Deregulation of miR-324/Kiss1/kisspeptin in early ectopic pregnancy: Mechanistic findings with clinical and diagnostic implications

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Abstract

Background: Ectopic pregnancy (EP) is a life-threatening condition, for which novel screening tools enabling early accurate diagnosis would improve clinical outcomes. Kisspeptins, encoded by *KISS1*, play an essential role in human reproduction, at least partially by regulating placental function and possibly embryo implantation. Kisspeptin levels are massively elevated in normal pregnancy and reportedly altered in various gestational pathologies. Yet, the pathophysiological role of *KISS1*/kisspeptin in EP has not been investigated previously.

Methods: Measurements of plasma kisspeptins and *KISS1* expression analyses in human embryonic/placental tissue were conducted in EP and their controls (women undergoing voluntary termination of pregnancy, VTOP) during early gestational window (<12-wks). Putative miRNA regulators of *KISS1* were predicted *in silico*, followed by expression analyses of selected miRNAs and validation of repressive interactions *in vitro*. Circulating levels of these miRNAs were also assayed in EP vs. VTOP.

Results: Circulating kisspeptins gradually increased during the first trimester of normal pregnancy, but were massively reduced in EP. This profile correlated with expression levels of *KISS1* in human embryonic/placental tissue, which increased in VTOP but remained suppressed in EP. Bioinformatic predictions and expression analyses identified miR-27b-3p and miR-324-3p as putative repressors of *KISS1* in human embryonic/placental tissue at <12-wks gestation, when expression of both miRNAs was low in VTOP controls, but significantly increased in EP. Yet, a significant repressive interaction was documented only for miR-324-3p, occurring at the predicted 3'-UTR of *KISS1*. Interestingly, circulating levels of miR-324-3p, but not of miR-27b-3p, were dramatically suppressed in EP, despite elevated tissue expression of the pre-miRNA, suggesting defective export in EP. A decision-tree model using kisspeptin and miR-324-3p levels was successful in discriminating EP vs. VTOP, with a receiver-operating characteristic (ROC) AUC of 0.95 ± 0.02 (95% CI).

Conclusions: Our results document a massive down-regulation of *KISS1*/kisspeptins in early stages of EP, likely via a repressive interaction with miR-324-3p. Our data identify circulating kisspeptins and miR-324-3p as novel biomarkers for accurate for screening of EP at early gestational ages.

Introduction

Ectopic pregnancy (EP) is defined as the implantation and development of a fertilized ovum elsewhere than in the uterine cavity. The fallopian tube is the most common site for ectopic implantation (1), which otherwise might occur also in the cervix, ovary, abdomen or even a scar of a previous caesarean section. Approximately, 1.5-2% of all reported pregnancies are extra-uterine (2). Despite substantial improvements in its timely management, EP remains the early pregnancy complication with the highest morbidity and mortality rates (2). EP can result in tubal rupture if not managed promptly, thereby compromising woman's health and future fertility (3). Although the overall incidence of EP increased by six-fold between the 1970's and 90's, its associated mortality has substantially fallen, largely due to better diagnosis and treatment before rupture (2). Nonetheless, EP still represents 9-13% of all pregnancy-related deaths in developed countries and is responsible for almost 75% deaths of the first trimester. The situation is much worse in developing countries; for instance, in Africa, women with EP encounter high fatality rates due to late diagnosis, where it accounts for up to 30% of pregnancy-associated deaths (4). Thus, there is an important clinical need to improve our understanding of the pathophysiological basis of EP and to develop better tools for accurate early screening and diagnosis of this condition.

It is assumed that EP is multifactorial in origin, with several contributing factors, including morphological or functional alterations of fallopian tube permeability, perturbed chemotactic tubal environment, and/or deregulated tubal motility; the major risks factors for EP being a history of tubal surgery and/or extra-uterine pregnancy (3). However, in more than half of cases of EP, no risk factor can be identified, suggesting the existence of additional, as yet unknown underlying mechanisms. Current diagnosis of EP is based mainly on transvaginal ultrasonography, together with serial measurements of the β-subunit of human chorionic gonadotropin (β-hCG) (5), although laparoscopy is often needed as method of certainty. Indeed, whilst ultrasound and β-hCG tests are extremely powerful for early detection of pregnancy, and identification of some of its eventual complications, their sensitivity and specificity substantially decrease in the case of pregnancies of unknown location (3), in which false positive or negative diagnosis may occur. In this context, it is especially crucial to avoid erroneous diagnosis of a pregnancy as non-viable, since it may lead to medical or surgical interventions that could eliminate or severely damage a healthy pregnancy (5). Accordingly, different biomarkers have been proposed in order to increase accuracy of EP diagnosis, which would allow a rapid triage of pregnancies at risk of extra-uterine location. These putative markers may include circulating levels of progesterone, estradiol, vascular-endothelial

growth factor-A (VEGF-A), inhibin-A and activin-A (6-11). Yet, despite promising results (11), there is little pathogenic information emerging from these studies, and no diagnostic test based on these markers is currently available in clinical practice for accurate biochemical identification of EP.

Kisspeptins, the products of the *KISS1* gene, have emerged in the last decade as master regulators of reproductive function in multiple species, including humans (12), as illustrated by the fact that patients with inactivating mutations of *KISS1* or the cognate kisspeptin receptor, *GPR54* (aka, *KISS1R*) suffer severe hypogonadism of central origin (13-15). Despite the overwhelming evidence supporting a major function of brain (hypothalamic) kisspeptin signaling in the control of reproduction, mainly via its capacity to stimulate gonadotropin-releasing hormone (GnRH) neurons (16), additional functions of kisspeptins have been reported at peripheral levels of the reproductive axis, including the ovary and the uterus (17, 18). On the latter, mounting evidence has demonstrated that the elements of Kiss1 system are expressed in human endometrium and placenta (18), and kisspeptins have been proposed to play an important role in endometrial gland development and function (19), as well as in human placentation (17, 18). Notably, expression and functional analyses have suggested that kisspeptin signaling is an important (negative) regulator of human trophoblast migration and invasion (20), as well as a (positive) regulator of embryo implantation (18). Altogether, these findings attest a relevant function of kisspeptins in human placentation, which is further suggested by the fact that circulating levels of kisspeptins dramatically increase during gestation (21), with abundant expression of *KISS1* in human trophoblast, especially in the first trimester (20). Accordingly, inappropriately low kisspeptin levels have been proposed as biomarker of gestational alterations, such as intrauterine growth restriction and preeclampsia (22, 23), and putative predictor of miscarriage risk (24). In fact, a very recent study comparing a well-defined, as yet limited (N=20), population of women with viable intrauterine pregnancy vs. women with confirmed spontaneous abortion suggested that kisspeptin levels during an early gestational window (weeks 6-10) might serve as good biomarker of pregnancy viability (25). However, to our knowledge, no single study has addressed potential dynamic changes in *KISS1*/kisspeptin in EP, and their eventual diagnostic utility in this condition.

The molecular mechanisms underlying EP remain largely unknown. Interestingly, recent studies have highlighted the potential contribution of deregulated microRNAs (miRNAs) as putative pathogenic and/or diagnostic factors in EP. MiRNAs are small, non-coding RNAs that can modulate (mainly repress) gene/protein expression, primarily via interaction with seed regions at the 3' untranslated region (3'-UTR) of target genes, thereby promoting mRNA degradation or

preventing protein translation (26). Deregulation of miRNA expression in embryonic/ placental tissues and the fallopian tube has been reported in EP (27-29). These studies have surfaced changes in the expression patterns of miRNAs of the let7 family, as well as mir-132, miR-145, miR149, miR-182, miR-196, miR-223, and miR-424 in ectopic implantation; yet, the putative mechanistic implications for these alterations are yet to be elucidated. In addition, changes in the circulating levels of some miRNAs have been associated to EP (30). Thus, miR-323-3p was reportedly increased in women with EP in one study (31), whereas additional studies have shown that circulating levels of miR-515-3p, miR-517a, miR-518b, miR-519d, miR-525-3p and miR-873 are significantly lower in patients with ectopic implantation (31-33). Considering that miRNAs in blood are in general stable and relatively easy to detect (30), these data hold promise for the identification of a novel class of non-invasive biomarkers of EP. Yet, to date, no single diagnostic test based on the determination of miRNAs (alone or in combination) has been validated as reliable biomarker of EP. Moreover, despite recent evidence on the epigenetic control of hypothalamic *Kiss1* in rodents (34), to our knowledge, no single report has documented the existence of miRNA-mediated regulation of human *KISS1*.

