Maternal Pregnancy-Associated Circulating MicroRNA predictors of infant birth outcomes are Determinants of Placental Maturation

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Conflict of Interest Statement

The authors have declared that no conflict of interest exists.
Abstract

We previously identified 11 miRNAs which were significantly elevated in blood plasma of pregnant mothers who subsequently gave birth to infants affected by prenatal alcohol exposure (PAE, Heavily Exposed Affected: HEa) compared to those with infants who were exposed but apparently unaffected (Heavily Exposed Unaffected: HEua) or unexposed (UE). These maternal HEa miRNAs were predicted to originate in part, as a paracrine placental signal to influence and coordinate placental epithelial-mesenchymal transition (EMT), a pathway essential for endometrial invasion and development. We now report that PAE inhibits expression of placental EMT pathway members in rodent and primate voluntary alcohol consumption models, with HEa miRNAs collectively mediating placental EMT inhibition. To directly investigate the interaction between HEa miRNAs and ethanol on placenta, we assessed their effects on human placental trophoblast cell lines. When administered together, HEa miRNAs retarded trophoblast cell cycle, significantly impaired expression of core EMT pathway members, and reduced invasiveness, pointing to their collective role in modulating placental growth and invasion deficits seen with PAE. HEa miRNAs additionally interfered with maturation-dependent intracellular calcium dynamics, while promoting syncytialization-dependent increases in placental hormone expression. Finally, a single systemic administration of the pooled murine-expressed HEa miRNA subpopulation, to pregnant mice, decreased fetal and placental growth and inhibited expression of EMT pathway mRNA transcripts in placenta. Taken together, our data suggests that, following PAE, HEa miRNAs interfere with placental development and may contribute to the pathology of Fetal Alcohol Spectrum Disorders.
Introduction

Alcohol use during pregnancy remains prevalent despite published prevention guidelines (1). A recent meta-analysis determined the global prevalence of alcohol use during pregnancy at 9.8% (2). Within the United States, a 2013 study found 18% of women reported alcohol consumption during pregnancy, and 6.6% engaged in binge-drinking episodes, which are particularly damaging to fetal development (3). In the state of Texas, we recently reported an average state-wide third-trimester rate of alcohol exposure of 8.4%, with rates as high as 17.7% in some localities (4). These data collectively suggest that prenatal alcohol exposure (PAE) is common.

PAE harms the developing embryo and fetus and can result in a constellation of adverse infant outcomes, including craniofacial dysmorphologies, growth retardation, and neurobehavioral abnormalities, collectively termed Fetal Alcohol Spectrum Disorders (FASDs) (5). Estimates for the prevalence of FASDs range from 2-5% of the school age population in the US and Western Europe (6) to 13.6-20.9% in South Africa (7). Estimates for the global prevalence of fetal alcohol syndrome (FAS), the most severe end of the FASD continuum, characterized by cranio-facial dysmorphologies, growth restriction, and CNS abnormalities, range from 0.15 to 0.3% (8, 9), but are much higher in specific populations, for example, as high as 5.9-9.1% in the Cape Coloured (mixed ancestry) community in South Africa (7).

Given the lifelong debility and economic burden associated with FASDs (10), there is an urgent need to develop sensitive and specific methodologies to identify affected children early in development, as well as develop interventions which can mitigate the effects of PAE on the developing fetus. In 2016, we reported that elevated levels of 11 distinct microRNAs (miRNAs)
in 2nd and 3rd trimester maternal circulation distinguished infants who were affected by in-utero alcohol exposure (Heavily Exposed Affected: HEa) from those who were apparently unaffected at birth by PAE (Heavily Exposed Unaffected: HEua), or those who were unexposed (UE) (11). We predicted that these HEa miRNAs (MIMAT0004569 [hsa-miR-222-5p], MIMAT0004561 [hsa-miR-187-5p], MIMAT0000687 [hsa-miR-299-3p], MIMAT0004765 [hsa-miR-491-3p], MIMAT0004948 [hsa-miR-885-3p], MIMAT0002842 [hsa-miR-518f-3p], MIMAT0004957 [hsa-miR-760], MIMAT0003880 [hsa-miR-671-5p], MIMAT0001541 [hsa-miR-449a], MIMAT0002869 [has-miR-519a-3p]) in addition to being biomarkers of infant outcome following PAE, influence signaling pathways crucial for early development, particularly the epithelial-mesenchymal transition (EMT) pathway (11).

Placental development involves maturation of the cytotrophoblasts at the tip of anchoring villi into invasive extravillous trophoblasts, as well as fusion of cytotrophoblasts into multinucleate, hormone-producing syncytiotrophoblasts (12). Maturation into extravillous trophoblasts, which invade the maternal decidua and remodel the uterine spiral arteries into low-resistance high-flow vessels that enable optimal perfusion for nutrient and waste exchange, requires cytotrophoblasts to undergo EMT (13). Impaired placental EMT, as well as orchestration of the opposing mesenchymal-epithelial transition pathway, has been found in conditions resulting from placental malfunction, primarily preeclampsia (14-19). While there have been no previous studies directly investigating the effects of PAE on placental EMT, a rodent study demonstrated that PAE, during a broad developmental window, reduced the number of invasive trophoblasts within the mesometrial triangle, a region of the uterine horn directly underlying the decidua (20). Furthermore, both human and rodent studies have found
PAE disrupts placental morphology, and interferes with cytotrophoblast maturation, as with preeclampsia (21-24). Disrupted trophoblast maturation, seen in these conditions, is associated with aberrant expression of placental hormones, primarily human chorionic gonadotropin (hCG) (25-28). Our study is the first to report that PAE interferes with expression of core placental EMT pathway members. We also provide evidence that a group of circulating maternal miRNAs, HEa.miRNAs, which predict adverse infant outcomes due to PAE, mediate some of PAE’s effects and interfere with EMT and cytotrophoblast maturation.
Results

**HEa**miRNAs are implicated in placental-associated pathologies

Outside of our report that elevated **HEa**miRNA levels predict infant outcomes following PAE, few other studies have investigated expression of these miRNAs in the context of PAE or other potentially toxic prenatal exposures (Figure 1A) (29-36). Given our prediction that **HEa**miRNAs interfere with signaling pathways governing fetal and placental development (11), we conducted a literature review of reports on **HEa**miRNA levels in gestational pathologies which, as with PAE, include aberrant placentation as an etiological factor (37-39). Surprisingly, placental and plasma levels of 8 out of 11 of these **HEa**miRNAs were significantly dysregulated in one or more of these gestational pathologies with expression of the majority of these 8 miRNAs altered in both fetal growth restriction and preeclampsia (Figure 1B) (33, 34, 36, 40-57), both of which are characterized by poor placental invasion as an etiological factor (58-64).

**HEa**miRNAs explain variance in infant growth outcomes due to PAE

Given the association of individual **HEa**miRNAs with gestational pathologies, we sought to determine if circulating **HEa**miRNAs levels could explain the variance in sex and gestational age-adjusted neonatal height, weight and head circumference in our Ukrainian birth cohort, which are growth measures sensitive to in utero environment (65). We found that 8 of the **HEa**miRNAs, each significantly explained between 7 to 19% of infant variation in these growth measures (Table 1). Furthermore, 7 of these miRNAs were also associated with fetal growth restriction and preeclampsia as identified by our literature review. Additionally, only 1 of the 3 miRNAs not previously-reported to be associated with gestational pathologies was correlated with measures of fetal growth and development in our dataset (Figure 1B). Interestingly, a
multivariate statistical regression model that accounted for levels of all 11 HEa miRNAs together, explained a far greater proportion of infant variance, between 24-31%, in all three growth measures than accounting for them individually (Supplementary Table 2).

**HEa miRNAs are transcribed preferentially in the placenta**

Data extracted from publicly available gene expression profiling datasets (66) show that HEa miRNAs as well as their unprocessed precursor transcripts, HEa pri-miRNAs, are enriched in placenta compared to other tissues, suggesting that the placenta itself transcribes these miRNAs and may be a significant contributory tissue to maternal circulating HEa miRNAs (Figures 1C and D). Since chorionic villi are immersed in maternal blood, it is possible that the placenta is both a source and a target of maternal HEa miRNAs. Moreover, since HEa miRNAs are also associated with gestational pathologies caused by poor placental invasion, these HEa miRNAs may also constitute an intra-placental paracrine signal to coordinate the (mal)adaptive response of tissues to PAE. We therefore assessed in rodent and primate models, whether PAE could result in impaired EMT, and if HEa miRNAs could explain the effects of PAE on placental EMT-associated gene expression.

**HEa miRNAs moderate PAE’s effects on EMT pathway members**

EMT, in trophoblasts, is characterized by the disappearance of epithelial markers like E-Cadherin and the appearance of the mesenchymal markers like the intermediate filament, vimentin, a process that is controlled by the expression of key mesenchymal determination transcription factors, Snail1 and 2 and TWIST, as extensively described (13, 17, 18, 67-70).
These five markers have been used extensively to assess EMT in a variety of model systems, so our studies utilized these markers to assess the effects of alcohol and HEa miRNAs on trophoblast EMT.

In the first analysis, using a murine model of PAE that mimicked moderate to binge-type alcohol consumption throughout early and mid-pregnancy, we fractionated GD14 placenta into three zones: the cytотrophoblast and syncytiotrophoblast rich labyrinth zone, the glycogen and spongiotrophoblast rich junctional zone, and the decidual zone comprising the endometrial contribution to the placenta (Figure 2A). Multivariate analysis of variance (MANOVA) for expression of these five core genes in the EMT pathway within placental trophoblasts, revealed a significant effect of ethanol exposure on EMT pathway member expression selectively within the labyrinth zone (Pillai’s trace statistic, $F(5,21)=6.85$, $p<0.001$, Figure 2B) but not within the junctional or decidual zones. Post-hoc univariate ANOVA indicated ethanol exposure specifically elevated $CDH1$ ($F(1,25)=7.452$, $p=0.011$), which encodes epithelial E-Cadherin, whereas expression of the pro-mesenchymal transcription factor $SNAI1$ was significantly reduced ($F(1,25)=21.022$, $p=0.0001$). We also observed a significant interaction between fetal sex and PAE on expression of $SNAI2$ ($F(1,25)=2.18$, $p=0.047$) and a trend towards decreased expression of the terminal mesenchymal marker $VIM$ (Vimentin, $F(1,25)=2.749$, $p=0.11$), while there was no effect on $TWIST$ expression (Figures 3A-3E). Consistent with our gene expression data, E-Cadherin protein levels were significantly elevated in the labyrinth zone of PAE placenta ($F(1,24)=31.63$, $p=0.0005$), while not in the junctional or decidual zones (Figure 3F and Supplementary Figures 3A and B). However, when we controlled for expression of the 8 mouse homologues of HEa miRNAs as a covariate, using multivariate analysis of covariance (MANCOVA), ethanol’s effect
on EMT became marginally nonsignificant (Pillai’s trace, $F_{(5,21)}=2.713$, $p=0.068$) (Figure 2C), suggesting that these miRNAs partially mediate effects of PAE on EMT pathway members in mice. Interestingly, PAE limited to the peri-conceptional period in rats also influenced expression of EMT core transcripts (Supplementary Figures 2B and 4A-4E).

