQ = 7.7; $P = 0.020$), and of hsa-miR-628-5p at 20 WG (RQ = 3.4; $P = 0.008$). An increase in hsa-miR-628-3p serum levels at 12 WG (RQ = 12.01; $P = 0.001$) and of hsa-miR-628-5p at 20 WG (RQ = 2.95; $P = 0.033$) was also observed in women who developed mild preeclampsia, and severe preeclampsia, respectively.
Conclusions: Serum hsa-miR-628-3p and hsa-miR-628-5p were differentially expressed between WWD-PE and controls, suggesting a participation of these miRNAs in the development of preeclampsia. Future studies are needed to validate hsa-miR628-3p and -5p as early predictors of preeclampsia.

1. Introduction

Preeclampsia (PE) is a pregnancy disorder, characterized by a new onset of hypertension (> 140 mmHg systolic or > 90 mmHg diastolic) after 20 weeks of pregnancy and the coexistence of proteinuria or other maternal organ dysfunction [1]. It is a leading cause of maternal and perinatal morbidity and mortality, with a worldwide incidence of 2–10% [2,3]. The pathophysiology of PE involves both maternal and fetal factors [4]. Abnormalities in the development of placental vasculature in early pregnancy, are considered to be a primary cause of placental hypoxia, increased oxidative stress (OS), release of inflammatory factors, endothelial dysfunction, and the subsequent development of clinical symptoms of PE [5,6]. Although in a normal pregnancy there is an increased susceptibility to OS (defined as a disturbance in the pro-oxidant–antioxidant balance in favor of the former) [7,8], in PE, OS increases both in the placenta and the maternal circulation as a result of the increase in free radicals and superoxide ions, generating injury of both placental cells and tissue and in some cases of

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the complete organ [9]. There are several mechanisms through which the cell responds to stress events; among these, reactive oxygen species (ROS)-metabolizing enzymes and heat shock proteins (HSP) [8]. In pregnancies complicated with PE, diminished placental function increases the apoptosis in placenta endothelial cells, where an increase of HSP90 protein levels has been reported when compared to that observed in normotensive controls, reflecting the imbalance of OS status in PE [7]. HSP90, the most abundant chaperone in eukaryotic cells accounting for 1–2% of cell proteins in most tissues, is involved in multiple biological processes such as cell proliferation, differentiation, and apoptosis [7]. Recently, Jieli *et al* provided evidence that HSP90 is a transcriptional target of the micro-RNA (miRNA) hsa-miR-628-3p in the lung cancer cell line A549. In their experiments the authors confirmed that hsa-miR-628-3p promotes apoptosis and inhibits cell migration by negatively regulating HSP90 [8]. Interestingly, *Lian* et al. shown that Runx2 (another target gene of hsa-miR-628-3p [10]) is transcriptionally regulated by HSP90 via the AKT/GSK-3β/β-catenin signaling pathway, and by which leads to apoptosis of osteosarcome cells via a caspase-3-dependent mechanism [11]. During early pregnancy (6–12 WG), the expression of RUNX2, MMP2/-9, GnRH (gonadotropin-releasing hormone), and the GnRH receptor in column extravillous trophoblast (EVT) cells correlate with the highly invasive behavior of these cells during the first trimester of pregnancy. Moreover, the co-expression of RUNX2, MMP2/9 in column EVT cells reflects trophoblast differentiation towards an invasive phenotype [12]. Accordingly, it seems likely that a dysregulation of hsa-miR-628-3p and therefore its targets genes (Runx2 or Hsp90) in early pregnancy, contributes with an abnormal trophoblastic invasion process and/or with increased placental apoptosis, and consequently, with the pathogenesis of PE. Under these premises and considering that potential participation of hsa-miR-628-3p in PE development, whose role has not been elucidated, the aim of this study was to evaluate the association between serum levels of miR-628-3p and -5p, and PE development.