In this context, the aims of our work were: (i) to evaluate potential changes in *KISS1* mRNA expression (at embryonic/placental tissue) and circulating kisspeptin levels in EP; and (*ii*) to highlight eventual regulatory mechanisms of *KISS1* expression in ectopic gestation, involving miRNAs. We have particularly targeted an early gestational window (<12-weeks), which is a clinically relevant time-frame for screening of patients for EP. Our data are the first to conclusively demonstrate a massive suppression of *KISS1*/kisspeptin levels in EP, which is linked to overt deregulation of its putative repressor, miR-324; a finding with potential mechanistic and clinical/ diagnostic implications.

Results

Embryonic/placental tissue expression of KISS1 and circulating levels of Kisspeptins in EP

Dynamic profiles of embryonic/placental *KISS1* expression and circulating levels of kisspeptins were compared between women with normal gestation undergoing voluntary termination of pregnancy (VTOP; taken as control pregnancies) and women suffering EP. For clinical reasons, we focused our analyses in a gestational window up to 12-weeks. Yet, for reference purposes, in the control group, additional samples from viable pregnancies up to 20 weeks of gestation were also included. Due to the limited number of samples available at early gestational ages, and in order to increase statistical power, samples from 4-, 5- and 6-weeks of gestation were grouped for analysis, as ≤6-week sample. Likewise, in some analyses, data from VTOP samples at the intervals 7-9, 10-12, 13-15 and 16-17 weeks of gestation were pooled together. For the same reasons, samples from control pregnancies at or beyond week-18 of gestation were grouped as 18-20 week samples.

Analysis of *KISS1* gene expression in embryonic/placental tissue from women undergoing VTOP revealed negligible expression in ≤6-week samples, with a massive increase thereafter, which reached a maximum >10,000-fold rise at week-12 of gestation. This was followed by a consistent, gradual decline in relative *KISS1* levels from gestational week-13 onwards, with relative expression levels that had returned to ≤6-week values in the ≥18-week sample (**Fig. 1A**). Circulating levels of kisspeptins followed a grossly similar profile during early gestation, with an ascending curve in the initial weeks of normal pregnancy, and concentrations that increased gradually up to gestational week-15; yet, they remained at a plateau state thereafter (**Fig. 1B**).

Similar analyses of *KISS1* expression and circulating kisspeptins were conducted in samples from women with EP, collected up to gestational week-12. When calculated as mean values during the whole study period (<12-week), both embryonic/placental *KISS1* gene expression and circulating kisspeptin levels were massively suppressed in women with EP vs. VTOP (**Fig. 1C**-**D**). To precisely monitor the timing of such suppression, data analysis was split in ≤6-, 7-, 8- and ≥9-week of gestation. In terms of *KISS1* mRNA in embryonic/placental tissue, expression levels were equally negligible in the ≤6-week groups from VTOP and EP; yet, from week-7 onward, control pregnancies showed higher *KISS1* mRNA levels than EP, although differences were in the limit of statistical significance up to week-9 of pregnancy (**Fig. 1E**). Circulating levels of kisspeptins followed globally similar profiles, although in this case, plasma concentrations of

kisspeptins were significantly lower in EP groups all through the study period, from ≤6-week until week-12 of pregnancy (**Fig. 1F**).

Embryonic/placental tissue expression of miR-324-3p and miR-27b-3p in EP

In an attempt to discover putative regulators responsible for the suppression of *KISS1* expression in EP, bioinformatics was applied to identify potential miRNA regulators, by identification of their corresponding seed regions in the 3'-UTR (or eventually 5'-UTR) of the *KISS1* gene. By searching different databases and implementing appropriate tools, three major putative candidates were identified: miR-137-3p and miR-324-3p, for which 8 and 7 nucleotides, respectively, from their predicted seed regions were found in the 3'-UTR of *KISS1*, and miR-27b-3p, with 7 nucleotides of its seed region complementary to *KISS1* promoter sequence (**Suppl. Fig. S1**). Expression analyses were subsequently applied to evaluate the levels of these miRNAs at early stages of gestation. These analyses documented that while miR-324-3p and miR-27b-3p are readily detectable in human embryonic/placental tissue during the first trimester of gestation, miR-137 is not, therefore suggesting negligible expression of this miRNA, which was excluded from further analyses.

Detailed expression analyses in embryonic/placental tissue from control pregnancies during the first 20-weeks of gestation demonstrated roughly similar expression profiles for both miR-324-3p and miR-27b-3p, with rather low relative expression levels during early stages of gestation, and sharp increase after week-10 (miR-324-3p) or week 13 (miR-27b-3p) of pregnancy (**Fig. 2A**-**B**). Similar analyses revealed that the expression levels of both miRNAs are significantly elevated in embryonic/placental tissue from EP that, when calculated as mean values during the whole study period (<12-week), represented a 4-fold (miR-324-3p) and 3-fold (miR-27b-3p) increase vs. VTOP levels (**Fig. 2C**-**D**). Timed analysis of such differences revealed that elevated expression of both miRNAs mainly concentrated at early stages, up to week-8 of gestation, and were already significant at the <6-week group (**Fig. 2E**-**F**).

Regulation of KISS1 Expression by miR-324-3p and miR-27b-3p

Based on bioinformatic predictions on the location of seed regions of miR324-3p and miR27b-3p at the 3'- and 5'-UTR of the *KISS1* gene, respectively, and the reciprocal changes in expression levels of these miRNAs and their putative target (*KISS1*/kisspeptins) in EP, we sought to demonstrate whether a direct repressive interaction actually exists, using proper luciferase reporter assays *in vitro*. For testing miR-324-3p/*KISS1* interactions, HEK-293T cells were co-transfected with Gluc-*KISS1*-3'UTR and miR-324-3p reporter plasmids; the former harboring the

KISS1 3'-UTR containing the putative miR324-3p seed region downstream the coding sequence of luciferase. As shown in **Fig. 3A**, over-expression of miR324-3p induced a significant 30% drop in luciferase activity, therefore demonstrating a direct interaction and negative post-transcriptional regulation of *KISS1* gene by miR-324-3p at its 3'-UTR. In contrast, given the predicted location of the seed region of miR-27b-3p at the 5'-UTR of *KISS1*, a reporter construct harboring the human *KISS1* promoter region upstream the luciferase coding sequence was used. HEK-293T cells were co-transfected with *KISS1*-promoter-GLuc and miR-27b-3p expression vectors. However, as shown in **Fig. 3B**, no significant changes in luciferase activity were found after miR-27b-3p over-expression in this heterologous cell system.

Circulating levels of miR-324-3p and miR-27b-3p in EP

Collectively, our results suggested that miR-324-3p and miR-27b-3p are deregulated in EP, and these changes (especially for miR-324-3p) may be mechanistically relevant for the observed suppression of *KISS1*/kisspeptin in ectopic gestations. To ascertain whether tissue alterations translate into detectable changes in the circulating levels of these miRNA, qPCR analyses were applied to plasma samples from VTOP and EP during the early gestational window. Circulating levels of miR-324-3p and miR-27b-3p were detectable in control (VTOP) pregnant women all through the study period, up to week-20 of gestation, with rather stable values (**Fig. 4A-B**); only a marginal increase in miR-27b-3p levels was detected in 13- to 15-week samples (**Fig. 4B**).

Integral plasma levels of miR-324-3p, calculated as mean values during the <12-week period, were significantly suppressed in EP vs. corresponding VTOP levels, with a marked 80% drop in mean concentrations (**Fig. 4C**). This substantial drop is in contrast with the observed increase in tissue levels of miR324-3p in EP, which are clearly elevated. To further document this discrepancy, the expression levels of the precursor, pre-miR324-3p, were assayed in <12-week samples from VTOP and EP. In line with the expression profile of mature miRNA, pre-miR324-3p levels in EP were significantly increased (**Suppl. Fig. S2**), therefore confirming the divergence between tissue expression and circulating levels of this miRNA. Opposite to miR-324-3p, mean plasma levels of miR-27b-3p were moderately increased in EP during the <12-week period (**Fig. 4D**), which parallels the expression data of the mature miRNA.

Timed analysis of the above changes revealed that circulating levels of miR-324-3p are consistently suppressed in EP all through the early gestational window (grouped at ≤6-, 7-, 8- and ≥9-week samples); yet, changes were in the limit of statistical significance at week-7 (**Fig. 4E**). In

clear contrast, no significant changes in the plasma levels of miR-27b-3p were detected between control and EP, except for a transient, modest increase in EP at gestational week-8 (**Fig. 4F**).

Circulating kisspeptin/miR-324-3p as new early biomarker of EP

Based on the above data, biostatistics tools were used to study the usefulness of circulating kisspeptins and miR-324-3p as early diagnostic biomarkers of EP, measured alone or in combination. For validation purposes, β-hCG levels, as gold-standard for biochemical diagnosis of EP, were also measured in a representative set of VTOP and EP patients of our cohort. As shown in **Suppl. Fig. S3**, β-hCG concentrations were markedly suppressed in EP vs. VTOP, when calculated as mean levels over the <12-week gestational period; a profile that resembles that of kisspeptins and miR-324-3p. Time analysis at the ≤6-, 7-, 8- and ≥9-week revealed similar trends, with a consistent suppression of β-hCG concentrations in EP patients, at all time-points studied.

