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Supplemental Information

MicroRNA-218-5p Promotes Endovascular Trophoblast Differentiation and Spiral Artery Remodeling

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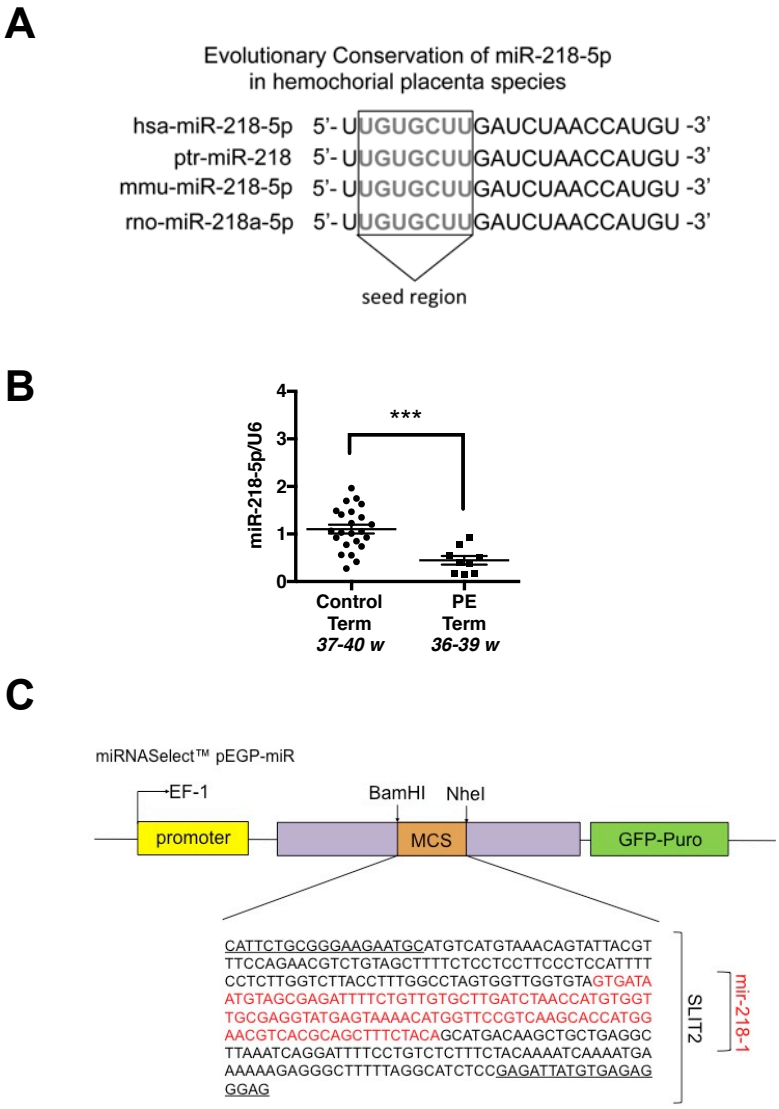


Figure S1. (A) Comparison of miR-218-5p across hemochorial placenta species (ptr: chimpanzee, mmu: mouse, rno: rat) shows complete conservation, including the full seeding region responsible for target complementarity. **(B)** Placentae from patients diagnosed with Preeclampsia delivered at term (34-39 weeks) were assessed for miR-218-5p levels and compared to pregnancies that were found to have no placental abnormalities. A significant decrease ($p<0.001$) of miR-218-5p was observed in PE-Term placenta group when compared to its healthy term control. **(C)** For the generation of mir218-1 stable cells, a portion of the Slit2 intron, bracketing the mir-218-1 stem-loop sequence, was cloned into the miRNASelect™ pEGP-miR expression vector. An immortalized human first trimester trophoblast cell line, HTR8/SVneo, was stably transfected with empty vector (EV) or mir-218-1 stem-loop construct to create stably expressing clones. Single colonies were picked after Puromycin selection for two weeks.

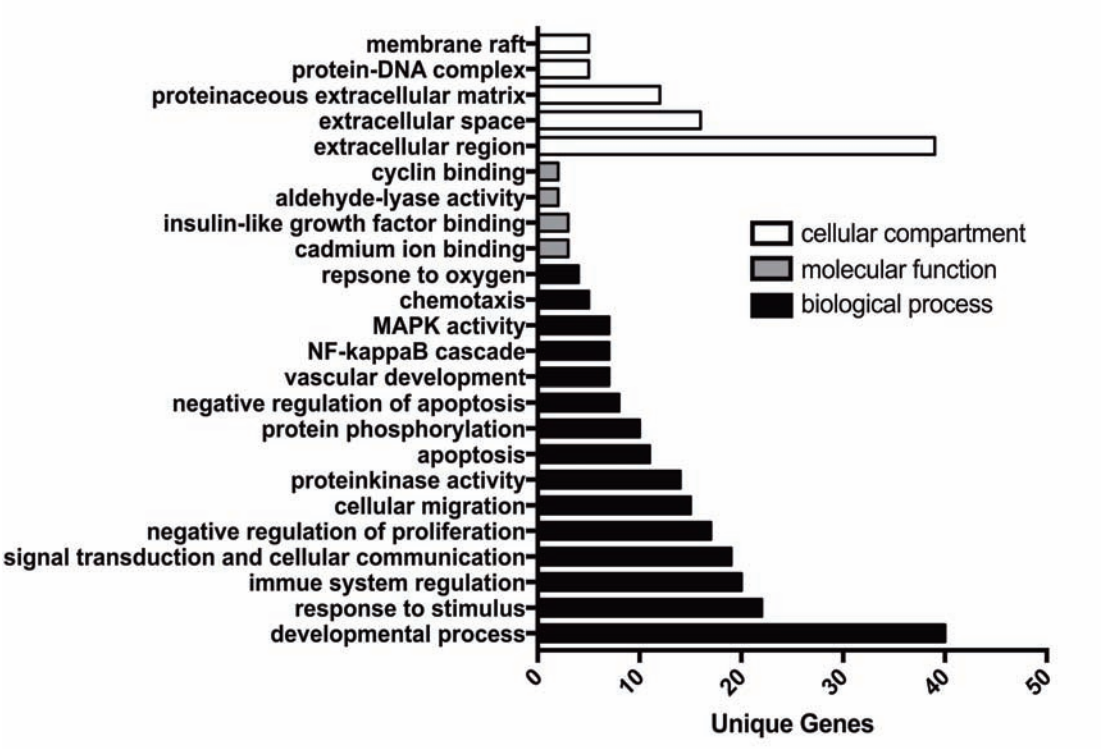


Figure S2. Gene ontology analysis of mir-218-1-regulated genes. RNA was extracted from Control and mir-218-1 overexpressing cells and cDNA microarray was performed. To help interpret these results each post-hoc test was divided into positive or negative fold change of at least 1.5 and a Benjamini and Yekutieli corrected ($p<0.3$) hypergeometric test, to look for enriched Gene Ontology categories that may overlap, was applied. Unique gene counts identified in each GO Term are represented on the x-axis.