2. Methods

2.1. Patients and biological samples

This was a retrospective nested cohort case-control study. Serum samples were provided by the Bank of Biological Samples of the Molecular Medicine Laboratory of the Academic Unit of Human Medicine and Health Sciences from the Universidad Autonoma de Zacatecas. These samples were obtained from pregnant women (at the 12th, 16th and/or 20th week of gestation (WG), and at the time of PE diagnosis) as part of a screening study for adverse pregnancy outcomes in Zacatecas, Mexico between November 2011 and January 2014. From a total of 588 participants, samples from sixteen pregnant women who developed PE (WWD-PE) during the follow-up period were selected and matched to 18 women in the cohort who had healthy pregnancies without complications (controls). Matching was based on age, nulliparity, body mass index (BMI), and a personal and family history of PE. From these participants, a total of 99 serum samples were included, 24 samples collected at 12 WG (6 from WWD-PE and 18 from controls), 28 at 16 WG (10 from WWD-PE and 18 from controls), 32 at 20 WG (14 from WWD-PE and 18 from controls), and the remaining 15 from the WWD-PE group at the time of PE diagnosis (Fig. 1).

2.2. PE definition

PE was diagnosed according to the guidelines of the International Society for the Study of Hypertension in Pregnancy [13]. Severe PE was considered as that in which the patient had a blood pressure ≥160 mm Hg systolic or ≥110 mm Hg diastolic on two occasions at least 6 h apart while the patient was at bed rest, and a proteinuria of 5 g or more in a 24-h urine specimen, or 3+ or greater in two random urine samples collected at least 4 h apart. PE was considered of early onset if it was present before 34 WG, and late if PE occurred later than 34 + 1 WG [5,14,15].

2.3. Ethical considerations

The protocol complied with the Declaration of Helsinki. The participants provided written informed consent, and Institutional Review Board approvals were obtained from the participating institutions: the Comité de Enseñanza, Investigación, Capacitación y Ética from Hospital de la Mujer Zacatecana (ID numbers: HMZ-520/281/11 and HMZ-5020/318/11) and the Comité de Bioética del Área de Ciencias de la Salud-UAZ (ID numbers: ACS/UAZ.Ofc. Nos. 0072009, 0062010).

2.4. MiRNA isolation and quantification from serum samples

Before miRNA isolation, the serum samples were thawed on ice and centrifuged at 13,300 rpm for 5 min to remove any trace of cell debris or sediment. Total RNA was isolated from 200 µl of serum using the miRNeasy Mini Kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's instructions. The RNA concentration was assessed using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Inc., USA). After RNA quantification, hsa-miR-628-3p and hsa-miR-628-5p expression was evaluated for each of the 45 PE samples, while only 25 serum samples from five controls were individually quantified at 12, 16, and 20 WG. One pooled sample from the 13 additional controls was also included for each time point. The aims of this pooling were to facilitate the technical difficulty in getting sufficient miRNA quantity from a simple patient and defray the cost of the experiments. The pool preparation was cautiously and it involved the use of the same RNA concentration of each subject in qRT-PCR reactions. On the same sense, the pool was prepared in the same manner every time that it was required. Quantification of the serum levels of hsa-miR-628-3p and hsa-miR-628-5p was carried out using Megaplex Pools from the TaqMan Human MicroRNA Array set v2.0 (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The cDNAs for the mature miRNAs were synthesized from 40 to 180 ng of total RNA. To enhance the ability to detect miRNAs with low expression, a 12-cycle pre-amplification round was included. The cDNA synthesis and pre-amplification steps were performed on a Veriti® 96-Well Thermal Cycler (Applied Biosystems). Quantitative real-time polymerase chain (qRT-PCR) reactions were carried out according to the manufacturer's instructions. The qRT-PCR reactions were done on an Applied Biosystems ViiA™ 7 Real-Time PCR System using the default cycling conditions. Quantification cycle (Cq) values were calculated using ViiA™ 7 Software, and the automatic baselines and thresholds were homogenized across multiple runs using Expression Suite software v1.0.3 (Applied Biosystems). The miRNA expression was considered positive if the amplification signal occurred before the 36th Cq.

