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Technical note

Trophoblast function is altered by decidual factors in gestational-dependant manner

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ABSTRACT

Inadequate implantation and placentation is associated with miscarriage and placental insufficiency. The decidual environment is thought to regulate trophoblast invasion, however this is poorly defined in humans. We aimed to determine the effect of decidualization on trophoblast function.

In vitro decidualized primary human endometrial stromal cells (HESC) significantly enhanced first-trimester extravillous trophoblast (EVT) (6–8-weeks gestation) adhesion, outgrowth/invasion. In EVTs from 10 to 12-weeks gestation this effect was absent (adhesion, invasion) or reversed (outgrowth). HESC conditioned media had no effect on trophoblast MMP9 production/activity.

Decidualization regulated EVT function in a gestational-dependent manner. This study highlights the importance of trophoblast-decidual synchrony.

1. Introduction

Inadequate implantation and placentation can lead to first-trimester miscarriage, placental insufficiency and other obstetric complications [1]. During implantation extravillous trophoblast (EVT) invade into the decidualized endometrium and upper third of the myometrium [2]. Decidualization describes endometrial stromal cell (human [H]ESC) differentiation into decidual cells, involving the reprogramming of HESC such that different genes are expressed at different stages of differentiation [3]. EVT released factors promote decidualization in vitro [4].

The decidua is thought to produce factors which regulate trophoblast invasion [2,5–8], however the precise effect of decidual cells and decidualization on EVT invasion is not understood. Abnormal EVT invasion and impaired decidualization are both associated with pregnancy pathologies including recurrent miscarriage, preeclampsia and placenta accreta [9–13]. In this study, we aimed to precisely define the effect of decidualized HESC factors on EVT function and determine whether impaired decidualization would affect EVT invasion.

2. Methods

Monash Health Human Research and Ethics Committee approvals #09317B and #06014C. Written informed consent was obtained from each patient before surgery.

Healthy women undergoing pregnancy termination for psychosocial reasons (amenorrhea 6–12 weeks) donated first trimester placenta. Cytotrophoblast were isolated by Percoll gradient as previously published [14].

Endometrial biopsies were collected by dilatation and curettage from fertile women (18–40 years) scheduled for tubal ligation or tubal patency testing during days 8–21 of a normalised 28-day menstrual cycle. Tissues had no obvious endometrial pathology. The women had no steroid treatment or other medication for ≥3 months before tissue collection. HESC were isolated by collagenase digestion and filtration [4] which results in a 97% pure stromal cell culture [15].

2.1. Culture conditions

All cells were cultured at 37 °C in a 5% CO_2 humidified culture incubator.

Isolated cytotrophoblast were cultured on Matrigel™ diluted 1:5 in DMEM/F12 to promote differentiation towards the EVT phenotype. HLAG (BD Biosciences) immunocytochemistry confirmed EVT purity [14]. EVTs were maintained in DMEM/F12 containing 10% Fetal Bovine Serum (FBS) and 1% antibiotics.

HESC were cultured and decidualized as previously described [14]. HESC conditioned media (CM) was collected on Days 2 (impaired