To determine if PAE’s effects on EMT pathway members in placenta are broadly conserved throughout mammalian evolution, we adopted a non-human primate (macaque) model of moderate to binge-type alcohol consumption. Placental tissues were isolated from GD85, GD110, and GD 135 placenta (Figure 2D), which spans the human equivalent of mid-second to mid-third trimester (Supplementary Figure 2C). There was a significant effect of ethanol exposure on expression of core EMT mRNA transcripts by MANOVA (Pillai’s trace statistic, $F_{(4,9)}=4.229$ $p=0.045$, Figure 3B). Consistent with our findings in mouse, post-hoc univariate ANOVA indicated that in primate placenta, ethanol exposure significantly increased $CDH1$ expression ($F_{(1,12)}=4.866$, $p=0.048$) whereas $VIM$ expression was significantly reduced ($F_{(1,12)}=12.782$, $p=0.0004$), suggesting that, as in the mouse, PAE also impairs EMT in the primate placenta. Interestingly, there was no effect on $SNAI2$ or $TWIST$ expression (Figures 3G-3J). As in mice, accounting for expression of $\text{HEa}miRNAs$ together as a covariate abolished the significant effect of PAE on EMT, though to a greater degree than mice (Pillai’s trace, $F_{(1,1)}=1.605$, $p=0.425$, Figure 2E). Interestingly, accounting for expression of individual $\text{HEa}miRNAs$ did not explain the effects of PAE on placental EMT, suggesting that $\text{HEa}miRNAs$ act in concert to mediate the effect of PAE on EMT in primate placenta (Figure 2F).

Collectively, our data suggests PAE induced impairment of EMT in the trophoblastic compartment of placentae is conserved between rodents and non-human primates and that
HEa miRNAs, particularly in primates, may moderate the effect of PAE on placental EMT.

Consequently, subsequent studies focused on the collective role of HEa miRNAs, either on basal or on alcohol-influenced placental trophoblast growth, invasion, and the maturation of physiological function.

HEa miRNAs impair EMT pathway member expression in a model of human cytotrophoblasts

To investigate whether HEa miRNAs collectively interfere with the EMT pathway, as suggested by our in vivo data, we measured expression of core members of the EMT pathway after transfecting HEa miRNA mimics and antagonirs into BeWO cytotrophoblasts.

Cytotrophoblasts normally undergo EMT to become invasive trophoblasts, which we also modelled using the HTR8 human extravillous trophoblast cell line. We initially overexpressed each of the 11 HEa miRNAs individually, to determine whether any of them could alter mRNA transcripts for EMT pathway members. However, we did not observe any significant effects due to overexpression of individual miRNAs (Supplementary Figure 5), consistent with our findings in the primate PAE model that individual miRNAs did not explain the effects of ethanol on EMT.

In contrast, transfection of pooled HEa miRNAs into cytotrophoblasts significantly increased CDH1 expression ($F_{(1,36)}=30.08$, $p<0.0001$). Interestingly, expression of the pro-mesenchymal transcription factors TWIST and SNAI1 were also significantly reduced, but only in the context of concomitant 320 mg/dL ethanol treatment, pointing to an interaction effect between HEa miRNAs and ethanol ($F_{(1,36)}=5.650$ and 5.146 respectively, $p=0.023$ and $p=0.029$, Figures 4A-D). Consistent with our qPCR data, transfection of HEa miRNAs also significantly increased E-cadherin protein expression ($F_{(1,20)}=33.86$, $p<0.0001$, Figure 4E). We were unable to detect
SNAIL transcript expression or vimentin protein expression in these cells, consistent with previous reports (71).

Whereas transfection of HEa miRNA mimics increased CDH1 expression, transfection of pooled antagonims to HEa miRNAs, significantly reduced CDH1 expression, only in the context of 320 mg/dL ethanol co-exposure (post-hoc Tukey’s HSD, p=0.005), consistent with an overall statistically significant interaction effect between ethanol exposure and HEa miRNA inhibition (F(1,36)=13.51, p=0.0008, Figure 4F). However, expression of TWIST was also decreased in this context and there was no significant difference in E-Cadherin protein expression relative to the control (Figure 4G-J). Thus, our data suggest that, increasing HEa miRNA levels impairs EMT pathway members in cytotrophoblasts whereas inhibiting their action has a more restricted effect on EMT pathway members.

HEa miRNAs impair EMT member expression in a model of human extravillous trophoblasts

We next investigated the effect of HEa miRNAs on EMT in HTR-8/SVneo extravillous trophoblast-type cells. Transfecting pooled HEa miRNA mimics into extravillous trophoblasts significantly decreased VIM expression (F(1,36)=28.43, p<0.0001). Expression of pro-mesenchymal transcription factors SNAIL was also reduced (F(1,36)= 64.88 respectively, p<0.0001). As with cytotrophoblasts, expression of SNAIL1 and TWIST were reduced only with 320 mg/dL ethanol co-exposure (post-hoc Tukey’s HSD, p =0.027 and p<0.0001 respectively) indicating an interaction effect (F(1,36)=4.21 and 5.18, p=0.048 and 0.029 respectively, Figures 5A-D). Consistent with our qPCR data, Vimentin protein expression was also significantly reduced (F(1,20)=9.535, p=0.006, Figure 5E). Interestingly, there was also a main effect of alcohol...
exposure on decreasing vimentin protein expression ($F_{(1,20)}=7.303, \ p=0.014$). We were unable to
detect expression of $CDH1$ transcript, or its E-Cadherin protein product, in extravillous
trophoblasts, consistent with previous reports (71).

In contrast to $\text{He}_\text{a}$$\text{miRNA}$ mimics, transfecting pooled antagonirs significantly increased
$VIM$ expression ($F_{(1,35)}=42.56, \ p<0.0001$). Likewise, there were interaction effects for $SNAI1$ and
$SNAI2$ ($F_{(1,35)}=10.31$ and $4.86, \ p=0.01$ and $p=0.034$ respectively), whereby antagonir
transfection increased expression of $\text{Snai2}$ in the context of $320\text{mg/dL}$ ethanol co-exposure
(post-hoc Tukey’s HSD, $p<0.0001$) and $\text{Snai1}$ under basal conditions (post-hoc Tukey’s HSD,
$p<0.0001$)(Figures 5F-I). Despite our qPCR data, we did not observe significant differences in
vimentin protein expression between treatment groups (Figure 5J). Collectively, our data
indicate that increased trophoblastic $\text{He}_\text{a}$$\text{miRNA}$ levels favors an epithelial phenotype, whereas
inhibiting their action promotes a mesenchymal phenotype.

Antagomirs prevent $\text{He}_\text{a}$$\text{miRNAs’}$ inhibition of EMT pathway members

We next investigated if pretreating trophoblasts with pooled $\text{He}_\text{a}$$\text{miRNA}$ antagonirs could
prevent inhibition of the EMT pathway caused by transfecting $\text{He}_\text{a}$$\text{miRNA}$ mimics. $CDH1$
expression in cytотrophoblasts transfected with control antagonir, and subsequently
transfected with control mimic, was not significantly different from those transfected with
$\text{He}_\text{a}$$\text{miRNA}$ antagonirs and $\text{He}_\text{a}$$\text{miRNA}$ mimics. However, $CDH1$ was significantly elevated in
cytotrophoblasts transfected with control antagonir and $\text{He}_\text{a}$$\text{miRNA}$ mimics (post-hoc Tukey’s
HSD, $n=10$ samples per group, $p=0.004$). Likewise, cells pre-transfected with $\text{He}_\text{a}$$\text{miRNA}$
antagomirs had significantly higher $SNAI1$ and $VIM$ expression than those pre-transfected with
control antagomir and subsequently transfected with \( \text{Hea} \text{miRNA} \) mimics (post-hoc Tukey’s HSD, n=10 samples per group, \( p=0.007 \) and \( p<0.0001 \) respectively) (Figure 6A-D).

Furthermore, VIM expression in extravillous trophoblasts pre-transfected with control antagomir, and subsequently transfected with control mimic, was not significantly different from those transfected with \( \text{Hea} \text{miRNA} \) antagomirs and \( \text{Hea} \text{miRNA} \) mimics. However, VIM, SNAI1, and SNAI2 expression were significantly reduced in cytotrophoblasts transfected with control antagomir and \( \text{Hea} \text{miRNA} \) mimics (post-hoc Tukey’s HSD, n=10 samples per group, \( p<0.0001 \), Figure 6E-H). Thus, our data suggest that pretreating cells with \( \text{Hea} \text{miRNA} \) antagomirs prevents inhibition of EMT pathway members resulting from transfection with \( \text{Hea} \text{miRNA} \) mimics in cytotrophoblasts and extravillous trophoblasts.