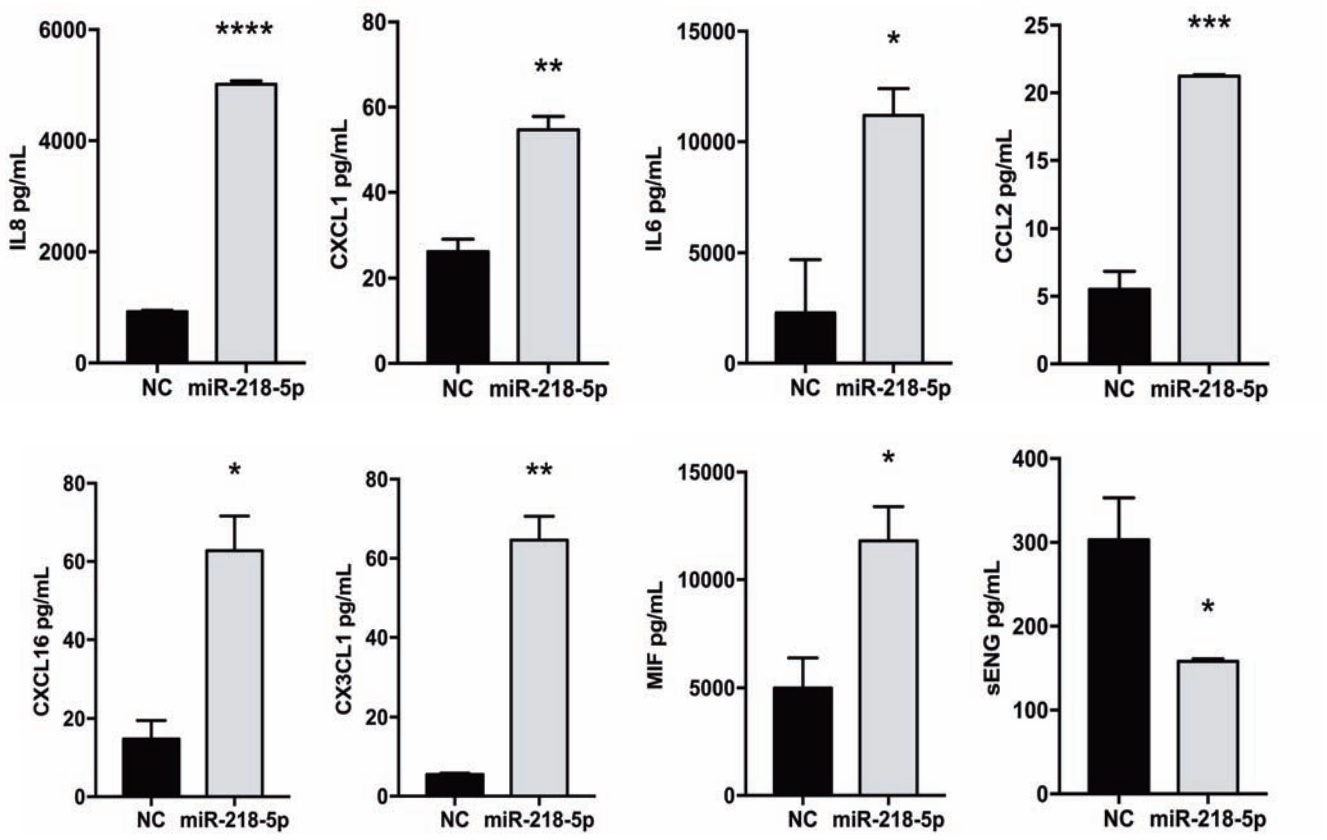


Figure S3. miR-218-5p upregulated the secretion of pro-invasive and pro-angiogenic factors and downregulated soluble Endoglin. First trimester placenta was treated with negative control or miR-218-5p (200nM) for 48h. The condition media was collected and used to assess the abundance of secretory proteins in the Bio-plex multiplex system. Upregulation of interleukin-8 (IL8), chemokine ligand 1 (CXCL1), interleukin-6 (IL6), Chemokine C-C motif ligand 2 (CCL2), chemokine C-X-C motif ligand 16 (CXCL16) C-X3-C motif chemokine ligand 1 (CX3CL1) and macrophage migration inhibitory factor (MIF) were observed in conditioned media of miR-218-5p treated tissues. Downregulation of soluble Endoglin (sENG) was detected in miR-218-5p conditioned media compared to control. Error bars represent SEM. Statistical significance was calculated using unpaired student t-test; * $p<0.05$, ** $p<0.005$, *** $p<0.0005$, **** $p<0.0001$.