Logistic regressions were applied to evaluate the prognostic power of kisspeptin, miR-324-3p, β-hCG, and their combinations in predicting EP, during the first 12 weeks of gestation. Logistic regression models were built using the R package caret and validated with repeated 10-fold cross-validation. Prediction scores demonstrated the capacity of kisspeptins and miR-324-3p to correctly discriminate between EP and VTOP groups. The overall predictive power of the models is shown in **Fig. 5A**, where ROC curves with prediction scores are presented (95% CI). Interestingly, the predictive power of the combination of kisspeptins/miR-324-3p was similar to that of β-hCG, and aggregation of miR-324-3p and β-hCG yielded ROC values close to the unit.

Based on these findings, a decision-tree model using miR-324-3p and kisspeptin levels was constructed using the J48 method implemented in the R package caret and validated with repeated 10-fold cross-validation (0.91 ± 0.01 AUC, 95% CI). In this model, presented in **Fig. 5B**, the first node is based on kisspeptin levels (≤228.7 pg/mL, n = 66, or ≥228.7 pg/mL, n = 43), the second node is based on miR-324-3p levels (≤98.69 counts, n = 35, or ≥98.59 counts, n = 31), and the third node is based on kisspeptin levels (≤85.75 pg/mL, n = 18, or ≤85.75 pg/mL, n = 13). In addition, using a similar approach, a decision-tree model incorporating also β-hCG levels was built; this is shown in **Suppl. Fig. S4**. In this model, the first node is based on β-hCG levels (threshold: 15,424 pg/mL), with a second level of discrimination for values below hCG threshold based on kisspeptin levels (threshold: 196.91 pg/mL), and a third level based on miR-324-3p (threshold: 43.46 counts). According to this model, for β-hCG levels over the threshold, discrimination is based on kisspeptin levels, with a nodal point at 85.75 pg/mL.

Discussion

Ectopic pregnancy affects up to 2% of all reported gestations and is endowed with considerable morbidity and even mortality, especially in developing countries (2, 4). The pathogenic basis of EP is not well understood. While current strategies for detection of EP, involving transvaginal ultrasonography and serial determinations of β-hCG, have proven reliable, false positives and negatives still occur. For instance, in nearly 20% of cases, EP patients display hCG profiles similar to those of intrauterine pregnancy, while in 10% of cases, hCG levels resemble those of early miscarriage (3). This has prompted the search for novel markers of EP, which may improve the sensitivity and specificity of current protocols for early detection of extra-uterine gestations. Yet, despite some promising findings (11), no alternative methods for biochemical triage of women at risk of EP at early stages of gestation are routinely in use in clinical practice. Likewise, the search for such novel biomarkers has not substantially expanded our understanding of the pathogenic mechanisms underlying ectopic placentation, which might help to define additional risk factors.

Human placentation is an intricate process of fetal-maternal interaction that shares some similarities with cancer migration and metastasis (35). In this context, kisspeptins, initially identified as potential metastasis-suppressing factors abundantly expressed in the placenta, were suspected as important players for the fine control of trophoblast invasion and placentation (17, 18). This *placental* dimension of kisspeptins was further attested by the proven elevation of kisspeptin levels already at early stages of human gestation (21). Accordingly, a number of gestational conditions putatively associated with abnormal placentation, ranging from intrauterine growth restriction to preeclampsia, have been associated with alterations in circulating kisspeptin levels (22, 23). Our current data unambiguously document for the first time that circulating kisspeptin levels are markedly decreased during the first trimester of gestation in EP, with a significant drop being already detectable at very early stages (≤6-weeks). This profile grossly correlated with the expression levels of *KISS1* in embryonic/placental tissue, in keeping with a potential placental source. Importantly, very few studies to date have correlated the expression profiles of *KISS1* and plasma kisspeptin concentrations during the early gestational period, and disparities between tissue expression and circulating levels have been described at later gestational ages in some pathologies, such as pre-eclampsia (36). In our study, the observed drop in *KISS1* expression might have an impact on the whole process of trophoblast invasion, which is inhibited by locally produced kisspeptins (20, 37), and might facilitate nidation at an ectopic site. Interestingly,

kisspeptins have been suggested also to facilitate initial embryo adhesion/implantation at proper endometrial sites (18, 38), while high tubal expression of Kiss1/kisspeptin has been proposed as mechanism to prevent ectopic implantation in rats (39). Whether early deregulation of kisspeptin production might facilitate adherence at extra-uterine sites in human pregnancy is yet to be elucidated.

All studies reported to date demonstrating alterations in kisspeptin levels or *KISS1* expression were associative in nature, and therefore did not provide mechanistic information for the observed changes. In contrast, our analyses also intended to identify potential mechanisms for deregulated *KISS1*/kisspeptin expression in EP. We particularly focused on the role of putative miRNA regulators of *KISS1* that may contribute to these changes. Our rationale was double: (a) deregulated expression of some miRNAs has been linked to EP (27-29, 31, 32); and (b) miRNAs have a potential diagnostic dimension, as they are amenable for detection in blood samples (30). To our knowledge, our study is the first to address the potential direct regulation of *KISS1* by miRNAs in a physiological setting. Notably, despite recent evidence for epigenetic modulation of the Kiss1 system in the brain, mainly in the context of pubertal maturation (40), the potential contribution of miRNA pathways in this phenomenon has not been documented to date. Based on a combination of bioinformatic and expression analyses, we identified two potential miRNA regulators of placental *KISS1* expression, namely miR-27b-3p and miR-324-3p, as they were predicted to have conserved seed regions at the UTRs of the *KISS1* gene and displayed detectable expression in embryonic/ placental tissue during early gestational periods. Furthermore, opposite to *KISS1* and kisspeptin levels, relative expression of both miRNAs was globally increased in EP vs. control pregnancies during this period, which is compatible with the predicted repressive action of these miRNAs on the expression/translation of its target gene, *KISS1*.

This putative regulatory role was further interrogated *in vitro*, using appropriate reporter assays. Of note, while most miRNA-target repressive interactions take place at the 3'-UTR (26), miRNA-binding sites have been identified also at the promoter region of certain genes, which may drive suppressive or stimulatory effects upon gene transcription (41, 42). This appeared to be the case for *KISS1*, for which a conserved seed region for miR-27b-3p was found in its 5'-UTR. However, luciferase promoter assays in HEK-293 cells, using a *KISS1*-promoter vector encompassing this site, failed to detect any significant impact of miR-27b-3p on *KISS1* promoter activity, at least in our heterologous system. In clear contrast, over-expression of miR-324-3p in HEK-293 cells was capable to repress *KISS1* expression, as denoted by decreased luciferase

activity using an expression vector including the 3'-UTR of *KISS1*. Giving the heterologous nature of the assay, it remains possible that the net magnitude of the observed changes might not be indicative of the real extent of this repressive interaction *in vivo*. Yet, our data unambiguously demonstrate for the first time the capacity of miR-324-3p to suppress *KISS1*, thus reinforcing the plausibility that the observed increase in miR-324-3p expression in ectopic embryonic/placental tissue is responsible for reduced expression of *KISS1*/kisspeptin levels in EP. Moreover, our findings collectively suggest that reduced kisspeptin levels in ectopic pregnancies are not merely due to defective placentation, but rather the consequence of an orchestrated deregulation of a miR-324-3p/*KISS1* pathway in ectopic gestational tissue.

To explore the potential diagnostic value of altered expression of miR-324-3p and miR-27b-3p in EP, we measured also the circulating levels of both miRNAs. Surprisingly, while only a rather modest increase in circulating miR-27b-3p was detected in EP, which was significant only at week-8 of pregnancy, the circulating levels of miR-324-3p were massively suppressed in ectopic gestations, with a significant reduction being already detectable at very early (<6-week) stages. The fact that not only the levels of this mature miRNA but also of its pre-miRNA were significantly increased in ectopic tissue suggests that the drop in circulating miR-324-3p concentrations in EP might be due to defective export of this miRNA from its embryonic/placental source, which may lead to accumulation of miR-324-3p; a phenomenon that may further contribute, via repressive interaction with *KISS1*, to the reduction in circulating kisspeptins in EP. In any event, from a diagnostic standpoint, the concomitant marked suppression of kisspeptin and miR-324-3p levels offers diagnostic possibilities, which were explored in our study. Thus, a biostatistical algorithm based on plasma levels of both factors allowed to construct a decision-tree model that was successful in discriminating EP vs. control pregnancy, with ROC AUC values that were roughly analogous to those offered by determination of β-hCG alone. Moreover, combination of β-hCG with these novel factors, especially miR-324-3p, permitted to further increase the discriminating power, with ROC data close to one and the added value that incorporation of complementary markers, such as kisspeptins and miR-324-3p, should permit to nullify the risk of false positives or negatives. All these features reinforce the potential of this method for biochemical triage of pregnancies at risk of ectopic gestation.