\( \text{Hea} \text{miRNAs} \) impair extravillous trophoblast invasion

Functionally, inhibition of the EMT pathway should reduce trophoblast invasiveness. Thus, we performed a transwell invasion assay using HTR8 extravillous trophoblasts transfected with \( \text{Hea} \text{miRNA} \) mimics and antagomirs. While ethanol exposure by itself did not impair trophoblast invasion (Supplementary Figure 6), there was a marginally significant interaction effect between ethanol exposure and \( \text{Hea} \text{miRNA} \) mimic transfection (\( F_{1,28}=3.418, \ p=0.075 \)). Thus, a planned comparison indicated that transfection with \( \text{Hea} \text{miRNA} \) mimics significantly reduced trophoblast invasion in the context of 320 mg/dL ethanol co-exposure, relative to the control mimics (\( t(14)=2.762, \ p=0.015 \)), consistent with our data demonstrating \( \text{Hea} \text{miRNAs} \) interfere with the EMT pathway (Figure 7A). Contrastingly, transfecting \( \text{Hea} \text{miRNA} \) antagomirs
increased invasion in the context of 320 mg/dL ethanol co-exposure, though this effect was only marginally significant (t(14)=1.805, p=0.093, Figure 7B).

HEa miRNAs retard trophoblast cell cycle progression

Given the proliferative nature of cytotrophoblasts, and the intimate relationship between EMT and cell cycle (72, 73), we assessed the effects of ethanol and HEa miRNAs on BeWO cytotrophoblast cell cycle. After pulse-labeling cells with the nucleic acid analog, EdU, for 1-hour, we found that individually transfecting 6 of the HEa miRNA mimics increased EdU incorporation (Unpaired t-test, p<0.05, FDR correction), suggesting an overall increased rate of DNA synthesis (Supplementary Figure 7A). Contrastingly, simultaneous transfection of HEa miRNAs significantly reduced EdU incorporation (F(1,26)=59.69, p<0.0001), mirroring the effects of increasing concentrations of ethanol (R²=0.304, p=0.012) (Supplementary Figure 7B and Figure 8A).

Consistent with the increased rates of DNA synthesis resulting from individual HEa miRNA mimic transfection, individual transfection of HEa miRNAs antagonirs generally reduced EdU incorporation, though only the antagonir to hsa-miR-760 did so significantly (t(110)=3.059, p=0.003, FDR correction) (Supplementary Figure 7A). Interestingly, simultaneous administration of antagonirs also reduced EdU incorporation, as observed with the pooled HEa miRNAs mimics (F(1,26)=34.83, p=0.0005, Figure 8B).

To further characterize the coordinated effect of HEa miRNAs on cytotrophoblast cell cycle, we pulse-labeled cells with EdU for 1-hour and, post-fixation, labelled them with 7AAD to segregate cells into three groups: G₀/G₁ (7AADlow, EDU-), S (EDU+), and G₂/M (7AADhigh, EDU-).
Both 120 mg/dL and 320 mg/dL ethanol exposures significantly decreased the proportion of cells in S-phase, while 320 mg/dL exposure increased the proportion of cells in G2/M-phase, consistent with the observed reduction in the rate of DNA synthesis (Supplementary Figure 7C).

Similar, to the effects of ethanol exposure, pooled hEa miRNA mimic administration also significantly decreased the proportion of cells in S-phase (F(1,28)=52.78, p<0.0001) while increasing the proportion of cells in G2/M-phase (F(1,28)=8.395, p=0.007) and exacerbated alcohol’s effects on the cell cycle (Figure 8C). Interestingly, pooled hEa miRNA antagonim administration also reduced the proportion of cells in S-phase (F(1,26)=14.98, p=0.0007) and increased the proportion of those in G2/M-phase (F(1,26)=12.38, p=0.002) (Figure 8D).

We sought to determine if pretreating cytotrophoblasts with pooled hEa miRNA antagonim could prevent the cytotrophoblast cell cycle retardation caused by transfecting hEa miRNA mimics. Cells transfected with the control or pooled hEa miRNA antagonim, and subsequently transfected with pooled hEa miRNA mimics, did not have significantly different cell cycle profiles or rates of DNA synthesis to each other but had significantly lower rates of DNA synthesis (ANOVA, F(2,12)=16.56, p=0.0004), and a decreased proportion of S-phase cells (F(2,12)=11.43, p=0.002) with an increased proportion of cells in G2/M phase (F(2,12)=11.47, p=0.002), compared to cells transfected with both the control antagonim and control mimic (Figures 9A and B). Collectively, our data indicate that transfection of these antagonims prevents further reduction in the rate of DNA synthesis, or cell cycle retardation, that would result from transfection with pooled hEa miRNA mimics.

hEa miRNAs have minimal effect on cell survival
We next sought to investigate whether ethanol- and HEa-miRNA-induced changes in cell cycle were related to an increase in cell death. Only the 320 mg/dL dose of ethanol exposure demonstrated a slight, but marginally significant effect, of increasing lytic cell death (t(18)=2.022, p=0.054), though there was no effect on apoptosis (Supplementary Figures 8A and B). However, the changes in cell cycle following transfection of individual or pooled HEa-miRNA mimics were not mirrored by changes in lytic cell death. Nevertheless, two HEa-miRNAs, hsa-mir-671-5p and hsa-mir-449a, did significantly increase apoptosis (Unpaired t-test, p<0.05, FDR correction) (Supplementary Figures 8C and D).

Contrastingly, transfection of 4 HEa-miRNA antagonists individually, significantly increased lytic cell death (Unpaired t-test, all p<0.05, FDR correction), with the antagonir to hsa-mir-491-3p also increasing apoptotic cell death (t(14)=3.383, p=0.004, FDR correction, Supplementary Figure 8C and D). Likewise, transfection of pooled HEa-miRNA antagonirs increased lytic cell death (F(1,36)=11.40, p=0.002) but did not cause increased apoptosis (Supplementary Figure 8E-H). Taken together, our data suggest that while ethanol exposure may increase cytotrophoblast death, increased levels of HEa-miRNAs have minimal effects on cell death, suggesting that their effect on cell cycle and the EMT pathway is independent of any effect on cell survival.

HEa-miRNAs modulate cytotrophoblast differentiation-associated Ca\textsuperscript{2+} dynamics. HEa-miRNAs’ effects on EMT pathway member expression, coupled with cell cycle retardation, indicates that HEa-miRNAs influence trophoblast maturation. Maturation of cytotrophoblasts into syncytiotrophoblasts is marked by dramatic changes in the cellular energetics profile (74). To model HEa-miRNAs’ effect on hormone-producing
syncytiotrophoblasts, we used a well-established protocol of forskolin induced syncytialization of BeWO cytотrophoblasts (75, 76). As expected, forskolin treatment induced fusion/syncytialization of cytотrophoblasts resulting in a greater average cell size in the forskolin + HEa miRNA mimics group ($F_{(1,386)}=4.386$, $p=0.037$). This suggests that the inhibition of EMT by these miRNAs may result in preferential syncytialization instead of differentiation to extravillous trophoblasts (Supplementary Figure 9A). Ethanol and forskolin treatment both increased baseline calcium levels, as indicated by the change in fluo-4 fluorescence ($F_{(1,426)}=5.593$ and 3.665 respectively, $p<0.0001$, Figure 10A, Supplementary Figures 9B-D). The effect of ethanol on baseline calcium was abrogated by HEa miRNAs while HEa miRNAs + forskolin was not significantly different to forskolin alone, indicating that forskolin and HEa miRNAs may be affecting similar calcium pathways. Syncytiotrophoblasts actively transport high amounts of calcium from maternal to fetal circulation, therefore forskolin-exposed trophoblasts likely have higher basal transport of calcium into the cells due to increased expression of plasma membrane channels (77). The conversion of trophoblasts to syncytiotrophoblasts is accompanied by an increase in endoplasmic reticulum, which could increase calcium buffering capabilities in response to ethanol-stress on the cells, thus HEa miRNA-induced syncytialization pathways may be protective against ethanol stress.

Adaptations to cellular stress can be seen in alterations to cellular energetics in response to ethanol, as ethanol-exposed BeWO cells showed decreased baseline and stressed oxygen consumption rates (OCR) ($F_{(1,28)}=15.55$ and 16.91, $p=0.0005$ and 0.0003 respectively) and increased extracellular acidification rates (ECAR) ($F_{(1,28)}=4.868$, $p=0.036$). However, HEa miRNAs had minimal effects on metabolic activity (Figures 10D-10G).
Extracellular ATP has been shown to inhibit trophoblast migration (78) and can directly stimulate increased intracellular calcium elevations through purinergic receptors ubiquitously present on trophoblasts (79). Both \textsuperscript{HEa}miRNA and ethanol administration significantly increased intracellular calcium in response to acute ATP administration ($F_{(1,426)}=10.34$ and $F_{(1,386)}=16.30$, $p=0.001$ and $p<0.0001$ respectively) (Figure 10B). This may be indicative of a lack of downregulation of purinergic receptors required in trophoblast migration as part of the interrupted EMT pathway. Forskolin-induced maturation decreased calcium response to ATP ($F_{(1,386)}=50.72$, $p<0.0001$) (Figure 10C) and prevented the \textsuperscript{HEa}miRNA-induced increase in ATP response. These data agree with previous studies showing increased nuclear trafficking of ionotropic receptor P2X7 and more localized P2X4 expression over placental development, which may decrease the overall calcium influx in response to ATP (80).

\textbf{\textsuperscript{HEa}miRNAs promotes syncytialization-dependent hormone production}

Transfection of \textsuperscript{HEa}miRNA mimics did not change \textit{CGA}, \textit{CGB}, or \textit{IGF2} transcript expression relative to the control in non-syncytialized trophoblasts. However, following syncytialization, \textsuperscript{HEa}miRNA mimics significantly increased expression of \textit{CGA} and \textit{CGB} (post-hoc Tukey’s HSD, $n=10$ samples per group, $p=0.001$ and 0.005 respectively). Consistent with our previous results, \textsuperscript{HEa}miRNA mimics also increased \textit{CDH1} expression in both cytotrophoblasts and syncytiotrophoblasts ($F_{(1,20)}=5.286$, $p=0.032$); there was also a main effect of syncytialization on \textit{CDH1} expression, as has been previously reported ($F_{(1,36)}=3.391$, $p=0.034$, Figures 11A-D).

Likewise, \textsuperscript{HEa}miRNAs increased E-cadherin protein expression ($F_{(1,20)}=5.286$, $p=0.032$), whereas forskolin decreased it ($F_{(1,20)}=10.24$, $p=0.005$) (Figure 11E). On the other hand, there was no
effect of HEαmiRNA antagonirs on CGA and CGB expression, although we did observe a decrease
in IGF2 transcript expression, following syncytialization, relative to controls (post-hoc Tukey’s
HSD, n=10 samples per group, p=0.001) (Figure 11F-I).