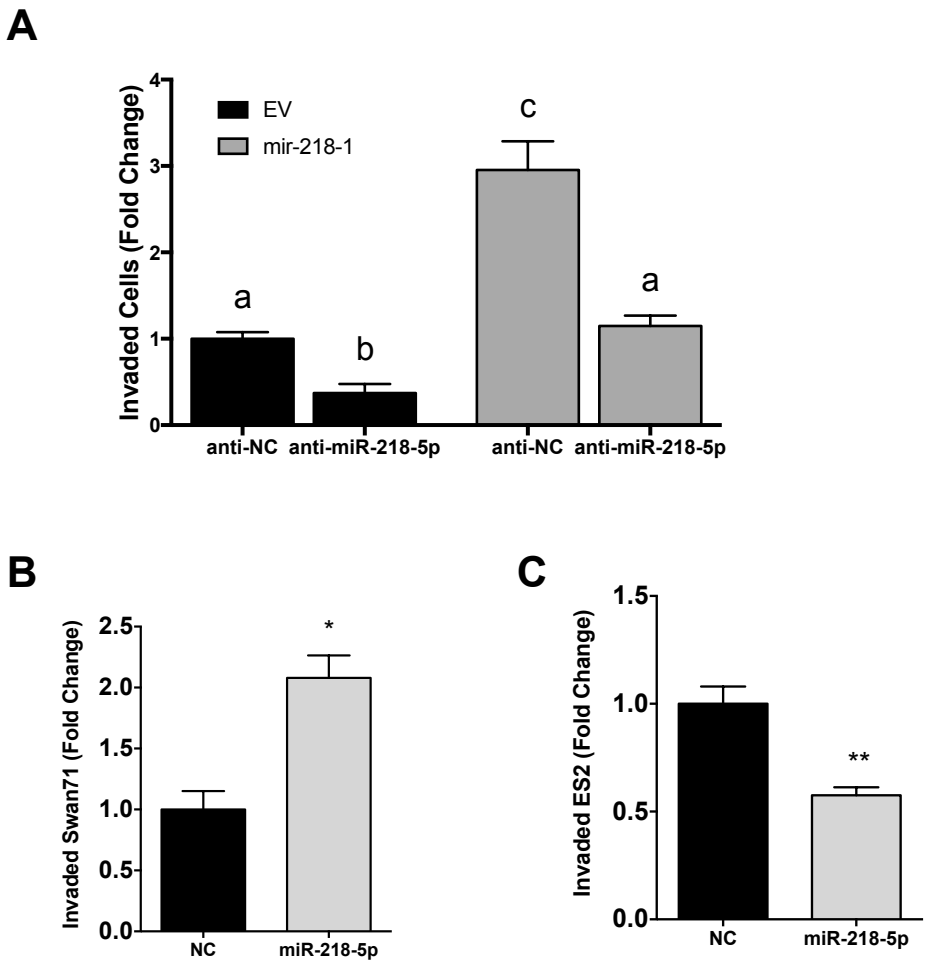


Figure S4. miR-218-5p has opposite effect on invasion in cancer cells. (A) mir-218-1 stable cells were used in a matrigel invasion assay as previously described. Transient anti-miR-218 transfection completely reversed the pro-invasive effect of mir-218-1. Different letters above bars denote statistical significance. **(B)** A first trimester immortalized cell line, Swan71**,** was transfected with miR-218-5p (100 nM) and seeded on matrigel-coated transwells. miR-218-5p significantly ($p<0.05$) increased the number of cells invaded through the transwells, as compared to control (NC) **(C)** miR-218-5p was transfected into ovarian cancer cells, ES2, and seeded on matrigel coated transwells. miR-218-5p significantly ($p<0.01$) downregulated the invasive ability of these cancer cells. A representative graph is shown from three independent experiments. A two-way ANOVA with Fisher's LSD test (A) or unpaired, two-tailed student t-test (B and C) was performed on GraphPad Prism. Error bars represent SEM.

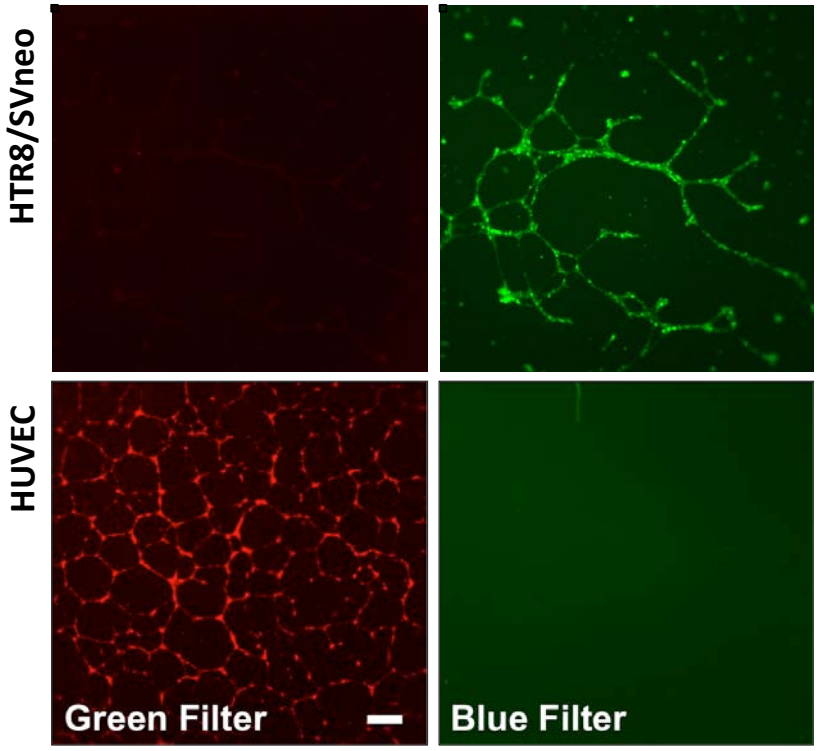


Figure S5. CellTracker™dye validation. To verify the specificity of stains, HTR8/SVneo cells were stained with CellTracker™ Green CMFDA and photos were taken with green filter (left panel) or blue filter (right panel). Same was performed for cell Tracker Red CMTPX dye and HUVEC. Scale bar: 500 µm