2.5. Data analysis

Categorical data analysis was carried out using the chi-squared test or Fisher's exact test and continuous variables were compared between the groups using the *t*-test or the Mann-Whitney *U* test, respectively. The relative quantification (RQ) of serum miRNAs was determined using the global normalization method, considering the control group as a reference with a 95% confidence level. During the obtaining of miRNA data, values from individual samples and that obtained from the pool were averaged, and the result was used in the statistical comparisons. Expression Suite software v1.0.3 (Applied Biosystems) was used to calculate delta Cq (ΔCq) values from the data generated.

3. Results

Table 1 shows the general characteristics of the study population. Mean maternal age in the study groups was 23.5 (± 5.1) and 23.4

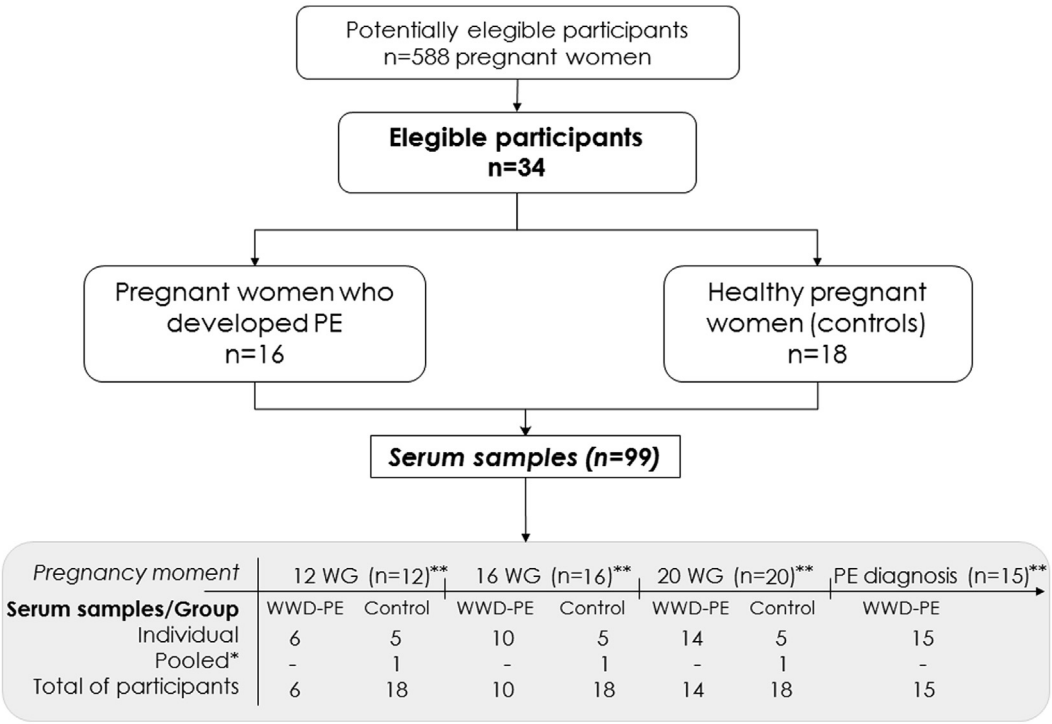

Fig. 1. Summary of the serum samples included in the study. A total of 99 serum samples from 34 participants were considered. In the controls one pooled sample was included for each stage of pregnancy. Each pooled sample (*) was prepared using 20 µl of serum from 13 additional controls. **Number of serum samples included in the qRT-PCR assays.

Table 1
General characteristics of the study population.