Given that HEαmiRNAs promotes syncytialization-dependent hormone production, we
next investigated maternal plasma levels of intact human chorionic gonadotropin (hCG) in our
Ukraine birth cohort. Plasma hCG levels were non-significantly increased in the second
trimester of HEα group mothers relative to their UE counterparts, consistent with previous
studies (81). During the third trimester, however, hCG levels remained significantly elevated in
HEα group mothers compared to the UE group (Median Test, n=23 samples in HEα group and
n=22 for HEua and HEa groups, p=0.03) (Figure 12). Furthermore, there was no significant
difference of gestational age at blood draw between the different groups indicating the
increased level of hCG in the HEα group was not confounded by gestational age at which blood
was sampled (Supplementary Figure 10) (82). Interestingly, both alcohol and hCG levels were
negatively associated with gestational age at delivery (GAD), with a significant interaction
between periconceptional alcohol exposure and hCG levels on GAD (Supplementary Table 3).
Taken together, our data suggests HEαmiRNAs may contribute to PAE-dependent increases in
hCG levels during pregnancy.

HEαmiRNAs reduce fetal growth

To investigate the functional consequences of elevated circulating HEαmiRNA levels, we
administered miRNA mimics for the 8-mouse homologue HEαmiRNAs, or a negative control
mimic, through tail-vein injection to pregnant mouse dams on GD10. On GD18, growth
parameters of male and female fetuses were assessed separately, and data from all same-sex fetuses from a single pregnancy were averaged into one data point. Dams administered HEa miRNA mimics produced smaller fetuses than those administered control mimics, according to all collected measures of fetal size: fetal weight ($F_{(1,17)}=9.92$, $p=0.006$), crown-rump length ($F_{(1,17)}=9.89$, $p=0.006$), snout-occipital distance ($F_{(1,17)}=9.09$, $p=0.008$), and biparietal diameter ($F_{(1,17)}=5.99$, $p=0.026$) (Figure 13E). Interestingly, placental weights were also significantly reduced in mice treated with HEa miRNA mimics ($F_{(1,17)}=6.92$, $p=0.018$) (Figure 13F).

Following tail-vein administration of two human-specific sentinel miRNAs, miR-518f-3p and miR-519a-3p, we found a high biodistribution of both miRNAs in the placenta, comparable to levels seen in the liver and spleen (Supplementary Figure 1A and 1B). Thus, to determine whether HEa miRNA’s effects on fetal growth could result from their actions on the placenta, we quantified the placental expression of core EMT members in the GD18 placentas of control and HEa miRNA fetuses. HEa miRNA administration significantly reduced expression of mesenchymal-associated transcript VIM ($F_{(1,14)}=14.23$, $p=0.002$) and SNAI2 ($F_{(1,14)}=5.99$, $p=0.028$) with a significant sex by HEa miRNA interaction effect on SNAI1 ($F_{(1,66)}=5.55$, $p=0.034$) and CDH1 ($F_{(1,14)}=6.01$, $p=0.028$) (Figures 14A-E). Interestingly, and in line with our in vitro findings whereby HEa miRNAs promoted syncytialization dependent cell fusion and hCG production, HEa miRNA administration significantly increased expression of the mRNA transcript for SynB, a gene that is important for syncytiotrophoblast maturation ($F_{(1,66)}=4.11$, $p=0.047$) (Figure 14F).
Discussion

In a prior study, we reported that gestational elevation of 11 maternal plasma miRNAs, in a Ukrainian clinical cohort, predicted which PAE infants would exhibit adverse outcomes at birth (11). These HEa miRNAs were elevated throughout mid and late-pregnancy, encompassing critical periods for fetal development, and were predicted to target key developmental pathways (11). Given that HEa miRNAs are placentally enriched and their dysregulation is associated with placental-associated gestational pathologies, we sought to determine if they also contributed to the pathophysiology of FASDs.

To mimic the alcohol consumption patterns in our Ukrainian cohort, we adopted moderate alcohol self-administration paradigms during mouse and macaque gestation. Though these species share a hemochorial placenta, fundamental differences in placental anatomy remain. Specifically, macaque trophoblasts exhibit deeper invasion into the uterine horn than their mouse counterparts (83-86). Despite these differences, we found PAE inhibited mesenchymal transition-associated mRNA transcripts in both our models, indicating a conserved effect of PAE on placental development. Additionally, we found that HEa miRNAs mediated the effects of PAE on core EMT pathway members in the placenta, and collectively though not individually, inhibited EMT in human cytotrophoblast and extravillous trophoblast culture models. The components of the EMT pathway were selected for assessment based on a substantial literature that implicates these as core EMT components (13, 17, 18, 67-70). However, analysis of their 3’UTRs indicates that these are unlikely to be the direct targets of HEa miRNA action. Additional studies will be needed to dissect out the signaling networks that connect HEa miRNAs to the assessed EMT components.
Interestingly, HEa miRNAs also promoted syncytialization (forskolin)-dependent hCG expression, mirroring the elevation of third trimester maternal hCG levels in the PAE group within our Ukrainian birth cohort. This late-gestation elevation of hCG levels may serve as a compensatory mechanism to prevent the preterm birth associated with PAE, as hCG during late gestation is hypothesized to promote uterine myometrial quiescence (87, 88). In support of this hypothesis, we found significant negative associations between both hCG levels and alcohol consumption with gestational age at delivery. Furthermore, there was a significant interaction between periconceptional alcohol exposure and hCG levels, with higher hCG levels corresponding to a smaller effect of alcohol exposure at conception on gestational age at delivery, indicating that hCG moderates the effect of alcohol on age at delivery (Supplementary Table 3).

Since HEa miRNAs collectively prevented trophoblast EMT, we hypothesized that, as a functional consequence, these maternal miRNAs would also inhibit fetal growth. When we delivered 8 out of the 11 HEa miRNAs known to be present in mouse, to pregnant dams during the period of placental branching morphogenesis and endometrial invasion, when EMT is particularly active, we found that HEa miRNAs reduced fetal growth. Importantly, ethanol exposure during this period has also been shown to result in fetal growth deficits and dysmorphia in rodent PAE models (89, 90) suggesting that maternal miRNA-mediated deficits in trophoblast invasion may mediate some of the effects of PAE on fetal growth. In support of this, we found placentas from the HEa miRNA treated group had impaired expression of core EMT pathway members. It is also feasible that HEa miRNAs disrupt fetal growth through placental vascular dynamics. The non-human primate tissue analyzed here was derived from animals that
were characterized in vivo using MRI and ultrasound imaging, which demonstrated that maternal blood supply to the placenta was lower in ethanol-exposed animals compared to controls, and that oxygen availability to the fetal vasculature was reduced (91). Thus, one possibility is that compromised trophoblast invasion contributes to impaired maternal blood flow in ethanol-exposed individuals, as we have also previously observed in mouse models (92).

Impaired nutrient uptake in the placenta, such as dysfunction in the amino acid, glucose, and fatty acid transport systems have also been implicated in gestational pathologies, such as intrauterine growth restriction (93). Furthermore, in vitro studies in trophoblasts have shown alcohol exposure selectively impairs a subset of these amino acid and fatty acid transport systems (94, 95) whilst in vivo, PAE in rodents alters expression of glucose transporters in the late gestation placenta (23). These placental dysfunctions could also have insidious effects on development that are not immediately detectable at birth. As an example, perturbations in placental iron transport by PAE may impair central nervous system development during the neonatal period, leading to cognitive deficits that last throughout adulthood (96, 97). Thus, further investigation is warranted to determine if HEa miRNAs contribute to this alcohol induced impairment in trans-placental nutrient transport, and ultimately whether this impairment of nutrient transport represents another avenue through which PAE disrupts fetal development.

It is likely that HEa miRNAs may mediate other pregnancy associated pathologies, aside from PAE. We identified numerous studies that reported increased circulating and placental levels of at least 8 out of 11 HEa miRNAs in gestational pathologies arising from placental dysfunction. For example, elevated levels of one HEa miRNA, miR-519a-3p, a member of the placentally-expressed C19MC family cluster, was reported in placentae of patients with pre-
eclampsia, recurrent spontaneous abortion, and intrauterine growth restriction (40, 41, 54, 55).

Interestingly, collective overexpression of the 59 C19MC miRNAs inhibits trophoblast migration, explaining their enrichment in the non-migratory villous trophoblasts and suggests their downregulation is necessary for maturation into invasive extravillous trophoblasts (98). Thus, a greater understanding of the placental roles of HEa miRNAs may also help disentangle the etiology of other pregnancy complications.

While our data suggest that the placenta is an important contributory source for circulating HEa miRNAs, the effects of HEa miRNAs likely extend beyond the placenta into other maternal tissues. Outside of the placenta, HEa miRNAs may circulate in association with lipoprotein complexes, or be encapsulated in extracellular vesicles (EVs) whose abundance dramatically increases during pregnancy (99, 100). Recent work has demonstrated that placental-derived EVs can regulate the maternal immune system as well as confer viral resistance to recipient cells, including endothelial cells (101-104). Interestingly, other studies also have identified endothelial cells as ready recipients for placental-derived EVs, suggesting placental-EVs and their cargo miRNAs participate in synchronizing the growth of the placenta with the accompanying maternal and fetal vascular remodeling (99, 105). While further investigation is warranted into the role of EVs and miRNAs in maternal-fetal communication, and to the extent they serve as paracrine/endocrine mediators for the placenta during development, targeting HEa miRNAs will likely have profound effects for both fetal and placental development in the context of FASDs and other gestational pathologies.

While we did not investigate the effects of PAE on EMT in non-placental organs, it is likely that PAE broadly disrupts EMT in multiple fetal compartments. Developmental ethanol
exposure has been shown inhibit the EMT-dependent migration of neural crest progenitors involved in craniofacial development, explaining the facial dysmorphology seen in FAS and FASDs (106, 107). Outside of its effects on the neural crest, PAE is significantly associated with various congenital heart defects, including both septal defects and valvular malformations (108-111). Septation of the heart, primarily atrial septation, is an EMT-dependent process, with endocardial cells delaminating and transdifferentiating into mesenchymal cells that populate the endocardial cushions as well as the muscular cap, which functions as the growing edge of the muscular atrial septum(112, 113). The mesenchymal cells from the endocardial cushions also form the cardiac valve progenitors. Thus, disruption of endocardial EMT could explain both the valvular and septal malformation associated with PAE.