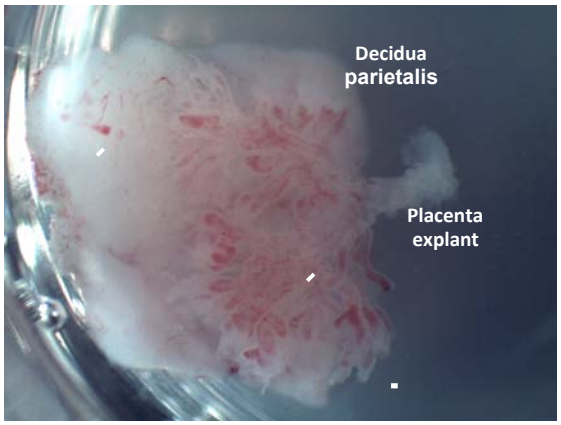
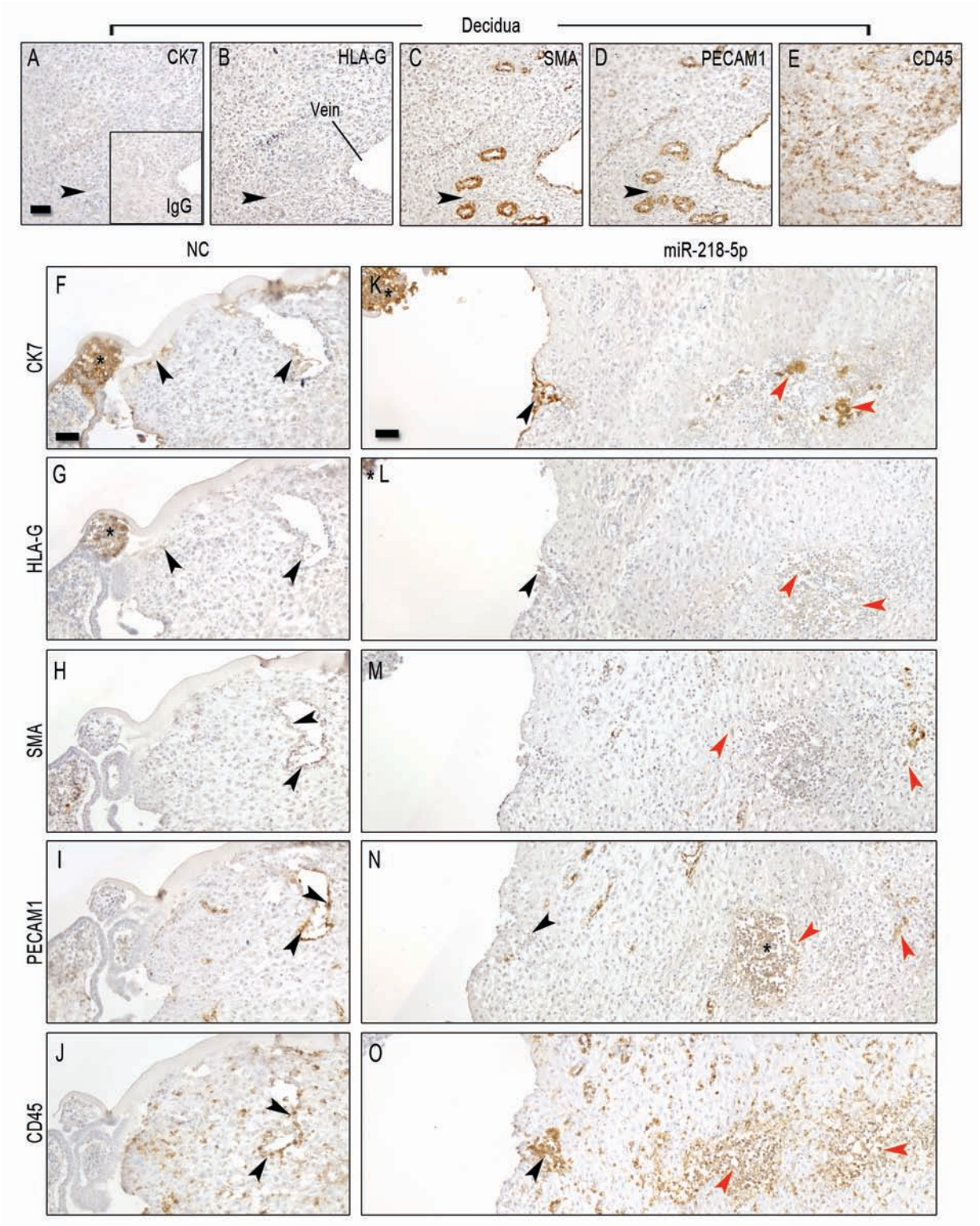


Figure S6. *Ex vivo* model of first trimester placenta-decidua co-culture. Patient-matched placenta and decidua between 8 and 9.5 weeks of gestation were used. Explants of decidua parietalis were placed, epithelial surface side up, on matrigel coated inserts. After 24h of treatment with oligos, placenta with anchoring villi were placed on top of the decidua and allowed to invade for 6 days. Scale bar: 250 µm