In summary, we provide herein conclusive evidence for a novel miR-324-3p/KISS1 regulatory pathway, which is altered and may have pathophysiological implications during early ectopic pregnancy. Our data are also endowed with a promising diagnostic dimension, as

determination of kisspeptins and/or miR-324-3p, alone or in combination with β-hCG, may allow improvement of current methods for non-invasive identification of EP, with superior sensitivity and specificity.

Methods

Ethical Approval

The present study was approved by the Institutional Review Board/Independent Ethics Committee of the Hospital Universitario La Fe, Valencia, Spain. Early embryonic tissue (mostly trophoblast) was collected after obtaining the corresponding informed consent from each patient, as described elsewhere (28, 29).

Sample collection

A total of 108 women with a normal ongoing pregnancy that desired a voluntary termination of pregnancy (VTOP) and 45 patients suffering from tubal ectopic pregnancy were recruited, following previous described criteria (28, 29). All individuals (EP and VTOP) included in this study signed an informed consent, and samples were properly dated according to the last menstrual period (week of pregnancy). The diagnosis of EP was based on clinical and physical examination, transvaginal ultrasound, and serial quantitative β-hCG levels, and confirmed by laparoscopy in which the tissue was removed, as described previously (28, 29). The EP patients did not receive methotrexate treatment, and laparoscopy was performed as follows: ectopic pregnancies selected for this study were un-ruptured gestations located in the isthmus or the proximal ampulla. The tube containing the ectopic pregnancy was grasped at both sides (approximately 1 cm away from the gestation site) and bipolar coagulation applied. Similarly, the adjacent mesentery was also coagulated. Then, salpingectomy was performed employing scissors; a longitudinal anti-mesenteric incision into the surface of the tube was made and mild pressure applied with two fingers to extract the gestational sac. Embryonic tissue was carefully separated from obvious blood clots or tubal tissue in the operating room under a stereomicroscope and was immediately placed in TRIzol reagent (*see below*), frozen and stored at -80ºC until use. A piece of each sample was sent to the Pathology Department (Hospital Universitario La Fe, Valencia), in order to provided histological confirmation of ectopic pregnancy and the absence of tubal tissue. The fetal dilation and evacuation method or fetal aspiration technique was performed in VTOP women to obtain the embryonic tissue.

RNA Extraction and Quantitative PCR

Total RNA was isolated from human embryonic samples from different stages of pregnancy (both control and ectopic gestations), using TRIzol reagent (Invitrogen, CA), following the manufacturer's protocol. The quality and concentration of the isolated RNA were determined by

spectrophotometry, following standard procedures. Real-time qPCR was performed on the samples using a Bio-Rad SFX 96 Real-Time System (Bio-Rad Laboratories, Hercules, CA), as described in detail elsewhere (43). For quantification of *KISS1* mRNA in embryonic samples, 1 µg of total RNA per tissue sample was treated with RQ1 RNAse-free DNAse-I (Promega, Madison, WY) and retro-transcribed (RT) in a 30 µl reaction using iScript™ Reverse Transcription Super-mix (Bio-Rad Laboratories). For real-time PCR amplification, we used SYBR Green qPCR Master Mix (Promega), with the following primer sequences: hKISS1-forward: 5′-AGC AGC TAG AAT CCC TGG G-3′, position 10698–10716 nt; hKISS1-reverse: 5′-AGG CCG AAG GAG TTC CAG T-3′, position 10947–10929 nt. The primer pair: hL19-forward: 5′-GAA ATC GCC AAT GCC AAC TC-3′ and hL19-reverse: 5′-ACC TTC AGG TAC AGG CTG TG-3′ was used for amplification of a 290-bp fragment of the mRNA of ribosomal L19 protein, which served as internal control for reaction efficiency and sample loading. PCR was initiated by one cycle of 95°C for 2-minutes, followed by 35 cycles of 15-seconds at 95°C, 30-seconds at 62°C, and 10-seconds at 72°C, followed by one final cycle of 72°C for 1-minute. Relative *KISS1* mRNA levels were normalized against the expression levels of L19 transcript.

For miRNA analyses, quantification of the expression levels of mir-324-3p and mir-27b-3p was performed according to "*miRCURY LNATM Universal RT microRNA PCR individual assay - Instruction manual v6.2*", as instructed by Exiqon. All miRNAs were reverse transcribed into cDNA in a single reaction step. The cDNA synthesis control (UniSp6) was added in the reverse transcription reaction giving the opportunity to evaluate the RT reaction. cDNA and SYBR Green qPCR Master Mix (Promega) were transferred to the qPCR plate with specifics primers (Exiqon). The average data obtained from plasma of control pregnancies was used as normalization reference and miR-191-5p was included as housekeeper in qPCR assays for each sample.

Bioinformatic analysis

Computational miRNA target prediction algorithms were applied to propose putative interactions between the UTR of *KISS1* and validated human miRNAs in available databases. Algorithms for predicting miRNAs which can putatively regulate *KISS1* were applied to the 3'-UTR and 5' promoter regions, with the following inclusion criteria: (a) miRNAs predicted for interaction at the 3'-UTR had to be identified at least in two of the following databases: http://www.targetscan.org/; http:// www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/; http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/; or http://www.microrna.org/microrna/home/; (b) miRNAs predicted for interaction at the promotor region had to be identified using the database http://mirwalk.umm.uni-

heidelberg.de; and (c) for all selected targets, conservation of at least 7 nucleotides at the seed region, complementary with *KISS1* sequence, was required.

Luciferase reporter assays

HEK-293T cells (human embryonic kidney cells) were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine and 1% (v/v) penicillin/streptomycin at 37 ºC in a humidified atmosphere containing 5% CO_2. Transient transfections were performed with Rotifect (Carl Roth, Karlsruhe, Germany) according to manufacturer's instructions. The expression vectors for Gluc-*KISS1*-3'UTR reporter, hsa-miR-324-3p and hsa-miR-27b-3p, and related reagents, were purchased from GeneCopoeia (Rockville, MD, USA), and luciferase assay performed using Secret-Pair Dual Luminescence Assay Kit according to the manufacturer's instructions (Promega, Madison, WI, USA). For *KISS1*-3'UTR reporter assays, HEK-293T cells co-transfected with *KISS1*-3'UTR reporter and hsa-miR-324-3p expression vectors. For *KISS1* promoter (5'-UTR) analyses, cells were co-transfected with a *KISS1*-promoter-Gluc reporter plasmid (KISS1p1339; a kind gift from Dr. Sabine Heger, University of Hanover, Germany) and the hsa-miR-27b-3p expression vector. Cells were collected in PBS after 24h and lysed following the instructions of the luciferase assay kit. Luciferase activity was measured using an Autolumat LB 9510 (EG&G Berthold, USA).

Assays for kisspeptins and β-hCG

Plasma kisspeptin levels were assayed in control (VTOP) and EP samples using a validated radioimmunoassay (44, 45); collection of blood samples was done in strict adherence to conditions known to preserve kisspeptins for proper immunometric detection (46). The kisspeptin antibody, GQ2, which was used at a final dilution of 1:3.500.000, has 100% cross-reactivity with human kisspeptin-54 and shorter fragments (kisspeptin-14 and -10), but <0.01% with any other related human RF-amide peptides (45). Kisspeptin-54 was labelled with ^{125}I using the Iodogen method. The assay was performed in duplicate using dilutions of neat plasma in 0.7 ml of 0.06 M phosphate buffer, with 0.3% BSA, incubated for 3 days at 4°C. Free and antibody-bound label were then separated by charcoal adsorption. The assay detected changes of 2 pmol/l of plasma kisspeptin IR with a 95% confidence limit. The intra- and inter-assay coefficients of variation were 8.3 and 10.2%, respectively.

For comparative purposes, the levels of β-hCG were also assayed in VTOP and EP samples. The hCG assay was carried out following the instructions of Human hCG ELISA Kit from Thermo-Fisher Scientific (Catalogue No# EHCG). This assay was applied to women with a normal

pregnancy that desired a voluntary termination (VTOP) during the first trimester of pregnancy as control (n=35) and to whole cohort of patients suffering from tubal ectopic pregnancy, EP (n=45).

Statistical analyses

Data of miRNA and kisspeptin levels are expressed as mean ± SEM. Two-tailed Student t tests were used to evaluate differences between case and control groups (single comparisons). ANOVA was used for statistical analyses of multiple data-points; when appropriate post-hoc, Newman-Keuls tests were applied to identify individual differences after multiple comparisons. All statistics were carried out with the use of Prism 7.0c (GraphPad Software, Inc.). P <0.05 was considered to be statistically significant.