Collectively, our data on HEa miRNAs suggest miRNA-based interventions could minimize or reverse developmental effects of PAE and other placental-related pathologies. miRNA-based therapeutic approaches have been advanced for other disease conditions. For example, Miraversen™, an anti-miR-122 antisense oligonucleotide, has been shown to be effective in reducing hepatitis-C viral burden (114). Additionally, a recent phase-1 clinical trial report indicated that liposomal delivery of a tumor-suppressor miRNA was well tolerated in human populations and showed evidence of therapeutic efficacy (115). However, we also observed with HEa miRNAs that the effects of combinations of miRNAs are not a sum of their individual effects. Functional synergy between clusters of co-regulated miRNAs may be a common feature in development and disease. For instance, in 2007, we presented early evidence that ethanol exposure reduced miR-335, -21, and -153 in neural progenitors and that coordinate reduction in these miRNAs yielded net resistance to apoptosis following ethanol exposure (116). In that
study, we also showed that coordinate knockdown of these three miRNAs was required to
induce mRNA for Jagged-1, a ligand for the Notch cell signaling pathway, an outcome that was
not recapitulated by knocking down each miRNA individually (116). More recently, combined
administration of miR-21 and miR-146a has been shown to be more effective in preserving
cardiac function following myocardial infarction than administration of either of these miRNAs
alone (117). While miRNA synergy has not been explored in detail, these data show that new
biology may emerge with admixtures of miRNAs, and that therapeutic interventions may
require the use of such miRNA admixtures rather than single miRNA molecules as have been
used in clinical studies to date. Our study was restricted to exploring the effects of the 11
HE\(\text{a}\) miRNAs on development, which represent only the significantly increased fraction of miRNAs
elevated in the HE\(\text{a}\) group maternal plasma within our Ukrainian cohort. Indeed, the next 5
most elevated miRNAs are also abundantly expressed in the placenta (data not shown),
indicating they may also mediate the effects of PAE on placental biology.

In conclusion, we have observed how a set of 11 miRNAs, predictive of adverse infant
outcomes following PAE, collectively mediate the effects of alcohol on the placenta. Specifically,
elevated levels of these miRNAs promote an aberrant maturational phenotype in trophoblasts
by inhibiting core members of the EMT pathway and promoting syncytialization-dependent
hormone production. Functionally, these miRNAs are clinically correlated with measures of
fetal development and directly cause intrauterine growth restriction when administered in vivo.
Our work suggests that a greater understanding for the role of HE\(\text{a}\) miRNAs during development,
and their role in coordinating the EMT pathway in the placenta and other developing tissues,
will benefit the understanding of FASDs and other gestational pathologies and potentially lead
to effective avenues for intervention.
Methods

Mouse model of PAE:
C57/BL6J mice (Jackson Laboratory, Bar Harbor, ME) were housed under reverse 12-hour dark / 12-hour light cycle (lights off at 08:00 hours). PAE was performed using a previously described limited access paradigm of maternal drinking (118, 119). Briefly, 60-day old female mice were subjected to a ramp-up period with 0.066% saccharin containing 0% ethanol (2 days), 5% ethanol (2 days), and finally 10% ethanol for 4-hours daily from 10:00–14:00 beginning 2 weeks prior to pregnancy, continuing through gestation (Supplementary Figure 2A). Female mice offered 0.066% saccharin without ethanol during the same time-period throughout pregnancy served as controls. Tissue from the labyrinth, junctional, and decidual zone of male and female gestational day 14 (GD14) placentae were microdissected, snap-frozen in liquid nitrogen, and stored at -80°C preceding RNA and protein isolation.

Mouse model for HEa miRNA overexpression:
For systemic administration of miRNAs, previously nulliparous C57/BL6NHsd dams (Envigo, Houston, TX) were tail-vein-injected on GD10 with either 50 μg of miRNA miRVana™ mimic negative control (Thermo Fisher, Waltham, MA, Cat No. 4464061) or pooled HEa miRNA miRVana™ mimics in In-vivo RNA-LANCEr II (Bioo Scientific, Austin, TX, 3410-01), according to manufacturer instructions. The 50 μg of pooled HEa miRNA mimics consisted of equimolar quantities of mmu-miR-222-5p, mmu-miR-187-5p, mmu-mir-299a, mmu-miR-491-3p, miR-760-3p, mmu-miR-671-3p, mmu-miR-449a-5p, and mmu-miR-204-5p mimics. For bio-distribution studies, 50 μg of pooled equimolar quantities of hsa-miR-519a-3p and hsa-miR-518f-3p mimics were
injected via tail vein. These human miRNAs were selected because no mouse homologs are known to exist and consequently, estimates for organ distribution of exogenous miRNAs in the mouse are unlikely to be contaminated by the expression of endogenous murine miRNAs. GD10 is a time point near the beginning of the developmental period of branching morphogenesis, immediately following chorioallantoic attachment, during which the placenta invades the maternal endometrium (120). At GD18, pregnancies were terminated with subsequent quantification of fetal weight, crown-rump length, snout-occipital distance, biparietal diameter, and placental weight (Figure 13A). Subsequently, tissue was snap-frozen in liquid nitrogen, and stored at -80°C preceding RNA isolation.

Rat model of PAE:

Outbred nulliparous Sprague-Dawley rats were housed under a 12-hour light/12-hour dark cycle. PAE in Sprague-Dawley was conducted according to our previously published exposure paradigm (23, 121). Briefly, dams were given a liquid diet containing either 0% or 12.5% ethanol (vol/vol) from 4 days prior to mating until GD4 (Supplementary Figure 2B). Dams had ad libitum access to the liquid diet 21-hours daily and consumed equivalent calories. Water offered during the remaining 3-hours of the day. On GD5, liquid diets were removed and replaced with standard laboratory chow. On GD20, placentas were immediately separated into the labyrinth and junctional zone, snap frozen in liquid nitrogen and stored at –80 °C preceding RNA isolation.

Non-human primate model of PAE:
As previously described in detail (91), adult female rhesus macaques were trained to orally self-administer either 1.5 g/kg/d of 4% ethanol solution (equivalent to 6 drinks/day), or an isocaloric control fluid prior to time-mated breeding. Each pregnant animal continued ethanol exposure until gestational day 60 (GD60, term gestation is 168 days in the rhesus macaque) (122).

Pregnancies were terminated by cesarean section delivery at three different time points; GD85, GD110, or GD135 (Supplementary Figure 2C). The macaque placenta is typically bi-lobed with the umbilical cord insertion in the primary lobe and bridging vessels supplying the fetal side vasculature to the secondary lobe (Figure 2D showing gross placenta anatomy) (123). Full thickness tissue biopsies (maternal decidua to fetal membranes) were taken from both the primary and secondary lobes of the placenta (Figure 2E showing H&E section of placenta).

Samples were immediately snap-frozen in liquid nitrogen and stored at -80°C preceding RNA isolation.

**Cell culture trophoblast models:**

BeWO human cytotrophoblastic choriocarcinoma cells and HTR-8/SVneo extravillous cells were sourced from ATCC (Manassas, VA, Cat No. CCL-98 and CRL-3271 respectively). BeWO cells were maintained in HAM’s F12 media containing penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% vol/vol fetal calf serum (FCS) at 37°C and 5% CO2. HTR8 cells were maintained in RPMI-1640 media with 5% vol/vol FCS, under otherwise identical conditions. Culture medium was replenished every 2 days and cells sub-cultured every 4-5 days.

BeWO cells were treated with 20 μm forskolin to induce syncytialization, as previously described (124, 125). BeWO and HTR8 cells were also subjected to four separate ethanol
treatment conditions: 0 mg/dL, 60 mg/dL (13 mM), 120 mg/dL (26 mM) or 320 mg/dL (70 mM).

To achieve HEa-miRNA overexpression and inhibition, Dharmacon miRIDIAN™ miRNA mimics and hairpin inhibitors [25 nM], or control mimic (Dharmacon, Lafeyette CO, Cat No. CN-001000-01-05) and hairpin inhibitor (Dharmacon, Cat No. CN-001000-01-05) [25nm], were transfected into subconfluent BeWO and HTR8 cells using RNAIMAX lipofection reagent (Thermo Fisher, Cat No. 13778).

Cell cycle analysis:
At 48-hours-post transfection, BeWO cells were pulsed with 10 μM EdU for 1-hour. Cells were immediately harvested, and cell cycle analysis was performed with the Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (Thermo Fisher, Cat No. C10420), in conjunction with 7-Amino-Actinomycin D (Thermo Fisher, Cat No. 00-6993-50), according to manufacturer instructions, using the Beckman Coulter® Gallios 2/5/3 Flow Cytometer. Data was analyzed using Kaluza software (Beckman Coulter, Brea, CA).

Cell death analysis:
BeWO cell culture was harvested 48-hours post transfection media was subjected to lactate dehydrogenase (LDH) detection using the Pierce™ LDH Cytotoxicity Assay Kit (Thermo Fisher, Cat No. 88953), according to manufacturer instructions, for lytic cell death quantification. The Promega Caspase-Glo® 3/7 Assay Systems (Promega, Madison, WI, Cat No. G8091) was used to quantify apoptotic cell death
Invasion assay:

At 24-hours post-transfection and/or ethanol exposure, HTR8 cells were serum starved for an additional 18-hours. Subsequently, HTR8 cells were seeded onto trans-well permeable supports precoated with 300 μg/mL Matrigel (Corning, Corning, NY, Cat No. 354248). After 24-hours, cells remaining in the apical chamber were removed with a cotton swab. Cells that invaded into the basal chamber were incubated with 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 3-hours, and the precipitate solubilized with 10% SDS in 0.01N HCl. Absorbance intensities were read at 570 nm in a Tecan Infinite® 200 plate reader.