Fig. 7A


Decidua
A
CK7
IgG
B
HLA-G
Vein
C
SMA
D
PECAM1
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CD45
NC
miR-218-5p
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CK7
G
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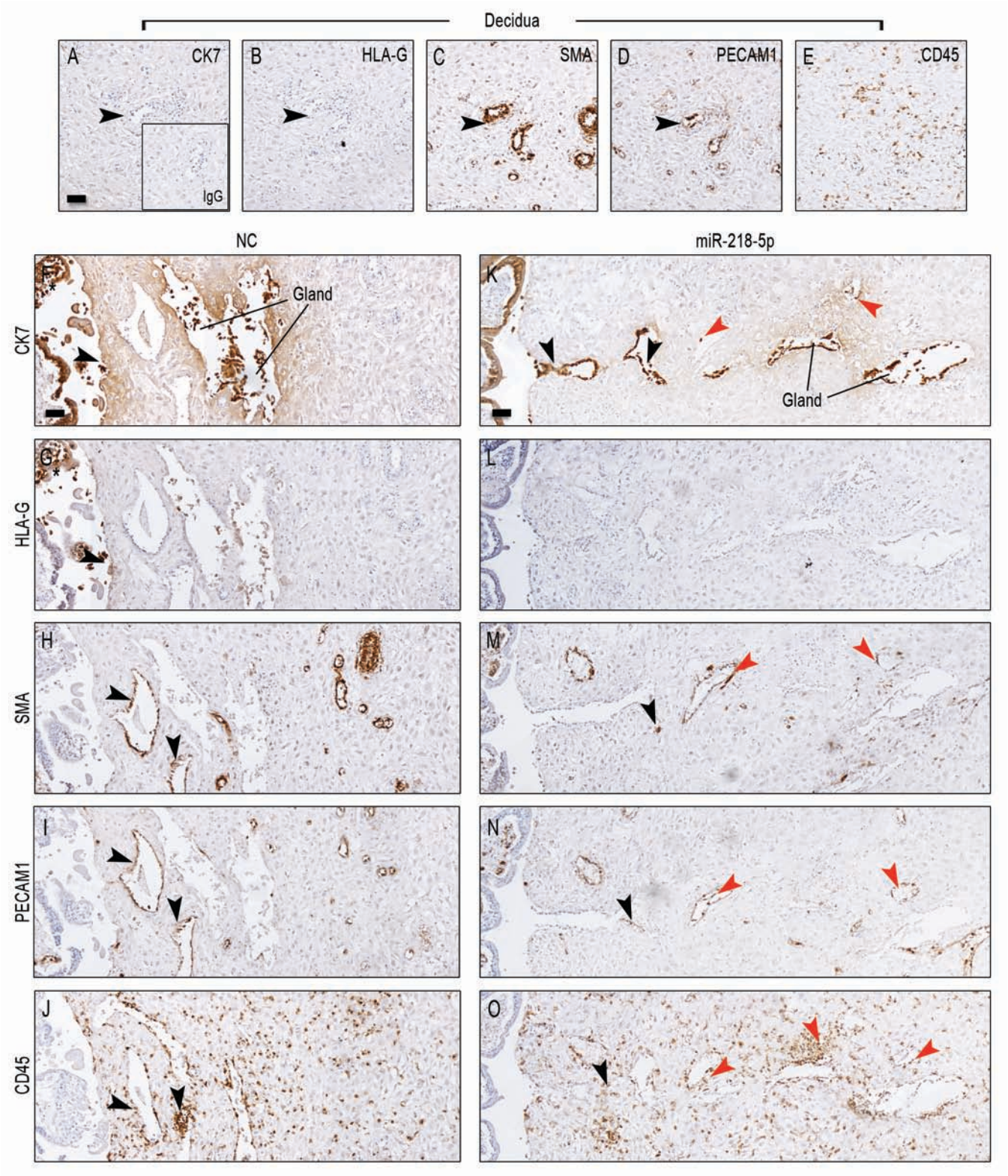
Fig. 7B


Decidua
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CK7
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PECAM1
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Figure S7. miR-218-5p accelerates spiral artery remodeling in two placenta/decidua co-cultures (7A and 7B).

(A-E) Investigation of markers for trophoblasts (CK-7 and HLA-G), spiral artery (SMA and PECAM1) and leukocytes (CD45) in decidua parietalis cultured alone. The tissue was negative for CK-7 and HLA-G (A and B, arrows). Tight, non-invaded arterioles stained positive for SMA and PECAM1 (C and D, arrows). Lymphocytes positive for CD45 were spread evenly throughout the tissue (E). **(F-J)** Placenta tissue pre-treated with non-targeting control (NC) was assessed for degree of vessel remodeling. EVTs positive for CK-7 (F) and HLA-G (G, asterisks) entered the proximal arteriole (arrows). Distal portion of the spiral arteriole stained for SMA (H) and PECAM1 (I) and leukocyte infiltration into the vessel wall is indicated by CD45 positive cells (J, arrows). In Fig. 7SA, the arteriole displayed widened lumens with invaded trophoblasts. Smooth muscle layers and endothelial layer were not intact. These findings suggest that the vessel is in an early "active phase" of vascular transformation. In Fig. S7B, the arteriole appeared at the priming stage for remodeling as the arteriole had been widened with leukocyte recruitment but smooth cell and endothelial layers were still intact and no trophoblast invasion took place. **(K-O)** Placenta tissue pre-treated with miR-218-5p shows an accelerated degree of vessel remodeling. Some CK7 (K) and/or HLA-G (O) positive EVTs (asterisk) entered the proximal portion of the arteriole (black arrows) and also invaded to the distal portions (red arrows). In Fig. S7A, only residual SMA (M) and PECAM1 (N) positive cells remained at both proximal (black arrows) and distal (red arrows) portion of the arteriole. Endovascular EVT at the distal site of remodeling are positive for PECAM1 (asterisk). Massive recruitment of leukocytes (O) to the sites of remodeling was evident. These observations suggest that both the proximal and distal portions of the arteriole were being actively remodeled. In Fig. S7B, while only a few residual cells positive for SMA (M) or PECAM1 (N) could be observed at the proximal site of remodeling (black arrow), the distal portions still showed smooth muscle (M, red arrows) and endothelial (N, red arrows) lining the vessel. Abundant recruitment of leukocytes (O) to the site of active remodeling (arrows) was observed. These data suggest that only the proximal portion of the arteriole was in active stage of remodeling while the distal region was still in a priming or early stage of remodeling. Scale bar: 50 μm

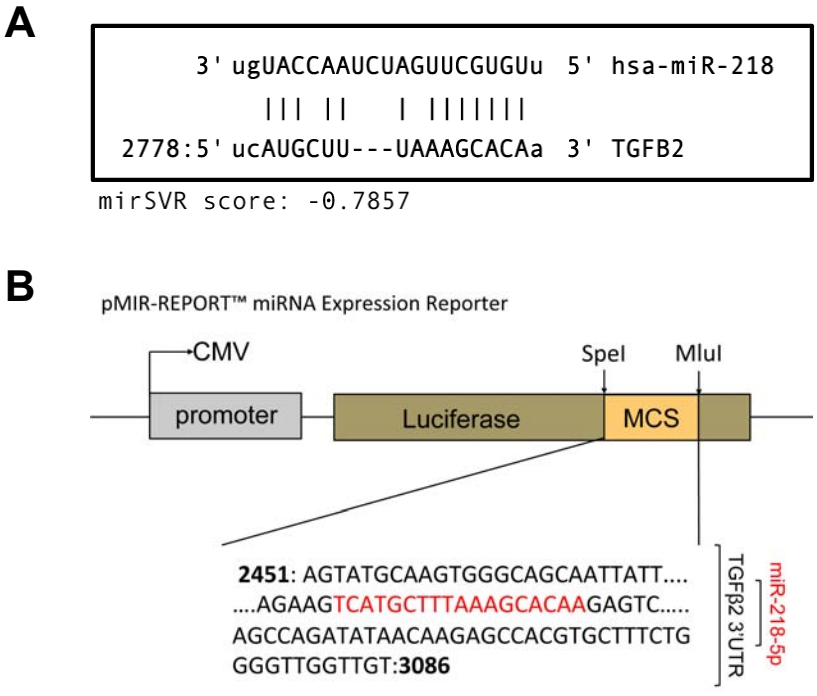

Figure S8. Generation of Luciferase-TGFB2 3'UTR reporter construct. (A) miRNA target prediction algorithm, MiRanda, shows a good miRSVR score (<-0.1) for the miR-218-5p targeting site on the TGFB2 3'URT. **(B)** Portion of the TGFB2 3'UTR containing the miR-218-5p binding region was cloned in the pMIR-REPORT™ miRNA Expression Reporter, downstream of the luciferase gene.