Data of patients collected during the first 10 weeks of gestation were used to test the ability of kisspeptins, miR324-3p and β-hCG to predict ectopic pregnancy. Different combinations of these variables were used after logarithmic transformation to build logistic regression models. Due to the limited number of cases data were not divided into training and testing sets. On the contrary, models were built and validated using repeated 10-fold cross-validation (47). *k*-fold cross-validation randomly divides the data into *k* blocks of roughly equal size. Each block is left out in turn while the other *k*-1 blocks are used to train the model that is then used to predict the class of the remaining "left-out" subsample. Finally, classification results are summarized into performance measures and averaged to get the overall resampled estimate.

Area under the curve, sensitivity, specificity, accuracy (total correct predictions/total samples) and Cohen's (un-weighed) Kappa statistic were computed for each model (kisspeptin, miR324-3p, β-hCG, β-hCG/miR-324-3p, β-hCG/kisspeptin, and kisspeptin/miR-324-3p).

Following the same validation procedure, a decision tree using miR-324-3p and kisspeptin was built. The C50 method (an extension of the C4.5 classification algorithm described in https://github.com/topepo/caret/) was used for this purpose. All models were built and validated using the R package caret, as defined in http://www.rulequest.com/see5-unix.html.

Authors Contributions

AAR played a major coordinating role in sample processing and in the conduction of molecular and hormonal analyses in tissue and plasma samples from ectopic pregnancy patients. He, in close collaboration with MSA, conducted also primary analyses of data and plotted the figures, and drafted the first version of the manuscript. MSA was involved in primary analysis of data and played a key role in miRNA bioinformatic and expression analyses. She also participated in plotting and discussion of data, and revised the first draft of the paper. FD, TL and MG actively participated in ectopic pregnancy sample recruitment, with a major role of FD in sample management and initial analyses; all participated also in discussion of data. HA was responsible for extensive biostatistical analysis of data and for application of mathematical algorithms for prediction. SSA was actively involved in miRNA analyses in ectopic pregnancy samples, while MLC and MAC were responsible for in vitro molecular analyses of miRNA-regulatory actions on *KISS1*. MFF, together with AP and MTS, participated in the design of the study and evaluation of results. ET, CPL and LS were actively involved in different (molecular and hormonal) analyses on the samples. AA and WSD were responsible for conduction of specific RIA for circulating kisspeptins, and actively participated in preparation and edition of the manuscript. AP and MTS jointly design the study. MTS integrated all the data and prepared the manuscript, which was thoroughly revised by AA, WSD and FD. All the authors contributed to manuscript preparation and take full responsibility for the work.

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Disclosure Statement

The authors have nothing to disclose in relation to the contents of this work.

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Figure Legends

Figure 1. *Expression of KISS1 in embryonic/placental tissue and plasma kisspeptin levels in normal (NP) and ectopic (EP) pregnancy*. In panels **A** and **B**, expression of *KISS1* and kisspeptin levels in NP (from VTOP) are presented, grouped in six gestational-age ranges: ≤6th, 7th-9th, 10th-12th, 13th-15th, 16th-17th and 18th-20th weeks of pregnancy. Analyses in EP were restricted to samples collected until week 12th of ectopic gestation. Integral mean levels of *KISS1* expression and plasma kisspeptin levels in samples from NP and EP up to week-12 of gestation are shown in panels **C** and **D**; EP values are expressed as normalized values against NP levels. In addition, the detailed temporal course of these changes is shown in panels **E** and **F**. Samples from week-5 -6 of gestation were grouped as ≤6-week, whereas those from week -9 to -12 were grouped as ≥9-week samples. Data are presented as mean ± SEM. For presentation, quantitative values were normalized to values from ≤6-week gestational samples. *, P<0.05; **, P < 0.01; and ***, P < 0.001 vs. corresponding values in NP (ANOVA followed by Newman-Keuls test).

Figure 2. *Expression of miR-324-3p and miR-27b-3p in embryonic/placental tissue in normal and ectopic (EP) pregnancy*. In panels **A** and **B**, expression levels of miR-324-3p and miR-27b-3p in embryonic/placental tissue from control pregnancies (NP; from VTOP) are presented, grouped in six gestational-age ranges: ≤6th, 7th-9th, 10th-12th, 13th-15th, 16th-17th and 18th-20th weeks of pregnancy. Analyses in EP were restricted to samples collected until week 12th of ectopic gestation. Integral mean levels of miR-324-3p and miR-27b-3p in samples from NP and EP up to week-12 of gestation are shown in panels **C** and **D**; EP values are expressed as normalized values against NP levels. In addition, the detailed temporal course of these changes is shown in panels **E** and **F**. Samples from week-5 -6 of gestation were grouped as ≤6-week, whereas those from week -9 to -12 were grouped as ≥9-week samples. Data are presented as mean ± SEM. For presentation, quantitative values were normalized to values from ≤6-week gestational samples. *, P<0.05; **, P < 0.01; and ***, P< 0.001 vs. corresponding values in NP (ANOVA followed by Newman-Keuls test).

Figure 3. *Potential interaction of miR-324-3p and miR-27b-3p with KISS1 in vitro*. HEK-293T cells were co-transfected with one of the following reporter plasmid: *KISS1*-3'UTR-Gluc (panel **A**) or KISS1-promoter-GLuc (panel **B**), together with a plasmid encoding the indicated miRNA (miR-324-3p or) miR-27b-3p, or a miRNA scrambled control. Luciferase activity was measured as relative light units (RLU) and is presented as % of inhibition with respect to control values for each miRNA. Data are presented as the mean ± SD of n = 3 experiments, done in triplicate. *, P < 0.05 (Student-t test).

Figure 4. *Plasma levels of miR-324-3p and miR-27b-3p in normal and ectopic (EP) pregnancy*. In panels **A** and **B**, plasma levels of miR-324-3p and miR-27b-3p in samples from control pregnancies (NP; from VTOP) are presented, grouped in six gestational-age ranges: ≤6th, 7th-9th, 10th-12th, 13th-15th, 16th-17th and 18th-20th weeks of pregnancy. Analyses in EP were restricted to samples collected until week 12th of ectopic gestation. Integral mean plasma levels of miR-324-3p and miR-27b-3p in samples from NP and EP up to week-12 of gestation are shown in panels **C** and **D**; EP values are expressed as normalized values against NP levels. In addition, the detailed temporal course of these changes is shown in panels **E** and **F**. Samples from week-5 -6 of gestation were grouped as ≤6-week, whereas those from week -9 to -12 were grouped as ≥9-week samples. Data are presented as mean ± SEM. For presentation, quantitative values were normalized to values from ≤6-week gestational samples. *, P<0.05; and **, P < 0.01 vs. corresponding values in NP (ANOVA followed by Newman-Keuls test).

Figure 5. *Plasma kisspeptin and miR-324-3p levels as diagnostic markers of EP*. In panel **A**, ROC curve analyses of kisspeptins, miR-324-3p, β-hCG, and their combinations in predicting EP. In lower-left panel, performance measures of the corresponding logistic regression models are presented. In addition, in panel **B**, a decision-tree model, generated using miR324-3p and kisspeptin levels, for predicting EP, is shown. For further details, see *Methods*.