Metabolic flux analysis and calcium imaging:

BeWO cells (10,000/well) were plated into Seahorse XF96 Cell Culture Microplates (Agilent Biotechnology, Cat No. 103275-100). The oxygen consumption rate (OCR), a measure of mitochondrial respiration, and extracellular acidification rate (ECAR), a measure of glycolysis, were measured using the Seahorse XFe96 flux analyzer (Seahorse Bioscience, North Billerica, MA). At the time of assay, cell culture medium was replaced with the appropriate pre-warmed Seahorse XF Base Medium (Agilent Biotechnology, Santa Clara, CA, Cat No. 102353-100). OCR and ECAR parameters were measured using the Seahorse XFp Cell Energy Phenotype Test Kit™ (Agilent Biotechnology, Cat No. 103275-100). Metabolic stress was induced by simultaneous treatment with 1μM Oligomycin and 0.125μM Carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone (FCCP).

BeWO cells were also plated onto glass coverslips in 24 well plates at a density of 30,000 cells/well. After exposure to ethanol and/or forskolin in culture, cells were prepared for calcium
imaging. After replacement of culture media with external imaging media (154 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 0.5 mM MgCl₂, 5 mM glucose, 10 mM HEPES, pH 7.4), cells were loaded for 35 minutes at 37°C with the calcium indicator dye fluo-4 AM (Thermo Fisher Scientific, Cat No. F14201), at a final concentration of 5µM fluo-4 AM in 0.1% DMSO. After incubation, cells were washed to remove remaining extracellular fluo-4 and imaged at 40x using confocal microscopy (FV1200-equipped BX61WI microscope, Olympus Corporation, Center Valley, PA). Time-lapse images were acquired at a frequency of 0.5Hz. Individual cells were manually outlined and area and mean fluorescence intensity were obtained for each cell (FIJI image processing package)(126). To determine the functional calcium range of each cell, at the end of imaging, cells were exposed to 5 µM ionomycin and 10 mM EGTA (0mM external Ca²⁺, \( F_{\text{range}} = F_{\text{ionomycin}} - F_{\text{EGTA}} \)). Baseline fluorescence was determined by averaging the lowest 5 consecutive fluorescence values during the initial 5 minutes \( (F_{\text{baseline}}) \) which was then expressed as a percentage of \( F_{\text{range}} \) \( (\Delta F_{\text{baseline}} = (F_{\text{baseline}} - F_{\text{EGTA}})/F_{\text{range}} \times 100) \). Maximal intracellular calcium response to 100 µM ATP was determined by averaging the highest 3 consecutive fluorescence values during ATP application \( (F_{\text{ATP}}) \) and determining the amount of fluorescence as a percentage of \( F_{\text{range}} \) \( (\Delta F_{\text{ATP}} = (F_{\text{ATP}} - F_{\text{EGTA}})/F_{\text{range}} \times 100) \).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis:

Total RNA was extracted from tissue, well as BeWO and HTR8 cells, using the miRNeasy Mini kit (Qiagen, Cat No. 217004). For miRNA qPCR assays, cDNA was synthesized from 200 ng of total RNA using the miRCURY LNA Universal RT cDNA synthesis kit (Exiqon, Cat No. 203301/Qiagen, Cat No. 339340, Germantown, MD) and expression was assessed using miRCURY LNA SYBR
Green (Exiqon, Cat No. 203401/Qiagen, Cat No. 339345). For mRNA qPCR assays, cDNA was synthesized from 500 ng of total RNA using the qScript™ cDNA Synthesis Kit (Quanta/Qiagen, Cat No. 95047). Gene expression analysis was performed using PerfeCTa SYBR Green FastMix (Quanta, Cat No. 95073) on the ViiA 7 Real-Time PCR System (Thermo Fisher Scientific). The data presented correspond to the mean 2^ΔΔCt after being normalized to the geometric mean of β-actin, Hypoxanthine-Guanine Phosphoribosyltransferase 1 (HPRT1), and 18s rRNA. Expression data for miRNA was normalized to the geometric mean of miR-25-3p, miR-574-3p, miR-30b-5p, miR-652-3p, and miR-15b-5p. For each primer pair, thermal stability curves were assessed for evidence of a single amplicon and the length of each amplicon was verified using agarose gel electrophoresis. A list of primers and their sequences is presented in Supplementary Table 1.

Western immunoblotting analysis:
Protein was extracted using 1X RIPA lysis buffer (Millipore Sigma, Burlington MA) supplemented with Halt protease inhibitor cocktail (Thermo Fisher Scientific). Tissue was homogenized using the Branson Sonifier 150. Protein concentration was determined using Pierce BCA protein assay kit (Thermo Fisher Scientific) and 30 μg of protein was loaded onto a 4-12% Bis-Tris (Invitrogen/Thermo Fisher Scientific, Cat No. NPO323BOX), size fractionated at 200 V for 35 minutes, and transferred to a PVDF membrane using the iBlot transfer system (Invitrogen/Thermo Fisher Scientific). Blots with protein from cultured cells were blocked with 5% nonfat dry milk in tris-buffered saline containing Tween®-20 (TTBS) for 1-hour and incubated overnight with primary antibody. The blot was then washed and incubated with an HRP-conjugated goat anti-rabbit or anti-mouse IgG (Invitrogen) at dilution 1:1000 for 1-hour,
then developed using PerkinElmer Western Lightning Plus Chemi ECL (PerkinElmer; Waltham, MA) and visualized using a CCD camera (Fluorchem Q, Alpha Innotech; San Leandro, CA). Blots with protein from homogenized tissue were dried overnight, rehydrated in methanol, stained with REVERT™ Total Protein Stain and developed with the Odyssey CLx Imaging System (LI-COR, Lincoln, NE). Blots were then blocked with Odyssey® Blocking Buffer (TBS) for 1h and incubated overnight with primary antibody. The blot was then washed and incubated with IRDye® 800CW secondary antibody (LI-COR, Cat No. 925-32210). The following antibodies were used: β-Actin HRP (Santa Cruz Biotechnology, Cat No. sc-47778); Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP (Thermo Fisher, Cat No. 62-6520); Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP (Thermo Fisher, Cat No. 65-6120); purified Mouse Anti-E-Cadherin (BD Biosciences, Cat No. 610181), Rabbit anti-vimentin antibody [EPR3776] (Abcam, Cat No. ab24647). Protein levels were quantified using the densitometric analysis package in FIJI image processing software (126).

ELISA:
The 2nd and 3rd trimester maternal plasma samples were collected as part of a longitudinal cohort study conducted in two regions of Western Ukraine as part of the Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD.org) between the years 2006 and 2011, as previously reported(11). Plasma, at a 1:1000 dilution, was subjected to hCG detection using Abcam’s intact human hCG ELISA kit (Cat no. ab100533) following the manufacturer’s protocol.

Literature Review
We conducted a literature review for HEαmiRNAs and their associated gestational pathology using the National Institute of Health’s Pubmed search interface. For each miRNA, the following search parameters were used:

\[ \text{[miRX OR miR X OR miRNA X OR miRNAX or miRNX]} \text{ AND MeSH Term} \]

where X represents the miRNA of interest and automatic term expansion was enabled. The following MeSH terms, and related search terms (in brackets), were used: Fetal Growth Retardation [Intrauterine Growth Retardation, IUGR Intrauterine Growth Restriction, Low Birth Weight, LBW, Small For Gestational Age, SGA], Premature Birth [Preterm Birth, Preterm Birth, Preterm Infant, Premature Infant, Preterm Labor, Premature Labor], Spontaneous Abortion [Early Pregnancy Loss, Miscarriage, Abortion, Tubal Abortion, Aborted Fetus], Pre-Eclampsia [Pre Eclampsia, Preeclampsia, Pregnancy Toxemia, Gestational Hypertension, Maternal Hypertension], and Maternal Exposure [Environmental Exposure, Prenatal Exposure]. Returned articles were subsequently assessed for relevance.

Secondary analysis of RNA sequencing data:

Expression levels of HEαmiRNAs in tissues were determined using the Human miRNA Expression Database and the miRmine Human miRNA expression database(66, 127). For expression analysis of HEαmiRNA pri-miRNAs, RNA sequencing data was used from NCBI’s sequence read archive (https://www.ncbi.nlm.nih.gov/sra). The accession numbers for the sequence files are: uterus (SRR1957209), thyroid (SRR1957207), thymus (SRR1957206), stomach (SRR1957205), spleen (SRR1957203), small intestine (SRR1957202), skeletal muscle (SRR1957201), salivary gland (SRR1957200), placenta (SRR1957197), lung (SRR1957195), liver (SRR1957193), kidney
(SRR1957192), heart (SRR1957191), whole brain (SRR1957183), adrenal gland (SRR1957124), bone marrow (ERR315396), colon (ERR315484), adipose tissue (ERR315332), and pancreas (ERR315479). Deep sequencing analysis was conducted using the Galaxy version 15.07 user interface according to the bioinformatics pipeline outlined in Supplementary Figure 1.

Statistical analyses:

Linear regression models were used to estimate associations between infant growth measures and miRNA expression levels, gestational age at blood draw, the interaction between subject-centered miRNA expression level and gestational age at blood draw, and child sex. Spearman correlations between infant growth measures and subject-centered miRNA expression levels were also calculated. Linear regression models were also used to estimate the associations between gestational at birth and log-transformed hCG levels, ethanol intake, the interaction between log-transformed hCG levels and ethanol intake, gestational at blood draw, and child sex. Statistical Analysis and graphs were generated with GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA), SPSS v24, or R version 3.3.1. Results are expressed as the mean ± SEM, or alternatively as box-and-whisker plots with the bounds of the box demarcating limits of 1st and 3rd quartile, a median line in the center of the box, and whiskers representing the total range of data. The overall group effect was analyzed for significance using 1-way MANOVA, 1-way or 2-way ANOVA with Tukey’s Honest Significance Difference (HSD) post-hoc testing when appropriate (i.e. following a significant group effect in 1-way ANOVA or given a significant interaction effect between experimental conditions in 2-way ANOVA), to correct for a family-wise error rate. A 2-tailed Student’s t-test was used for planned comparisons. For experiments
characterizing the individual effects of HEa miRNAs against the control miRNA or antagonirs, individual 2-tailed Student’s t-test with 5% FDR correction was applied to account for multiple comparisons. All statistical tests, sample-sizes, and post-hoc analysis are appropriately reported in the results section. A value of p < 0.05 was considered statistically significant, and a value of 0.1 < p < 0.05 was considered marginally significant.