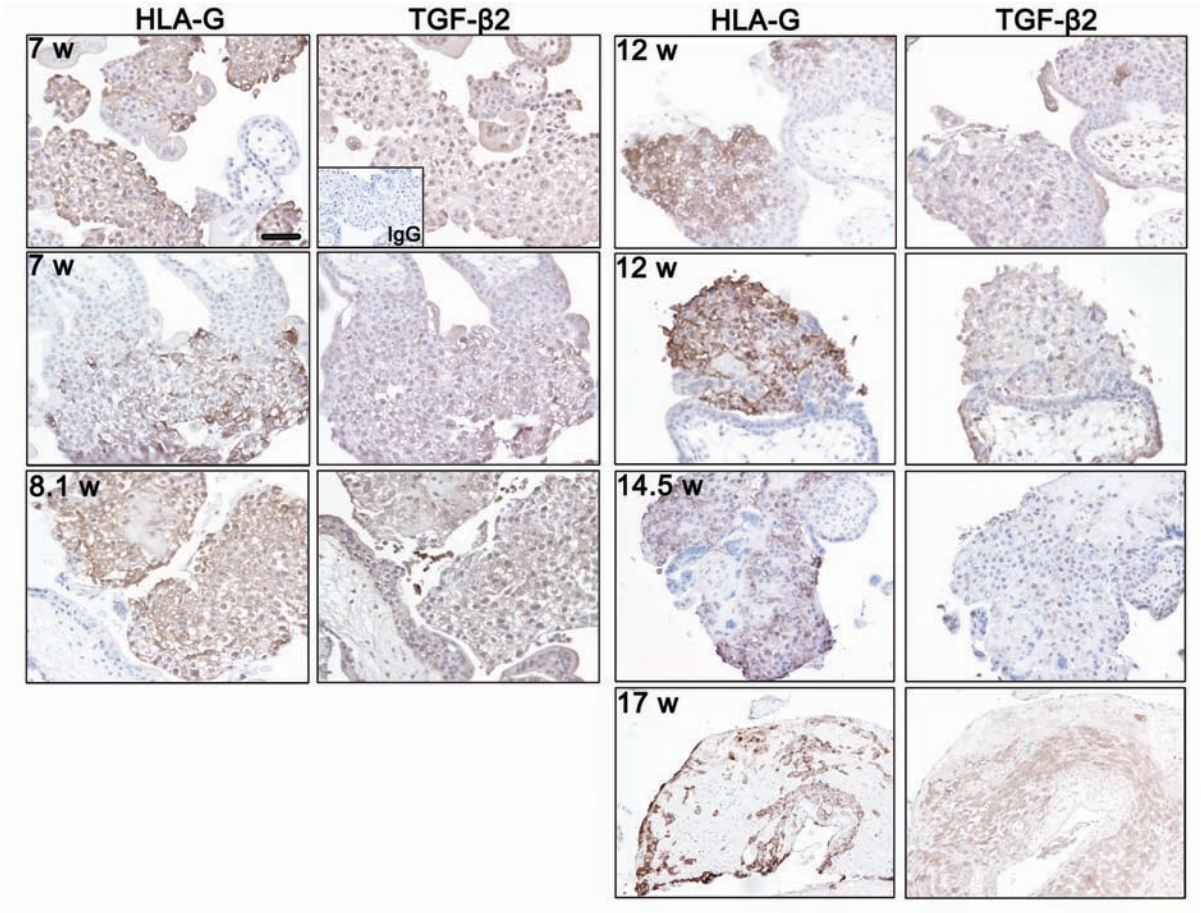

Figure S9. TGF-β2 expression in EVTs of healthy placenta across early gestation. Placenta from elective termination between 7-17 weeks of gestation were probed for HLA-G (EVT marker) and TGF-β2. TGF-β2 signal was lower in EVT from 12-17 w placenta compared to early gestation samples. Scale Bar: 100 μm

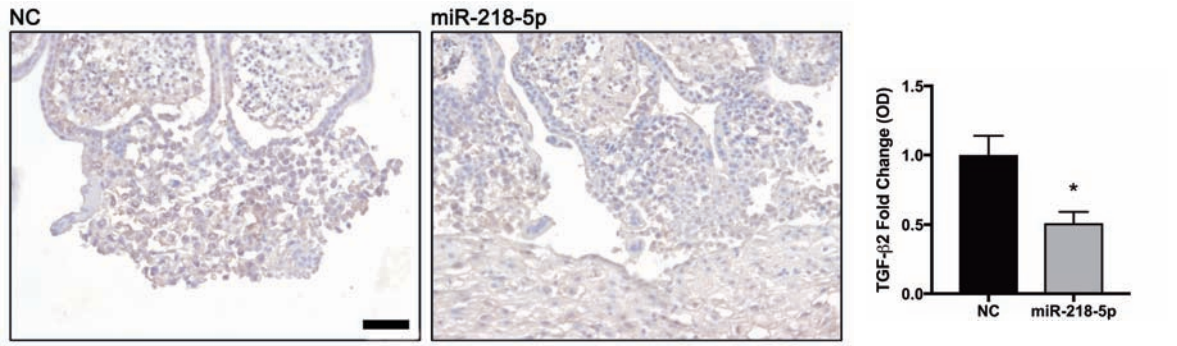

Figure S10. TGFb2 in EVT columns from placenta-decidua co-culture. Slides from co-culture in Figure 5 were selected and probed for TGF-β2. TGF-β2 staining was weaker in EVT of columns from miR-218-5p pretreated placenta. DAB staining was quantified in ImageJ using the Fiji package. Micrographs (n=5) were H DAB colour deconvoluted and mean grey value of villous columns were measured and normalized to NC OD (log10(255/mean)). Placenta pretreated with miR-218-5p had a significantly ($p<0.05$) weaker DAB signal in EVT containing columns compare to NC treated placentas. Representative images of CK-7 and HLA-G are found in the main text Figure 5 F+G (NC) and K+L (miR-218-5p). Scale Bar: 100 μm