Variable	WWD-PE (n = 16)	Control (n = 18)	*P* value
Maternal age (mean ± SD)	23.5 ± 5.1	23.4 ± 5.8	0.913
Body mass index (mean ± SD)	28.1 ± 5.5	27.2 ± 4.9	0.639
Number of pregnancies (median, range)	2 (1–5)	1 (1–4)	0.387
Nulliparous n (%)	6 (37.5)	9 (50.0)	0.699
Personal history of PE n (%)	1 (6.3)	1 (5.5)	1.0
Family history of PE n (%)	1 (6.3)	1 (5.5)	1.0
Family history of hypertension n (%)	13 (81.2)	13 (72.2)	1.0
Family history of Diabetes Mellitus type II n (%)	8 (50)	13 (72.2)	0.176
Smoking during pregnancy n (%)	0 (0.0)	0 (0.0)	1.0
*Systolic blood pressure (mmHg)	102.2 ± 12.3	103.2 ± 10.9	0.841
*Diastolic blood pressure (mmHg)	62.2 ± 14.8	69.1 ± 11.1	0.215
*Glucose (mg/dl)	82.3 ± 6.9	81.1 ± 11.0	0.796
*Eritrocytes ($\times 10^6$/µl)	4.4 ± 0.5	4.7 ± 0.4	0.139
*Hemoglobine (g/dl)	13.0 ± 1.4	14.2 ± 0.8	0.063
*Leucocyte count ($\times 10^3$/µl)	9.2 ± 1.4	9.1 ± 2.1	0.898
*Total Cholesterol (mg/dl)	187.8 ± 57.8	184.6 ± 35	0.869
*High Density Lipoprotein (mg/dl)	67.9 ± 10.8	60.7 ± 10.7	0.142
*Triglycerides (mg/ml)	110.1 ± 18.3	139.4 ± 38.6	0.057
*Urine proteins (mg/dl)	Negative	Negative	–

SD: Standard deviation. *Laboratory data obtained at 16 WG from 10 WWD-PE and 18 controls.

(± 5.8) years for the WWD-PE and the controls, respectively. Previous to PE diagnosis, there were no differences between the study groups in clinical findings such as systolic blood pressure (SBP), diastolic blood pressure (DBP), glucose, hemoglobin, among others ($P > 0.05$). From the PE cases, 25% of patients received a PE diagnosis before 34 WG (early PE), and 31% of the cases were sub-classified as severe PE. At the time of the PE diagnosis, the mean SBP and DBP was 151.3 mmHg and 100 mmHg, respectively. In the PE group, protein values in urine were between 300 and 619.3 mg/dl. As expected, no differences in risk factors between PE cases and controls were observed (Table 1).

The results of the RQ of serum levels of the miRNAs hsa-miR-628-3p and hsa-miR-628-5p at different evaluated WG are shown in Fig. 2. The RQ values for hsa-miR-628-5p were 4.04, 0.563, and 3.45 at 12, 16, and

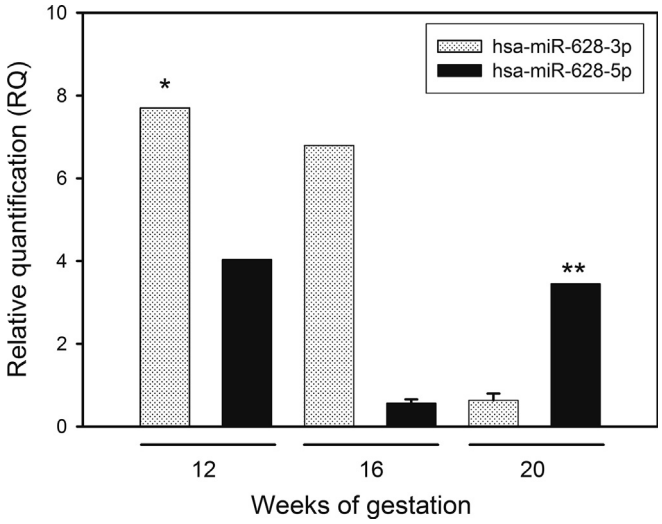

Fig. 2. Relative quantification analysis of serum hsa-miR-628-3p and hsa-miR-628-5p. Hsa-miR-628-3p and hsa-miR-628-5p serum levels were quantified in the study population at 12, 16 and 20 WG. The number of samples from WWD-PE at each pregnancy time were 6 at 12 WG, 10 at 16 GW and 14 at 20 GW, respectively. Delta Cq values from the data generated were used to calculate the relative quantification (RQ) and *P* values considering the control group as a reference. *P*-value from each comparison was calculated using two-tailed Student's *t*-test. *$P = 0.02$. **$P = 0.008$.