Study approval:

Human study protocols were approved by Institutional Review Boards at the Lviv National Medical University, Ukraine, and the University of California San Diego as well as Texas A&M University in the USA. Research was conducted according to the principles expressed in the Declaration of Helsinki with written informed consent received from participants prior to inclusion in the study. All rodent experiments were performed in accordance with protocols approved by the University of New Mexico Institutional Animal Care and Use Committee (IACUC), and the Texas A&M University IACUC. All procedures involving non-human primate research subjects were approved by the IACUC of the Oregon National Primate Research Center (ONPRC), and guidelines for humane animal care were followed. The ONPRC abides by the Animal Welfare Act and Regulations enforced by the US Department of Agriculture.

Author contributions:

AT, RM and CC conceived of and planned the study. AT, AM, and NS designed and conducted cell culture studies, and AT conducted in vivo, murine miRNA overexpression studies and analyzed tissues from mouse, rat and primate PAE models. AA developed the mouse PAE model.
and LA and KM developed the rat PAE model and provided tissues. VR, NN, CK and KG developed the non-human primate model of PAE and provided RNA from microdissected tissues. AW and CC performed statistical analyses of human studies. AT, AM and RCM collaborated on all other statistical analyses. AT, AM, AW, NS, AA, VR, NN, CK, KG, CC, and RM collaborated in preparing the manuscript.
Table 1: HeA miRNAs are significantly correlated with independent measures of infant size

The correlation of 2nd and 3rd trimester maternal plasma HeA miRNA levels with independent measures of infant size. HeA miRNAs and their significantly correlated sex and gestational age-adjusted growth parameters appear in red. *p<0.05, **p<0.01.

| MMAT # | miRNA      | Trimester | Weight | Height | Head Circumference |
|--------|------------|-----------|--------|--------|--------------------|
|        |            |           | Sig.   | R²     | p                  | Sig.   | R²     | p                  |
| MMAT0004569 | hsa-miR-222-3p | 2         | 0.621  | 1.224  | 0.051             | 0.066  | 9.572  | 0.179             | 0.805  | 1.732  | 0.104             |
| MMAT0004561 | hsa-miR-187-5p | 2         | 0.462  | 6.347  | 0.088             | 0.17   | 12.407  | 0.074           | 0.134  | 15.903  | 0.103             |
| MMAT0005887 | hsa-miR-299-3p | 2         | 0.582  | 1.113  | 0.029             | 0.066  | 6.296  | 0.203           | 0.039  | 8.65   | 0.1               |
| MMAT0004765 | hsa-miR-491-5p | 2         | 0.172  | 3.61   | 0.112             | 0.849  | 2.033  | 0.055           | 0.024  | 12.529  | 0.156             |
| MMAT0004948 | hsa-miR-885-5p | 2         | 0.142  | 4.227  | 0.174             | 0.044  | 7.657  | 0.231           | 0.059  | 1.36   | 0.115             |
| MMAT0002842 | hsa-miR-518-3p | 2         | 0.246  | 2.517  | 0.134             | 0.918  | 2.134  | 0.118           | 0.007  | 14.511  | 0.216             |
| MMAT0004957 | hsa-miR-760 | 2         | 0.059  | 6.314  | 0.195             | 0.22   | 4.966  | 0.079           | 0.055  | 10.158  | 0.185             |
| MMAT0003880 | hsa-miR-671-5p | 2         | 0.133  | 7.24   | 0.111             | 0.578  | 5.284  | 0.031           | 0.073  | 10.704  | 0.107             |
| MMAT0001541 | hsa-miR-241 | 2         | 0.191  | 11.564 | 0.104             | 0.718  | 5.851  | 0.072           | 0.173  | 10.036  | 0.068             |
| MMAT0002859 | hsa-miR-204-5p | 2         | 0.028* | 12.377 | 0.184             | 0.272  | 4.973  | 0               | 0.131  | 7.095   | 0.108             |
| MMAT0002869 | hsa-miR-519a-3p | 2         | 0.034* | 7.975  | 0.153             | 0.403  | 6.83   | 0.012           | 0.063  | 8.181   | 0.096             |
| MMAT0004569 | hsa-miR-157-5p | 3         | 0.875  | 0.993  | -0.046            | 0.018* | 10.705  | -0.186          | 0.577  | 4.656   | -0.31             |
| MMAT0004561 | hsa-miR-157-5p | 3         | 0.536  | 2.055  | 0.049             | 0.37   | 2.029  | -0.109          | 0.704  | 3.497   | 0.002             |
| MMAT0003880 | hsa-miR-518-3p | 3         | 0.511  | 0.762  | 0.035             | 0.514  | 1.769  | 0.072           | 0.87   | 3.786   | 0.077             |
| MMAT0004498 | hsa-miR-885-3p | 3         | 0.824  | 3.155  | -0.028            | 0.2    | 12.122  | -0.121          | 0.747  | 4.188   | -0.081            |
| MMAT0002842 | hsa-miR-518-3p | 3         | 0.877  | 0.148  | 0.029             | 0.102  | 4.686  | -0.158          | 0.376  | 5.009   | 0.032             |
| MMAT0004569 | hsa-miR-157-5p | 3         | 0.015  | 2.098  | 0.109             | 0.421  | 1.715  | 0.016           | 0.245  | 7.817   | 0.182             |
| MMAT0004957 | hsa-miR-700 | 3         | 0.398  | 1.396  | 0.141             | 0.781  | 0.716  | 0.322           | 0.207  | 0.052   | 0.172             |
| MMAT0003880 | hsa-miR-671-5p | 3         | 0.055  | 8.715  | 0.155             | 0.367  | 3.521  | -0.133          | 0.076  | 8.196   | 0.15              |
| MMAT0001541 | hsa-miR-448a | 3         | 0.995  | 0.085  | -0.006            | 0.082  | 0.876  | -0.151          | 0.002  | 12.022  | 0.135             |
| MMAT0002859 | hsa-miR-204-5p | 3         | 0.019* | 11.672  | 0.23            | 0.208  | 5.689  | 0.022           | 0.050** | 18.693  | 0.319             |
| MMAT0002859 | hsa-miR-519a-3p | 3         | 0.391  | 2.62  | 0.043             | 0.302  | 5.917  | -0.151         | 0.106  | 9.266   | 0.118             |
Figure 1

1A) Venn diagram on number of miRNAs reported to be associated with alcohol or other prenatal exposures.

1B) Venn diagram on number of miRNAs reported to be associated with different gestational pathologies. Inset colored circles represent the corresponding sex and gestational age-adjusted growth parameters these miRNAs were correlated with. Of the 22 studies queried, 11 (50%) utilized unbiased screenings for miRNA expression.

1C) Heatmap of mature miRNA expression and 1D) pri-miRNA expression across different tissues resulting from secondary analysis of publicly available RNA-sequencing data. Legend depicts row-centered Z-score.

Figure 1: miRNAs are placentally enriched and associated with gestational pathologies

1A) Venn diagram on number of miRNAs reported to be associated with alcohol or other prenatal exposures.

1B) Venn diagram on number of miRNAs reported to be associated with different gestational pathologies. Inset colored circles represent the corresponding sex and gestational age-adjusted growth parameters these miRNAs were correlated with. Of the 22 studies queried, 11 (50%) utilized unbiased screenings for miRNA expression.

1C) Heatmap of mature miRNA expression and 1D) pri-miRNA expression across different tissues resulting from secondary analysis of publicly available RNA-sequencing data. Legend depicts row-centered Z-score.
Figure 2: HEa miRNAs mediate the effect of PAE on EMT pathway members in mouse and macaque placentas.

2A) Histological image of GD14 mouse placenta. Outlined in red is the labyrinth zone, blue is the junctional zone, black is the decidual zone. Inset is a high magnification image of the labyrinth zone.

2B) MANOVA of gene expression of core EMT pathway members in different regions of the mouse placenta in control and PAE mice (n=29 samples).

2C) MANCOVA of gene expression of core EMT pathway members in the mouse placental labyrinth zone before (Basic Model) and after accounting for the expression of HEa miRNAs (n=29 samples).

2D) Gross anatomy photograph of the primary (left) and secondary (right) lobes of a GD135 macaque placenta. Outlined in red is an individual cotyledon from the secondary lobe. Inset is a full thickness hematoxylin and eosin stained histological section of a representative cotyledon with the fetal membranes outlined in black, villous tissue outlined in red and maternal decidua in blue.

2E) MANCOVA of gene expression of core EMT pathway members in placental cotyledons of PAE and control macaques, accounting for the expression of HEa miRNAs collectively (n=23 samples).

2F) MANCOVA of gene expression of core EMT pathway members in macaque placentas after accounting for expression of HEa miRNAs individually (n=23 samples).
Figure 3: PAE interferes with EMT pathway member expression in mouse and macaque placentas

Expression of 3A) CDH1, 3B) VIM, 3C) SNAI1, 3D) TWIST, and 3E) SNAI2 in the placental labyrinth zone of PAE and control mice (n=5-12 samples per group).

3F) Densitometric quantification of E-Cadherin expression in the labyrinth zone of PAE and control mice as well as representative blot of E-Cadherin expression (top) and total protein expression (bottom, n=5-12 samples per group).

Expression of 3G) CDH1, 3H) VIM, 3I) SNAI2, and 3J) TWIST transcripts in PAE and Control macaque placental cotyledons (n=3-5 samples per group).

Results are expressed as the mean ± SEM, LDR=Molecular Weight Ladder; ANOVA: significant main effect of PAE [Ɛp<0.05, ƐƐƐp<0.001], significant interaction effect (sex by PAE, ['p<0.05]). For post-hoc analysis, ***p<0.001 by Tukey’s HSD.
Figure 4: HEa miRNAs interfere with EMT pathway member expression in BeWO cytotrophoblasts

Expression of 4A) CDH1, 4B) VIM, 4C) TWIST, and 4D) SNAI1 transcripts 4E) and densitometric quantification of E-Cadherin protein levels in BeWO cytotrophoblasts following HEa miRNAs or control miRNA overexpression with or without concomitant 320 mg/dL ethanol exposure.