Table S1A. Peking University Third Hospital Patient Data Summary

	Control	PE	P value#
N	15	15	
Gestational age (weeks)	37.56±0.2	36.67±0.27	0.020
Maternal age (years)	32±1.1	29.27±1.48	0.126
Maternal Weight before (Kg)	56.29±1.12	56±2.27	0.913
Maternal Weight after (Kg)	72±2.19	71.2±2.71	0.822
Maternal Height (cm)	160.36±1.12	158.53±1.09	0.254
Systolic BP before (mmHg)	119.23±1.37	118.57±2.06	0.795
Diastolic BP before (mmHg)	77.69±1.22	79.29±1.27	0.375
Systolic BP after (mmHg)	116.43±1.33	153.33±3.86	0.0001
Diastolic BP after (mmHg)	76.43±1.33	99.00±2.40	0.0001
Proteinuria (g/24h)	0	3.27±0.6	0.0001
50g GLU*	7.71±0.47	6.64±0.31	0.069
Baby Weight (g)**	3331.07±74.69	2662.14±127.54	0.0001

* 50g oral glucose challenge test.

** one mother from the PE group was not included for she gave birth to twins, a male baby at 2080g and a girl baby at 2210g.

determined by t-test for different parameters between Control and PE groups.

Table S1B. Mount Sinai Hospital - BioBank Patient Data Summary*

	1st Trimester	2nd Trimester	Preterm Control	Term Control	Term PE
N	13	9	23	24	9
Gestational age (weeks)	9±0.62	16±0.77	30±0.14	38±0.52	37±0.33
Maternal age (years)			32±1.17	33±0.76	34±2.74
Sex Male			12	15	6
Sex Female			11	9	3
Birth Weight (g)			1638±119.6	3363±74.5	2914±135.6
Max Systolic (mmHg)			n/a	n/a	159±4.75
Max Diastolic (mmHg)			n/a	n/a	101±0.98
Proteinuria (dip stick)			n/a	n/a	1-4
AST (u/L)			n/a	n/a	23±2.48
ALT (u/L)			n/a	n/a	16±2.11
Creatinine (μM)			n/a	n/a	58±2.18
Uric Acid (μmol/L)			n/a	n/a	356±21.29
Platelets (u/L)			n/a	n/a	201±19.51

* All data are Mean ± SEM

Table S2. List of primers, siRNAs, miRNAs, and antagomirs used in this study

Name	**Sequence: 5'** to **3'**
mir-218-1	**F:** GGATCCCATTCTGCGGGAAGAATGC
	R: GCTAGCCTCCCTCTCACATAATCTC
TGFB2 3'UTR	**F:** AGGACTAGTAGTATGCA-AGTGGGCAGCAA
	R: ACTACGCGTACAACCAACCCCAGAAAGCA
Cyc1	**F:** CAGATAGCCAAGGATGTGTG
	R: CATCATCAACATCTTGAGCC
VE-cad	**F:** GCCAGTTCTTCCGAGTCACA
	R: TTTCCTGTGGGGGTTCCAGT
PECAM1	**F:** ATTGCAGTGGTTATCATCGGAGTG
	R: CTCGTTGTTGGAGTTCAGAAGTGG
MMP1	**F:** GTCTCACAGCTTCCCAGCGA
	R: ATGGCATGGTCCACATCTGC
IL1b	**F:** AATCTGTACCTGTCCTGCGTGTT
	R: TGGGTAATTTTTGGGATCTACACTCT
IL8	**F:** CAGAGACAGCAGAGCACACA
	R: GGCAAAACTGCACCTTCACA
ECSCR	**F:** ACAACTCCCAGCCCACAATG
	R: GTGGTCAGACTTAGACCGCC
CXCL1	**F:** CAGGGAATTCACCCCAAGAACA
	R: GGATGCAGGATTGAGGCAAGC
ITGA1	**F:** GCTGGCTCCTCACTGTTGTT
	R: CACCTCTCCCAACTGGACAC
TGFB2	**F:** ATTGATGGCACCTCCACATATA
	R: ACGTAGGCAGCAATTATCCTG
miR-218-5p	**F:** TTGTGCTTGATCTAACCATGT
	R: N-Code Universal Primer
U6 snRNA	**F:** CGCAAGGATGACACGCAAATTC
	R: N-Code universal primer (Invitrogen MIRC-50)
siTGFB2	**5'-** ACCAAATACTTTGCCAGAAACTATtt **-3'** **3'-** ttTGGTTTATGAAACGGTCTTTGATA **-5'**
NC (non-targeting control)	**5′ -** UUCUCCGAACGUGUCACGUtt **-3′** **3′ -** ttAAGAGGCUUGCACAGUGCA **-5′**
hsa-miR-218-5p mimic	**5'-** UUGUGCUUGAUCUAACCAUGUtt **-3'** **3'- tt**AACACGAACUAGAUUGGUACA **-5'**
Anti-NC (non-targeting control)	mirVana™ miRNA Inhibitor, Negative Control #1
Anti-miR-218-5p	hsa-miR-218-5p mirVana™ Inhibitor