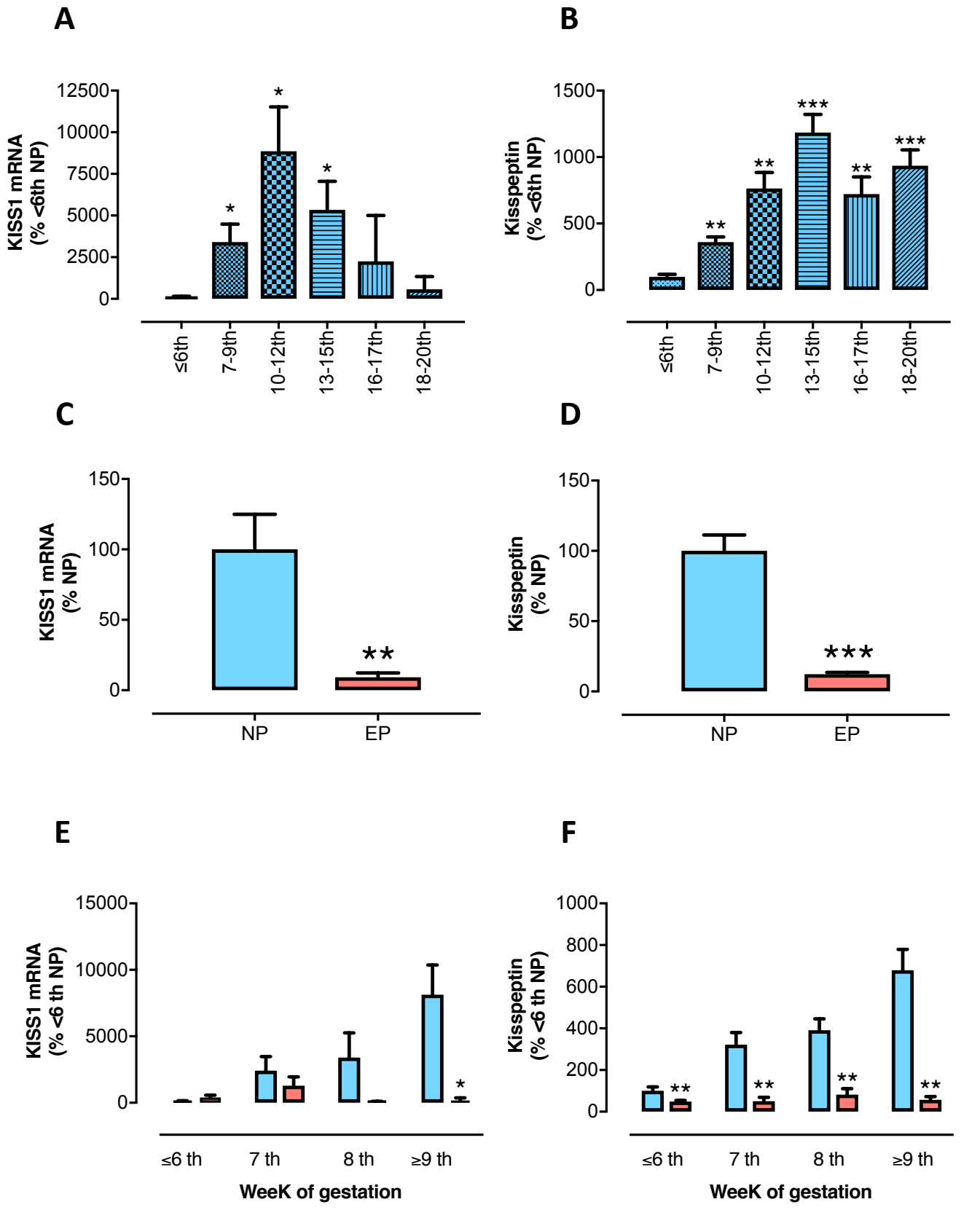

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Figure 1

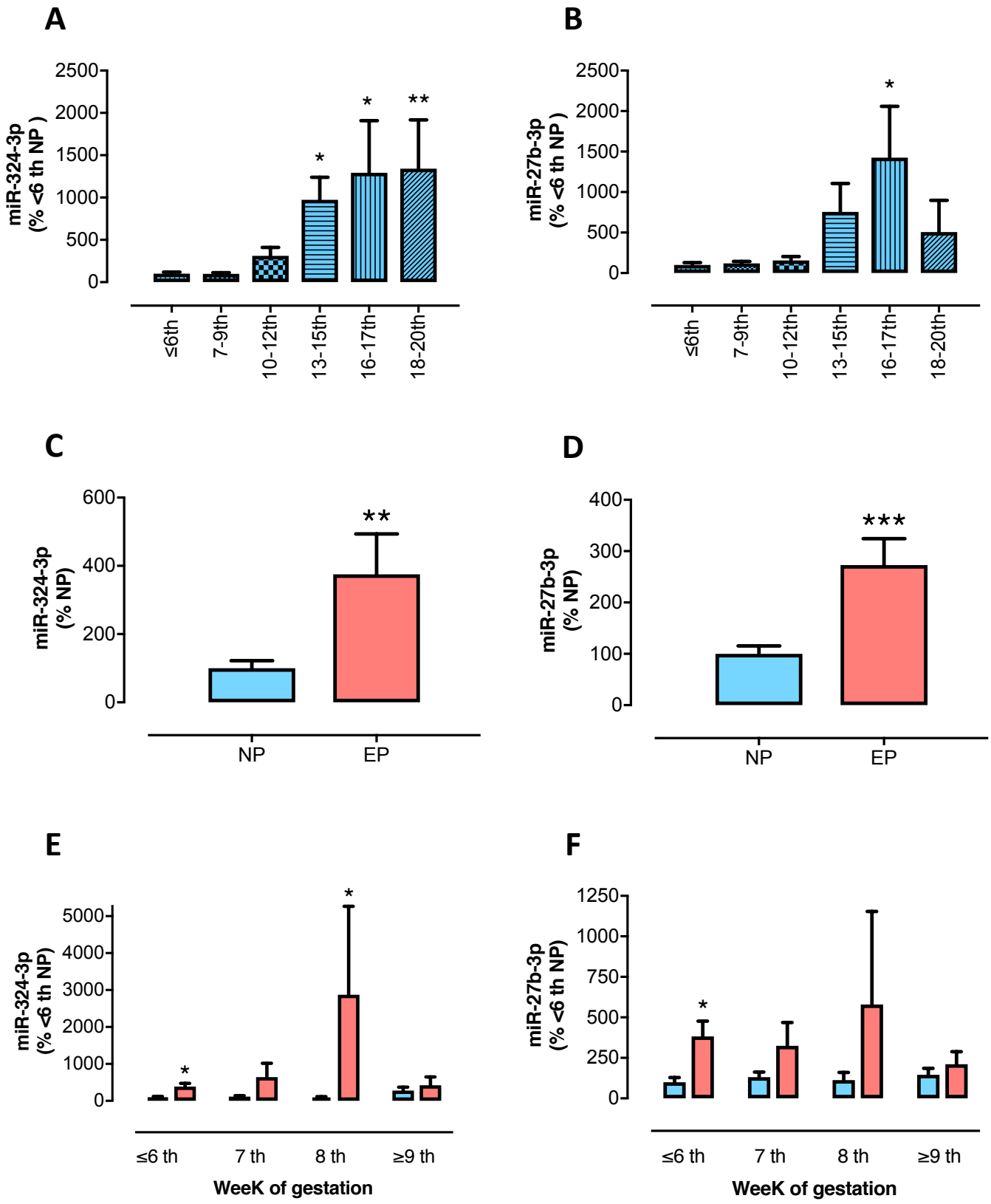

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Figure 2

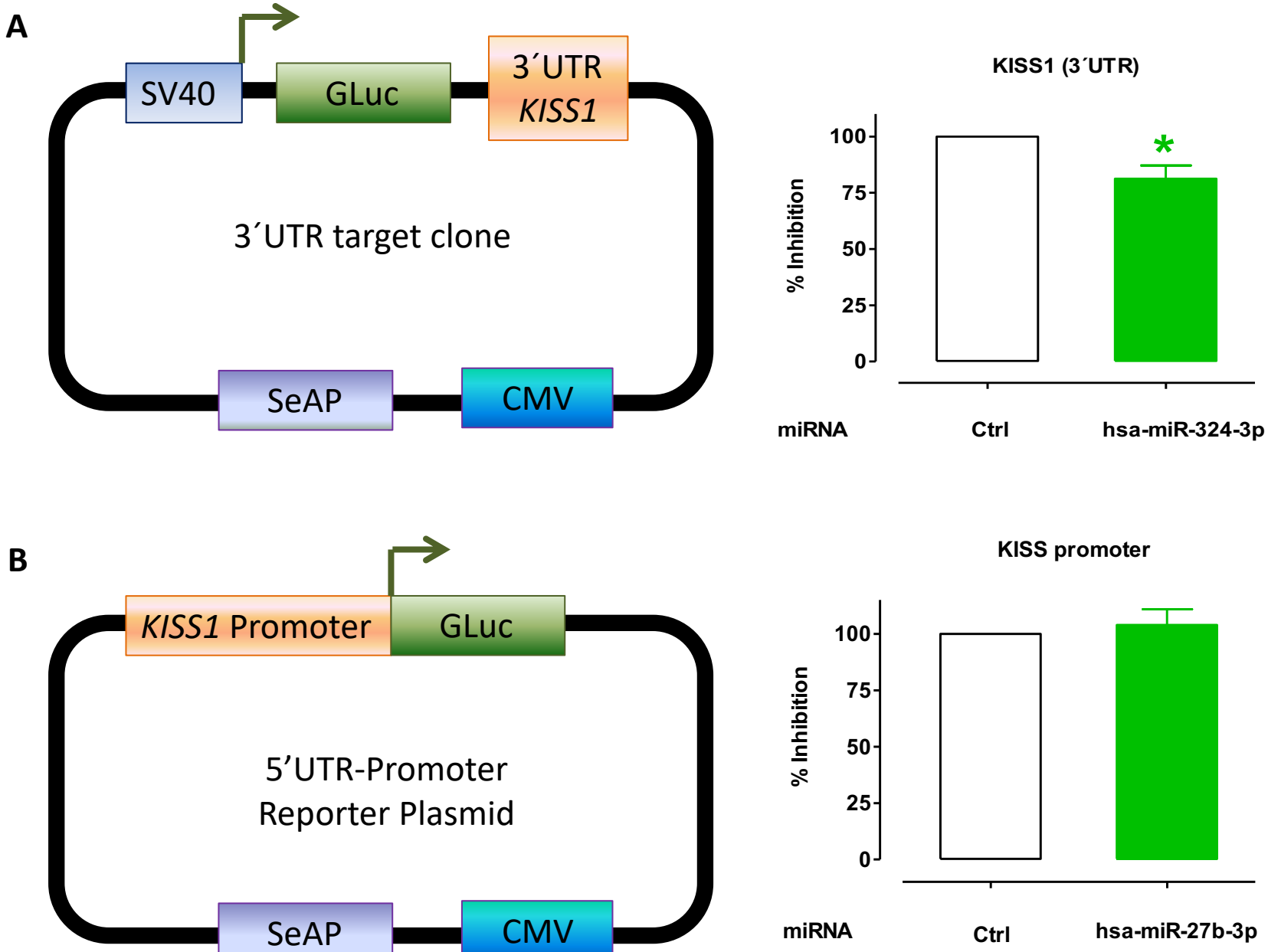

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Figure 3

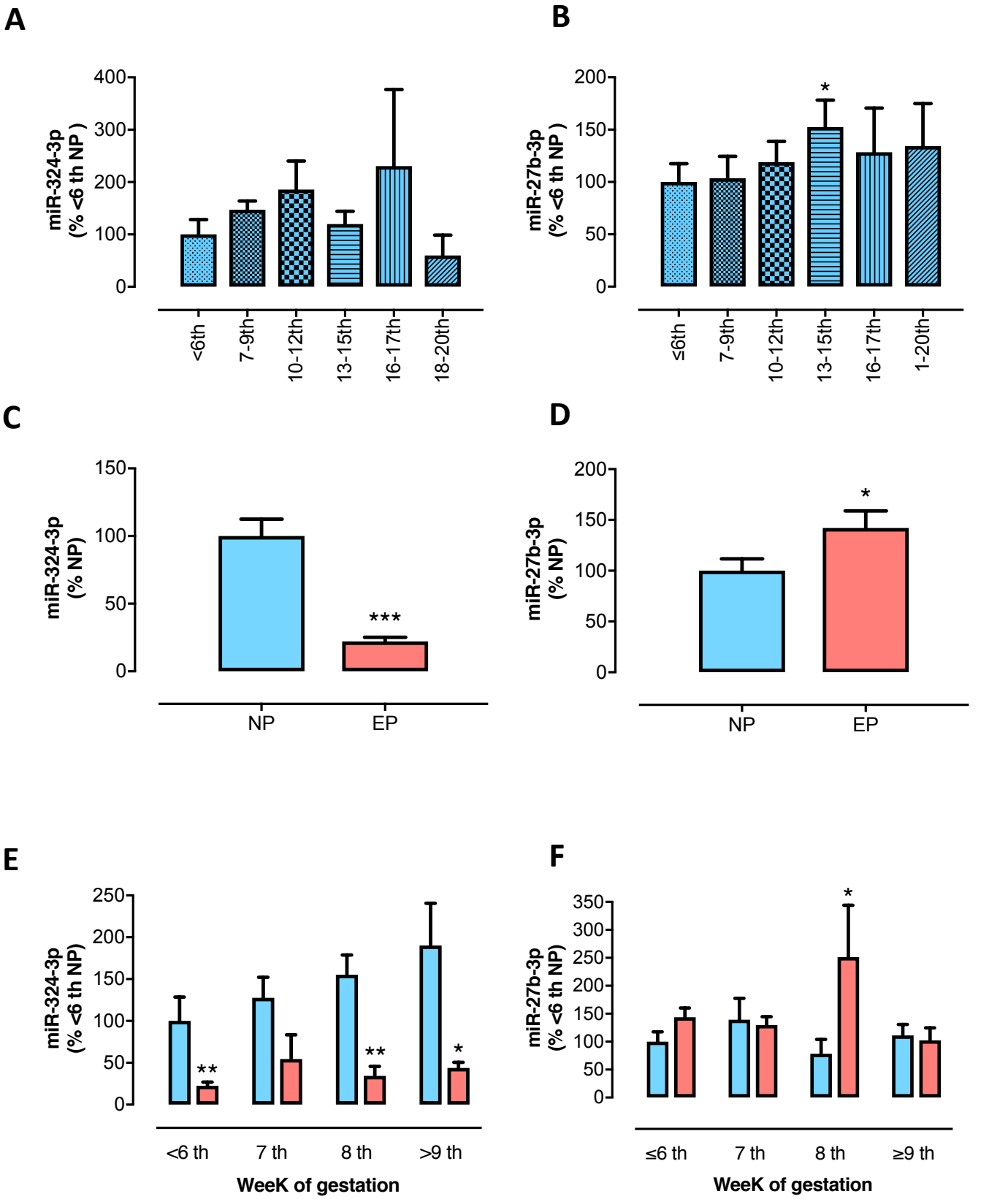

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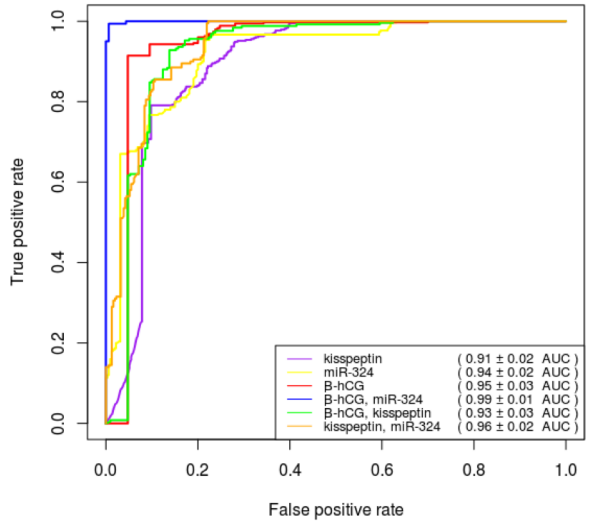
Figure 4

ROC Curve



	AUC	Sens	Spec	Accuracy	Kappa
Kiss	0.909	0.830	0.824	0.827	0.652
Mir324	0.941	0.907	0.767	0.839	0.675
Hcg	0.947	0.905	0.943	0.927	0.844
Hcg , Mir324	0.997	0.998	0.945	0.970	0.942
Hcg , kiss	0.934	0.858	0.902	0.880	0.758
Kiss , Mir324	0.955	0.898	0.855	0.880	0.751

Tree-based Decision Model

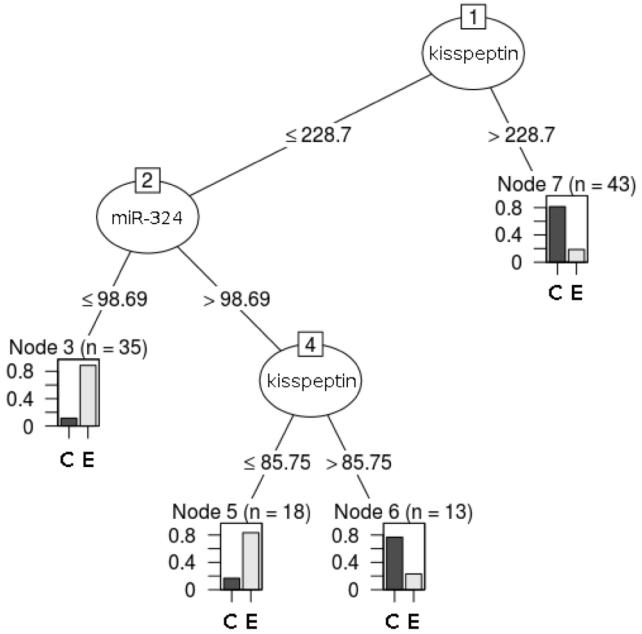


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Figure 5

Supplemental Figure Legends

Suppl. Figure S1. *Bioinformatic prediction of putative miRNA regulators of human KISS1*. Bioinformatic tools were applied to identify conserved seed regions of miRNAs that may operate as regulators of *KISS1*. Three major candidates were identified: miR-324-3p and miR-137-3p, with predicted seed sequences at the 3'-UTR of *KISS1*, and miR-27b-3p, with a predicted seed region at the promoter (5'-UTR) of the gene. Details about location and sequence of these recognition sites are included.

Suppl. Figure S2. *Expression of pre-miR-324-3p in embryonic/placental tissue in normal and ectopic (EP) pregnancy*. Integral mean expression levels of pre-miR-324-3p in samples from NP and EP up to week-12 of gestation are shown; EP values are expressed as normalized values against NP levels. *, $P < 0.05$ (Student-t test).