20 WG, respectively, whereas that the RQ values observed for hsa-miR-628-3p were 7.70 and 0.637 at 12 and 20 WG. Considering the control group as a reference, there was an increase in the serum levels of hsa-miR-628-3p in WWD-PE at 12 WG ($P = 0.020$). Differences in the levels of serum RQ of hsa-miR-628-5p at 20 WG were also observed between study groups ($P = 0.008$). In controls, serum levels of hsa-miR-628-3p at 16 WG were detected in only one patient (Fig. 2) and therefore the comparison of hsa-miR-628-3p serum levels between WWD-PE and controls was not carried out.

In our study only five WWD-PE donned the complete set of blood samples (12, 16 and 20 WG). To evaluate the effect of the sample size, these five patients were separated and the statistical analysis was newly carried out (data not shown). The significant *P* value was maintained for the hsa-miR-628-3p at 12 WG with slight modifications ($P = 0.02$ *vs* $P = 0.027$). The most highlighted differences were found at 20 GW for the hsa-miR-628-5p because the statistical significance was not observed for WWD-PE ($P = 0.008$ *vs* $P = 1$). On the same sense, at this time, a significant down modulation of miR-568-5p was observed in the controls ($P = 0.035$), reflecting the impact of the sample size (n = 14 WWD-PE in the first analysis *vs* n = 5 WWD-PE in this last analysis) on the statistical findings.

To evaluate differential gene expression of the miRNAs of interest in relation to the severity of the clinical manifestations of disease, the study participants were stratified as women who develop mild PE (WWD-Mild-PE), women who developed severe PE (WWD-Severe-PE), and controls. The results of these comparisons are displayed in Table 2. Considering the control group as a reference, a 12-fold increase in the hsa-miR-628-3p serum levels was observed in the WWD-Mild-PE at 12 GW ($P = 0.001$). Differences between hsa-miR-628-5p serum levels at 20 WG in the WWD-Severe-PE group were also observed (RQ = 2.95; $P = 0.033$). There were no differences in circulating levels of hsa-miR-628-3p and -5p between WWD-Mild-PE and WWD-Severe-PE at the moment of PE diagnosis (Table 2). The comparisons between the miRNAs of interest according to the onset of disease could not be carried out because of the low number of patients diagnosed as early onset PE.

4. Discussion

This study evaluated the association between serum hsa-miR-628-3p and -5p and PE development. The expression level of hsa-miR-628-3p and -5p was quantified at 12, 16, 20 WG, and at the time of PE diagnosis. Our results showed that compared with healthy pregnant women, there was an increment of 7.7 times in the hsa-miR-628-3p serum levels in WWD-PE at 12 WG (first trimester). Differences of hsa-miR-628-5p at 20 WG (second trimester) above three-fold were also observed, being higher in WWD-PE. No studies have carried out a determination of serum hsa-miR-628-3p and/or -5p expression profiles at different time points in early pregnancy; however, even though some previous reports evaluated the utility of serum miRNAs as early markers of PE, alterations in the levels of circulating miR-628 were not reported [16,17].

Is well known that the human miR-628 gene is located on chromosome 15q21.3 (www.genecards.org) [18] nevertheless, the participation of miRNAs 628-3p and -5p in human diseases has remained relatively unexplored (Fig. 3A–D). Hsa-miR-628-3p regulates cell processes by targeting mRNA of Hsp90 and Runx2 [8,10]. According to the negative regulatory effects on HSP90, hsa-miR-628-3p promotes apoptosis and inhibits cell migration in the lung cell line A549 [8]. HSP90 transcriptionally regulates the mRNA of Runx2 via the AKT/GSK-3β/β-catenin signaling pathway, which leads to apoptosis of osteosarcome cells by a caspase-3-dependent mechanism [11]. In human placenta, abundant expression of RUNX2 during early pregnancy (6–12 WG) together with GnRH, the GnRH receptor, and MMP-2/9 in column EVT cells, spatiotemporally correlated with the highly invasive behavior of these cells during the first trimester of pregnancy, and during trophoblast differentiation towards an invasive phenotype [11]. Accordingly, the serum upregulation of hsa-miR-628-3p in early pregnancy of WWD-PE may reflect a negative regulation of mRNA of Hsp90/Runx2 and their low cell levels at 12 WG could contribute to abnormal trophoblastic migration/invasion and/or with a deregulated cell apoptosis process, and therefore, with PE pathogenesis (Fig. 3E). Regarding hsa-miR-628-5p, there are very few reports exploring its functional role. It has been reported as downregulated in prostate cancer [19] and decreased levels of miR-628-5p has also been postulated as a consequence of cell exposure with IL-3, G-CSF (granulocyte colony-stimulating factor), and GM-CSF (granulocyte-macrophage colony-stimulating-factor), in leukemic progenitors might be responsible for promoting increased FOXO3a expression in acute myeloid leukemia [20]. FOXO3a is a predicted target of miR-628-5p and it functions as a trigger for apoptosis through expression of genes necessary for cell death (www.genecards.org) [18]. In our study, an increased serum level of hsa-miR-628-5p at 20 WG preceded the origin of clinical manifestations of PE. Considering the role of cytokines and pro-inflammatory factors such as IL-3, M-CSF, and GM-CSF in PE pathogenesis [21,22], this may reflect of the abnormal systemic response to the abnormal placental implantation process. Additional studies are needed to confirm these hypotheses and to investigate the cell or tissue