Table S3. Primary Antibodies and Staining Reagents

Antibody	Company	Cat No.	Species	Dilution/ Concentration	Diluent
Cytokeratin-7	Dako	M7018	Mouse	1:100	PBS
PECAM1	Dako	M0823	Mouse	1:100	PBS
SMA	Dako	M0851	Mouse	1:100	PBS
CD45	Dako	M0701	Mouse	1:100	PBS
HLA-G	ExBio	11-499	Mouse	1:300	PBS
Ac-α-tubulin	Sigma-Aldrich	T6793	Mouse	1:500	1% BSA-PBST
Calcein AM	Corning	354217	N/A	1 μM	Serum-free media
CellTracker™ Green CMFDA	Sigma-Aldrich	C2925	N/A	1 μM	Serum-free media
CellTracker™ Red CMTPX	Invitrogen	C34552	N/A	1 μM	Serum-free media

Supplemental Materials and Methods

Patient Sample Diagnostic and Exclusion Criteria

Placentas were collected from informed consented pregnant patients attending the Peking University Third Hospital. Patients who developed renal disease, transient hypertension during pregnancy, gestational diabetes, spontaneous abortion, intrauterine fetal death, fetal chromosomal or congenital abnormalities or pregnancies conceived by fertility treatment were all excluded from this study. The placentas were collected within 1 h of caesarean birth, and specimens at the chorionic plate and basal plate were separately taken from the placenta disc near the position of umbilical cord insertion. The specimens were snap-frozen in liquid nitrogen and subjected to RNA extraction. Preeclampsia (PE) was diagnosed when patients had no history of preexisting or chronic hypertension, but they showed systolic blood pressure of >140 mm Hg or diastolic blood pressure of >90 mm Hg on at least 2 occasions, accompanied by significant proteinuria (>2 g per 24 h in 2 samples collected at >4 h intervals) after 20 weeks of gestation. Normal or uncomplicated pregnancy (Control) was defined as a previously normotensive woman who did not suffer from complications during pregnancy and who delivered a healthy neonate with a weight adequate for a gestational age. The clinical characterization of the samples is listed in Table S1A.

Placental and decidual tissue samples from first and second trimester (6-20 weeks of gestation) were collected with informed consent from healthy patients undergoing elective termination of pregnancy at the Morgentaler Clinic, Toronto. The research nurse excluded all samples with known HIV or Hepatitis infection and any classified as a missed miscarriage. Preterm placenta (26-36 weeks of gestation) were collected from women in spontaneous preterm labor delivered either by caesarian section for fetal distress or vaginal delivery (11 had clinical signs of chorioamnionitis diagnosed by placental pathology). Third-trimester healthy term placentas (37-40 weeks) were from vaginal delivery or elective cesarean sections with Appropriate for Gestation Age babies.

Microarray

A gene expression analysis was conducted at the Princess Margaret Genomics Centre on a mir-218-1 overexpressing cell line and a control EV cell line, using the Human HT-12 V4 BeadChip, which comprises a total of 47323 probes. Data was checked for overall quality using R (v3.0.2) with the Bioconductor framework and the LUMI package installed. Data was imported in GeneSpring v12.6 for analysis. During import, the data was normalized using a standard (for Illumina arrays) quantile normalization followed by a “per probe” median centred normalization. Only probes that were above the 20th percentile of the distribution of intensities in 100% of any of the groups were allowed to pass through the filtering. A one-way ANOVA with a Benjamini-Hochberg FDR corrected $p<0.05$ showed 4106 significantly varying probes. Heat maps were created using the Multi Experiment Viewer (MeV) Microarray Software Suite.

Transwell Migration and Invasion Assay

Transwell inserts with 8 μm pores (Costar, Corning Inc) were coated with 150 μg/ml matrigel in Serum Free media (Cultrex Reduced Growth Factor BM extract-PathClear, Trevigen) and allowed to polymerize overnight at 37°C/5% CO_2. Cells were gently removed from culture plates using Accutase (Innovative Cell Technologies), and seeded at a density of 20,000 cells per well in serum-free RPMI-1640 media. As a chemotactic agent, 10% serum containing media was seeded on the outside of the transwell. After 24 h, membranes were fixed for 2 min in 100% methanol and stained using Harleco Hemacolor Staining Kit (EMD Chemicals). Similarly, for migration assays 10,000 cells per well were seeded on 8 μm pore transwell membranes without any matrigel coating. Invaded or migrated cells were counted with ImageJ[1] and the invasion/migration index was calculated as a fold of invaded/migrated cells in the treated groups compared to the control group. Each experiment was repeated in a minimum of three independent trials.

LDH Assay

Conditioned media was collected at the end of EVT outgrowth assays. Samples were processed using the 96-well format CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega) according to the manufacturer's procedure. A non-conditioned media control was used to subtract background from each reading. A high-LDH control (placental explant incubated in provided lysis solution) was used to determine % cytotoxicity of each sample.

ELISA

HTR8/SVneo cells stably transfected with mir-218-1 or its empty vector were seeded on 12-well plates at 100,000 cells/well in 10% FBS containing RPMI-1640 media. Conditioned media was collected after 72 h and spun down at 500 g for 5 min. TGF-β2 was measured using the Human TGF-β2 Quantikine ELISA Kit (R&D Systems) following the manufacturer's recommended procedure.

Cell Proliferation Assay

Cells were seeded on 6-well plates at the density of 50,000 cells per well in 10% FBS RPMI-1640 media and cultured for 24 h, 48 h and 72 h. Cells were then detached and stained with trypan blue. The number of live and dead cells were counted using a hemocytometer. Each time point was done in triplicate and repeated three independent times.

Detection of acetylated α-tubulin by immunofluorescence

EV and mir-218-1 overexpressing cells were seeded in a monolayer on glass coverslips and allowed to attach overnight. Cells were wounded down the middle with a tip, and covered in serum-free media for 16 h. Cells were fixed in ice cold Acetone:Methanol (1:1 solution) for 20 min then washed three times in 1X PBS. Cells were permeabilized with 0.2% Tween for 15 min then blocked in 1% BSA-PBS-T for 1 h. Cells were probed for acetylated alpha tubulin (Table S2) for 2 h at room temperature, followed by secondary anti-mouse antibody for 1 h. Coverslips were mounted on slides, and pictures were taken on the Zeiss LSM 700 Confocal Microscope.

Supplemental References

1. O'Brien J, Hayder H, Peng C. Automated Quantification and Analysis of Cell Counting Procedures Using ImageJ Plugins. *J Vis Exp* 2016:e54719–e54719.