Suppl. Figure S3. *Plasma levels of β-hCG in normal and ectopic (EP) pregnancy*. In panel **A**, integral mean plasma levels of β-hCG in samples from NP and EP up to week-12 of gestation are shown; EP values are expressed as normalized values against NP levels. In addition, the detailed temporal course of these changes is shown in panel **B**. Samples from week-5 -6 of gestation were grouped as ≤6-week, whereas those from week -9 to -12 were grouped as ≥9-week samples. Data are presented as mean ± SEM. For presentation, quantitative values were normalized to values from ≤6-week gestational samples. *, $P<0.05$; and ***, $P < 0.001$ vs. corresponding values in NP (ANOVA followed by Newman-Keuls test).

Suppl. Figure S4. *Decision-tree model for triage of EP*. A decision-tree model, generated using a sequential combination of β-hCG, kisspeptin and miR324-3p kisspeptin levels, for predicting EP, is depicted. For further details, see *Methods*.

Specie	mRNA	Region	miRNA	Seed Leght	Sequences
Human	Kiss1 (5'-3')	3' UTR	miR-137-3p (3'-5')	7	5' GGAGCTTCCAACCCGAGGCAATAA 3' 3' GAUGCGCAUAAGAAUUCGUUAUU 5'
Human	Kiss1 (5'-3')	3' UTR	miR-324-3p (3'-5')	8	5' GGGCGCAG-GTGCGGGGCAGTGAA 3' 3' GGUCGUCGUGGACCCCGUCACCC 5'
Human	Kiss1 (5'-3')	Promoter	miR-27b (3'-5')	8	5' TAGCCCCTCTGCC--TTCA-GAGA 3' 5' UUC-AC-AG-UGGCUAAGUUCUGC 3'

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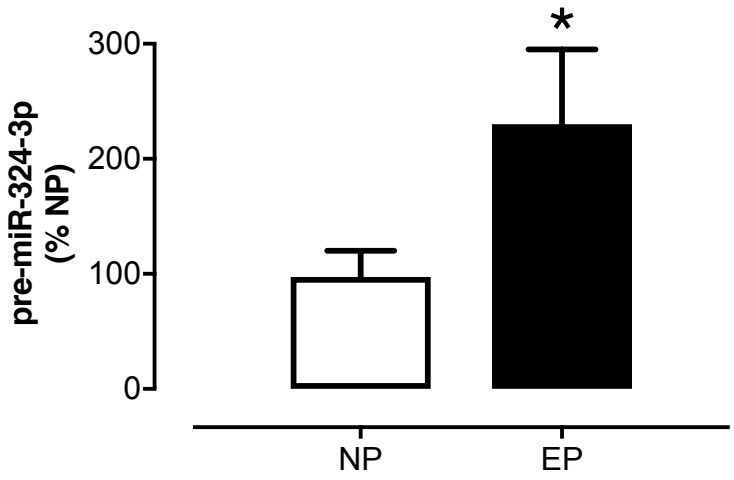
Suppl. Figure S1



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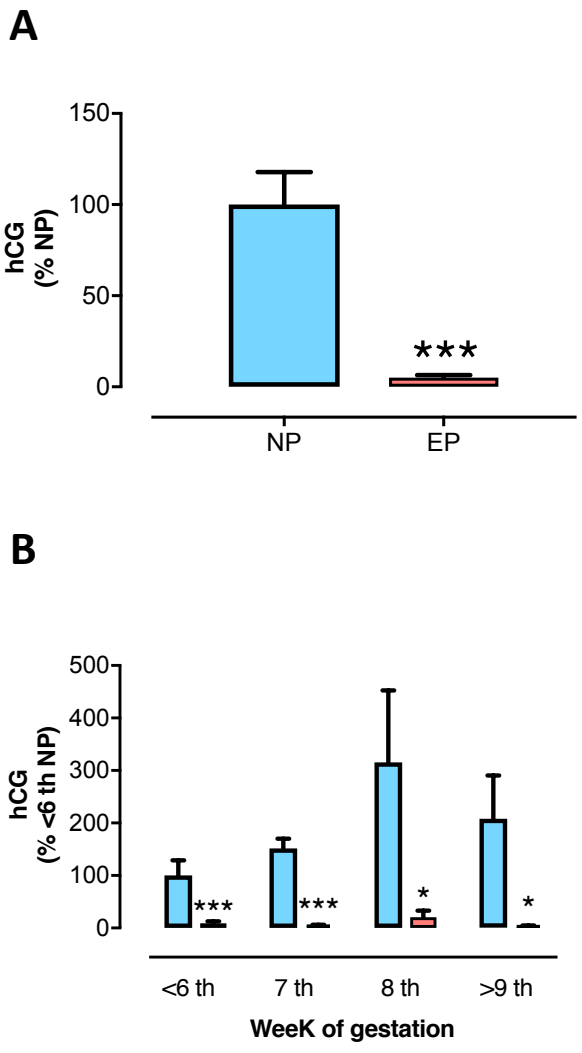
Suppl. Figure S2



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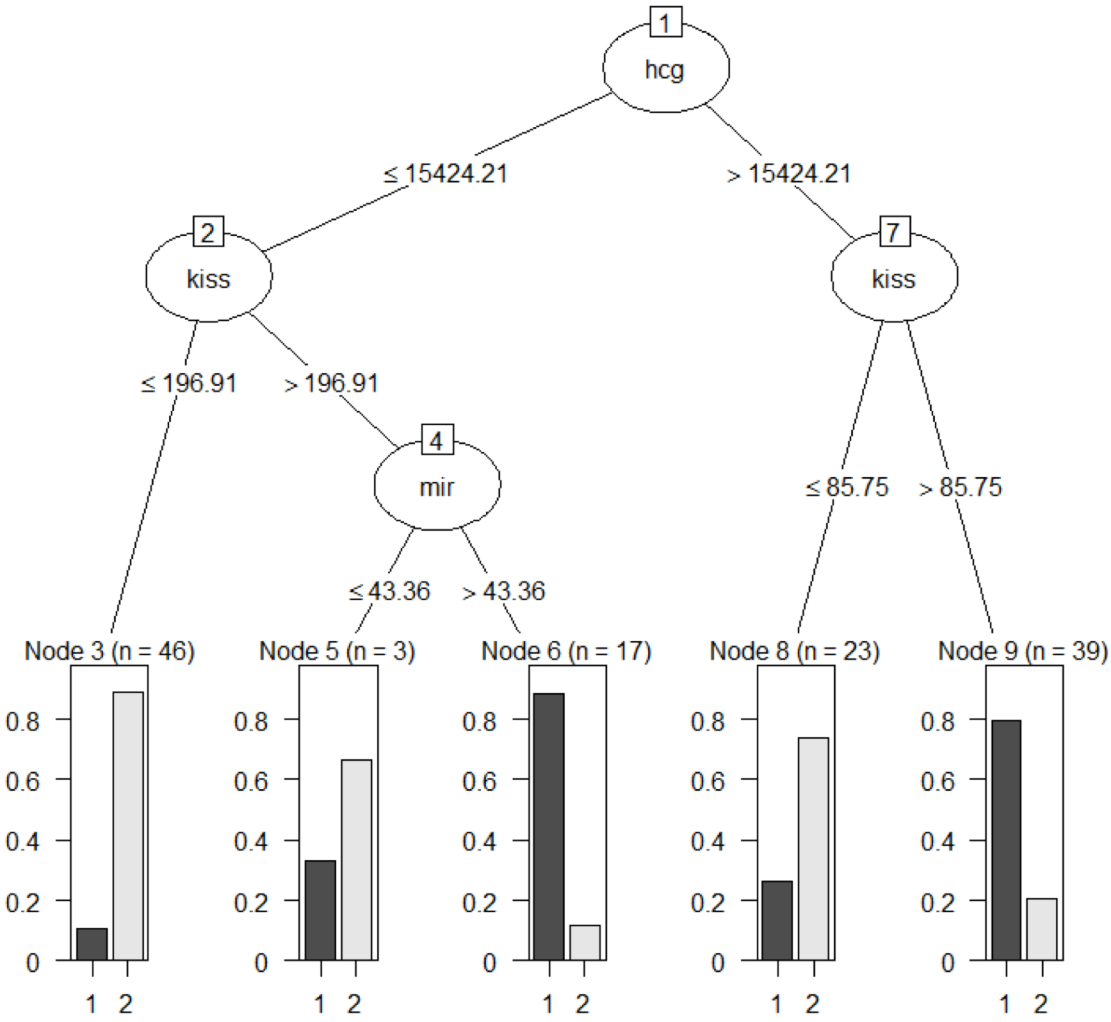
Suppl. Figure S3



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Suppl. Figure S4