Table 2
Relative quantification of serum hsa-miR-628-3p and hsa-miR-628-5p in the study groups, classified as mild or severe PE.

Gestational age	Regulation group	Target	RQ	RQ min	RQ max	*P*-value
12 WG	Mild PE	hsa-miR-628-3p	12.01	6.93	20.79	**0.001**
		hsa-miR-628-5p	–	–	–	–
	Severe PE	hsa-miR-628-3p	3.11	0.053	183.626	0.332
		hsa-miR-628-5p	8.281	0.857	80.032	0.321
16 WG	Mild PE	hsa-miR-628-3p	12.77	7.635	21.365	ND[†]
		hsa-miR-628-5p	1.185	0.354	3.971	0.719
	Severe PE	hsa-miR-628-3p	3.348	0.267	41.915	ND[†]
		hsa-miR-628-5p	0.457	0.167	1.254	0.377
20 WG	Mild PE	hsa-miR-628-3p	0.911	0.506	1.642	0.58
		hsa-miR-628-5p	2.676	0.801	8.937	0.094
	Severe PE	hsa-miR-628-3p	0.742	0.054	10.147	0.64
		hsa-miR-628-5p	2.954	1.507	5.789	**0.033**
PE diagnosis	Severe PE[*]	hsa-miR-628-3p	1.861	1.241	2.79	1
		hsa-miR-628-5p	0.892	0.199	3.988	1

P-values were calculated from the comparison of serum levels of the miRNAs evaluated in the study, considering the control group as reference. [*]PE diagnosis, data were obtained considering the mild PE group as reference. [†]ND: No determined (the miRNA amplification was not observed in the controls). Significant *P*-values are highlighted in bold.