4F) Expression of CDH1, 4G) VIM, 4H) TWIST, and 4I) SNAI1 transcripts 4J) and densitometric quantification of E-Cadherin protein levels in BeWO cytotrophoblasts following HEa miRNAs or control hairpin inhibitor transfection with or without concomitant 320 mg/dL ethanol exposure. Results are expressed as the mean ± SEM, LDR=Molecular Weight Ladder, n=10 samples per group; ANOVA: significant main effect of HEa miRNA transfection [####p<0.0001], significant interaction effect (HEa miRNA by 320mg/dL ethanol, [*p<0.05, ***p<0.001]). For post-hoc analysis *p<0.05, **p<0.01 by Tukey’s HSD.
Figure 5: 

HEa miRNAs interfere with EMT pathway member expression in HTR8 extravillous trophoblasts

Expression of 5A) SNAI2 5B) VIM 5C) TWIST and 5D) SNAI1 transcripts 5E) as well as densitometric quantification of Vimentin protein levels in HTR8 extravillous trophoblasts following HEa miRNAs or control miRNA overexpression with or without concomitant 320 mg/dL ethanol exposure.

Expression of 5F) SNAI2 5G) VIM 5H) TWIST and 5I) SNAI1 transcripts 5J) as well as densitometric quantification of Vimentin protein levels in HTR8 extravillous trophoblasts following HEa miRNA or control hairpin inhibitor transfection with or without concomitant 320 mg/dL ethanol exposure.

Results are expressed as the mean ± SEM, LDR=Molecular Weight Ladder, n=10 samples per group; ANOVA: significant main effect of HEa miRNA transfection [##p<0.01, ####p<0.0001], significant main effect of 320mg/dL ethanol exposure [‡p<0.01], significant interaction effect (HEa miRNA by 320mg/dL ethanol, [‘p<0.05, †p<0.01]). For post-hoc analysis *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 by Tukey’s HSD.
Figure 6: Antagomirs prevent \textsubscript{HeA}miRNA induced impairment of EMT core pathway members

Expression of 6A) CDH1 6B) VIM 6C) TWIST and 6D) SNAI1 Transcripts following control or \textsubscript{HeA}miRNA hairpin inhibitor transfection followed by control or \textsubscript{HeA}miRNA overexpression in BeWO cytotrophoblasts.

Expression of 6E) CDH1 6F) VIM 6G) TWIST and 6H) SNAI1 Transcripts following control or \textsubscript{HeA}miRNA antagomir transfection followed by control or \textsubscript{HeA}miRNA overexpression in HTR8 extravillous trophoblasts.

In subheadings: C denotes control miRNA mimic or hairpin whereas T denotes \textsubscript{HeA}miRNA mimic or hairpin inhibitor. Results are expressed as expressed as the mean ± SEM, n=10 samples per group; ANOVA: significant treatment effect [###p<0.0001, ####p<0.0001]. For post-hoc analysis, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by Tukey’s HSD.
Figure 7: $\text{HE}_\text{A}$ miRNA impair extravillous trophoblast invasion

Transwell invasion of HTR8 extravillous trophoblasts following transfection with 7A) $\text{HE}_\text{A}$ miRNA mimics or 7B) hairpin inhibitors with or without concomitant 320 mg/dL ethanol exposure. Results are expressed as the mean ± SEM; n=10 samples per group; *p<0.05 by Unpaired T-test
Figure 8: HEa miRNA cause cell cycle retardation in trophoblasts

8A) Degree of EdU incorporation following control and HEa miRNA overexpression or 8B) control and HEa miRNA hairpin inhibitor transfection with or without concomitant 320 mg/dL ethanol exposure in BeWO cytotrophoblasts. Results are expressed as the mean ± SEM. 8C) Box and whisker plot for the proportion of cells in G0/G1, S, or G2/M phase of the cell cycle following control and HEa miRNA overexpression or 8D) control and HEa miRNA hairpin inhibitor transfection with or without concomitant 320 mg/dL ethanol exposure. Bounds of box demarcate limits of 1st and 3rd quartile, line in middle is the median, and whiskers represent the range of data. Representative flow cytometry experiment images are shown on the right. n=10 samples per group; ANOVA: significant main effect of HEa miRNA transfection [##p<0.01, ###p<0.001, and ####p<0.0001].
Figure 9: Antagomirs prevent HEa miRNA-induced cell cycle retardation

9A) Degree of EdU incorporation following control or HEa miRNA hairpin inhibitor transfection followed by control or HEa miRNA overexpression in BeWO cytotrophoblasts. Results are expressed as the mean ± SEM.

9B) Box and whisker plot for the proportion of cells in G0/G1, S, or G2/M phase of the cell cycle following control or HEa miRNA hairpin inhibitor transfection followed by control or HEa miRNA overexpression in BeWO cytotrophoblasts. Bounds of box demarcate limits of 1st and 3rd quartile, line in middle is the median, and whiskers represent the range of data. Representative flow cytometry experiment images are shown on the right.

In subheadings: C denotes control miRNA mimic or hairpin whereas T denotes HEa miRNA mimic or hairpin inhibitor. n=5 samples per group; ANOVA: significant treatment effect [###p<0.001]. For post-hoc analysis, **p<0.01 by Tukey’s HSD.
Figure 10: **miRNAs modulate differentiation-associated Ca\(^{2+}\) dynamics but have minimal effect on the cellular energetics profile**

10A) Time-lapse confocal images of BeWO cytotrophoblasts loaded with fluo-4 Ca\(^{2+}\) indicator dye under indicated treatment conditions. Arrowhead indicates a fused, multinuclear cell, scale bar is 50µm.

10B) Box and whisker plot of intracellular calcium levels following acute ATP administration in BeWO cytotrophoblasts with control and miRNA overexpression with or without concomitant 320 mg/dL ethanol exposure. Bounds of box demarcate limits of 1\(^{st}\) and 3\(^{rd}\) quartile, line in middle is the median, and whiskers represent the range of data.

10C) Box and whisker plot of intracellular calcium levels following acute ATP administration in BeWO cytotrophoblasts with control and miRNA overexpression with or without 20 µm forskolin treatment.

10D) Baseline oxygen consumption rate (OCR), 10E) baseline extracellular acidification rate (ECAR), 10F) stressed OCR, and 10G) stressed ECAR in BeWO cytotrophoblasts with control and miRNA overexpression with or without concomitant 320 mg/dL ethanol exposure. Metabolic stress was induced by treatment with 1µm Oligomycin and 0.125µM (FCCP). Results are expressed as expressed as the mean ± SEM.

n=10 samples per group; ANOVA: significant main effect of 320 mg/dL ethanol exposure \([*p<0.05, **p<0.001]\), significant interaction effect (miRNA by 320 mg/dL ethanol, \([*p<0.05, **p<0.01, and ***p<0.0001]\)). For post-hoc analysis, *p<0.05, **p<0.01, ***p<0.001, and ***p<0.0001 by Tukey’s HSD.
**Figure 11:** HEa miRNAs promote syncytialization dependent hCG production

Expression of 11A) CGA, 11B) CGB, 11C) IGF2, and 11D) CDH1 transcripts 11E) and densitometric quantification of E-Cadherin protein levels in BeWO cytotrophoblasts following HEa miRNAs or control miRNA overexpression with or without 20 μm forskolin treatment. 

Expression of 11F) CGA, 11G) CGB, 11H) IGF2, and 11I) CDH1 transcripts 11J) and densitometric quantification of E-Cadherin protein levels in BeWO cytotrophoblasts following HEa miRNAs or control hairpin inhibitor transfection with or without 20 μm forskolin treatment.

Results are expressed as the mean ± SEM, LDR=Molecular Weight Ladder, n=10 samples per group; ANOVA: significant main effect of HEa miRNA transfection [####p<0.0001], significant interaction effect (HEa miRNA by 320mg/dL ethanol, [†p<0.05]). For post-hoc analysis, *p<0.05, **p<0.01 by Tukey’s HSD.
Figure 12: PAE elevates 3rd trimester maternal hCG

Box and whisker plot of 2nd and 3rd trimester maternal hCG levels in UE, HEua, and HEa group mothers of our Ukrainian birth cohort. Bounds of box demarcate limits of 1st and 3rd quartile, line in middle is the median, and whiskers represent the range of data. Results are expressed as the mean ± SEM, n=22-23 samples per group; *p=0.03 (Mood’s Median Test, \( \chi^2 = 7.043, \text{df}=2 \)).
Figure 13: HEa miRNAs restrict fetal growth

13A) Schematic for measures of crown rump length (CRL), biparietal diameter (BPD), and snout-occipital distance (SOD).

13B) Fetal weight, 13C) crown-rump length, 13D) biparietal diameter, 13E) snout-occipital distance, 13F) and placental weight at GD18 following administration of control (Ctrl) and HEa miRNA mimics to pregnant C57/Bl6 dams on GD10. Dots represent median measures of fetal size and placental weights from male and female offspring in independent litters. There were no significant differences in litter sizes [Ctrl: 8.2 and HEa miRNAs: 8.5] or sex ratios [Ctrl: 0.86 and HEa miRNAs: 1.21] between treatment conditions (p>0.5 for all measures).

Results are expressed as the mean ± SEM, n=5-6 separate litters per treatment condition; ANOVA: significant main effect of HEa miRNA administration [#p<0.05 and ##p<0.01].
**Figure 14:** HEα miRNAs interfere with core EMT pathway members in the placenta

Expression of 14A) CDH1 14B) VIM 14C) TWIST 14D) SNAI1 and 14E) SynB transcripts in GD18 placenta following administration of control (Ctrl) and HEαmiRNA mimics to pregnant C57/Bl6 dams on GD10.

Dots represent median expression values of male and female offspring in independent litters. Results are expressed as the mean ± SEM, n=5-6 separate litters per treatment condition, ANOVA: significant main effect of HEαmiRNA administration [#p<0.05, ###p<0.001], significant interaction effect (fetal sex by HEαmiRNA administration, [†p<0.05]). For post-hoc analysis, *p<0.05 by Tukey’s HSD.
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