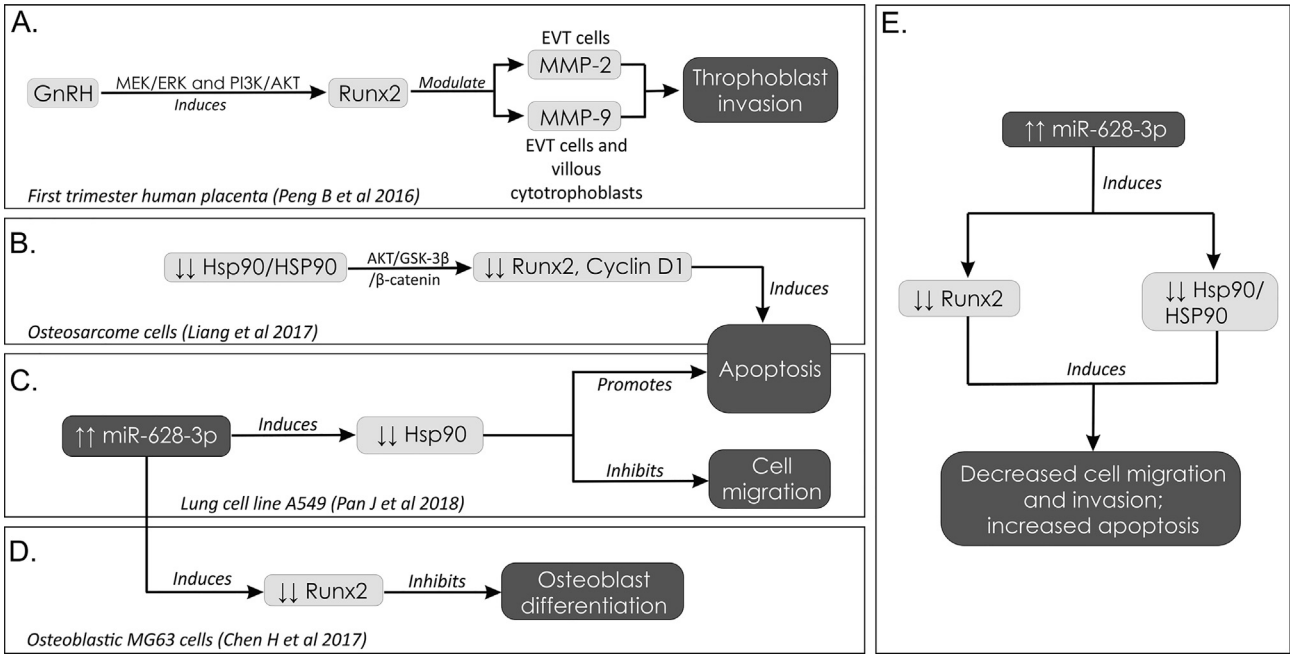

Fig. 3. Modulation of cell function through hsa-miR-628-3p, Runx2, and Hsp90. (A) In human placenta, peak levels of MMP-2 and -9 are detected at 6–12 WG, paralleling the high expression of GnRH and its receptor in first trimester. Abundant expression of GnRH/GnRH receptor, RUNX2 and MMP2/9 in column EVT cells is spatiotemporally correlated with the highly invasive behavior of these cells during the first trimester of pregnancy. Co-expression of RUNX2, MMP2/9 in column EVT cells reflects trophoblast differentiation towards an invasive phenotype [12]. (B) The mRNA and protein expression of Runx2 is suppressed by the inhibition of HSP90 (or HSP90 knockdown) through the AKT/GSK-3β/β-catenin signaling pathway, which leads to apoptosis of osteosarcome cells via a caspase-3-dependent mechanism [11]. (C–D) hsa-miR-628-3p negatively regulates both Hsp90 (cell line A549) and Runx2 (osteoblastic MG63 cells), promoting apoptosis and inhibiting cell migration by negatively regulating HSP90 (C) [8] and inhibiting osteoblast cell differentiation by targeting Runx2 (D) [10]. (E) Increased circulating levels of hsa-miR-628-3p in early pregnancy may reflect an abnormal regulation of cell migration, invasion, and apoptosis, through Runx2 and/or Hsp90 down regulation in placenta and/or other tissues.

origin of hsa-miR-628-3p and -5p.

In our study, differences between the circulating levels of hsa-miR-628-3p and/or -5p obtained with and without PE classification at 12 and 20 WG were identified. However considering the low number of participants in each subgroup after sub-stratification (WWD-Mild-PE/WWD-Severe-PE), these associations between the miRNAs evaluated and severity criteria of disease should be considered cautiously. In the same sense, in our study, there was a low number of participants with a diagnosis of early PE and therefore the modulation of hsa-miR-628-3p and/or -5p considering PE temporality criteria could not be carried out, with this being a study limitation.

Although future studies should be performed to increase the number of participants to validate and postulate hsa-miR628-3p and -5p as early predictors of PE, our results provide molecular basis for the understanding of PE pathogenesis.

5. Conclusion

Serum hsa-miR-628-3p and hsa-miR-628-5p were differentially expressed between WWD-PE and controls suggesting a participation of these miRNAs in PE development. Future studies are needed to validate hsa-miR628-3p and -5p as early predictors of PE.

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Declaration of interest statement

The authors report no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.preghy.2019.03.012.

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