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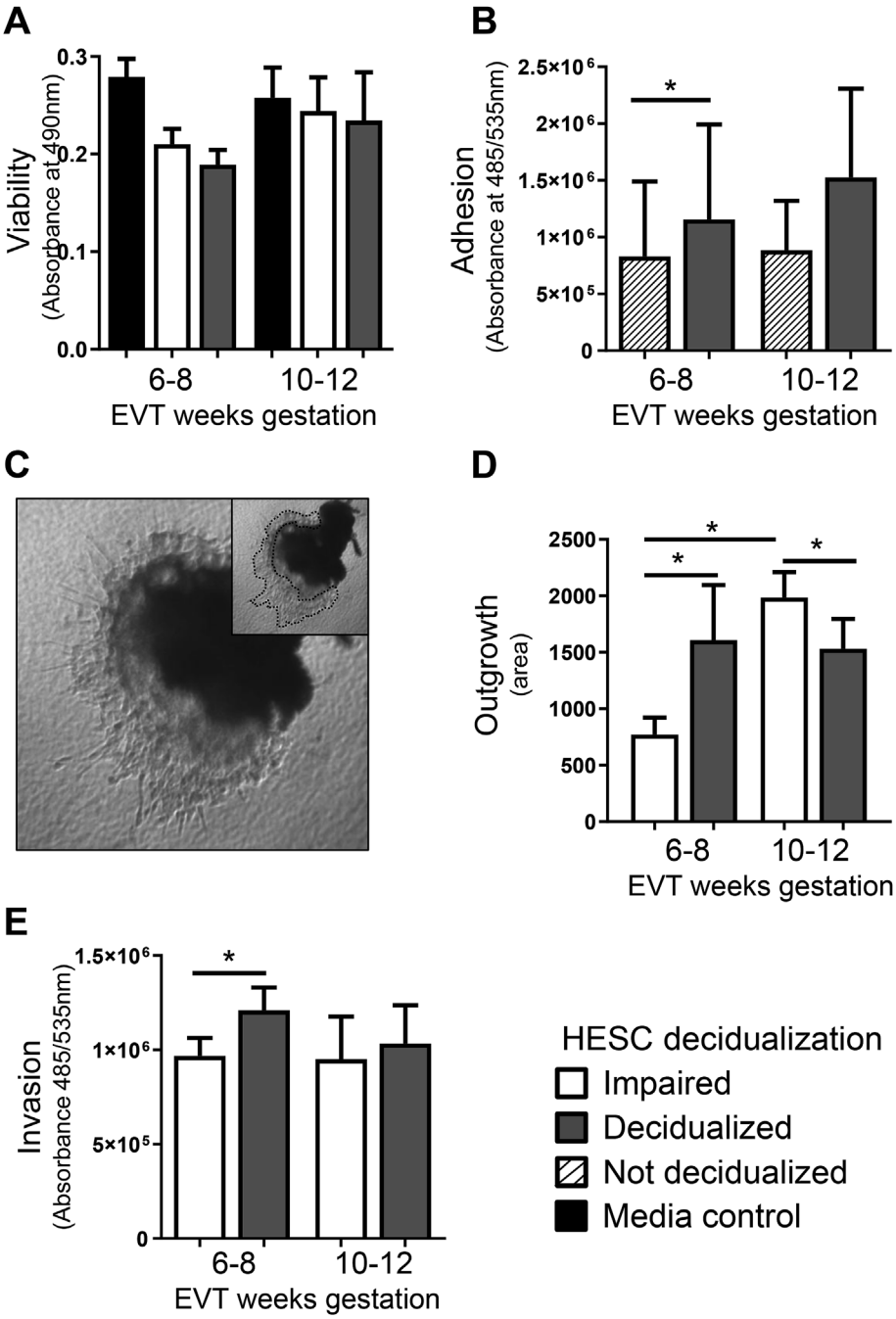

Fig. 1. Decidualized HESC regulation of EVT function was gestation week dependent.
A. HESC CM had no effect on isolated EVT viability (n = 3/group). Statistical test: Wilcoxon matched pairs sign rank test.
B. Isolated EVT adhesion to decidualized HESC was significantly enhanced compared to non-decidualized stromal cells in the 6–8 week EVTs (n = 6/group) but not 10–12 week EVTs (n = 5/group; triplicate replicates included for each treatment group/placenta). Statistical test: Wilcoxon matched pairs sign rank test.
C. Representative figure showing EVT outgrowth from villous tip anchored to collagen drop. Insert shows outlined (dotted line) area of EVT outgrowth.
D. HESC CM regulated EVT outgrowth from placental villous tips (6–8, n = 3/group; 10–12, n = 5/group; villous tips were cultured in quadruplicate replicates/treatment group for each individual placenta). Decidualized HESC CM significantly enhanced 6–8-week EVT outgrowth, but impaired 10–12-week EVT outgrowth. Statistical test: paired *t*-test.
E. Invasion of isolated EVT was significantly enhanced by decidualized HESC CM only in the 6–8week EVTs (6–8, n = 6/group; 10–12, n = 4/group; triplicate replicates performed for each treatment group/placenta). Statistical test: paired *t*-test.
EVT, extravillous trophoblast; HESC, Human endometrial stromal cells; Data shows mean ± SEM; *, $p < 0.05$.

decidualization) and 14 (complete decidualization) as previously described, pooled (n = 5/6) and snap frozen until use in functional assays. Prolactin secretion confirmed decidualization [14].

2.2. Viability assay

The Cytotoxicity Detection Kit^{PLUS} (Lactate Dehydrogenase [LDH]) (Roche Applied Science) was used as per the manufacturer's instructions.

2.3. Cell-cell adhesion assay

Confluent HESC were decidualized as above or treated with 17β-oestradiol alone for 14d. 1×10^5 EVT labelled with 4 μM Calcein-AM were seeded on top of confluent HESC (in triplicate) for 90min and adhesion measured as previously published [16].

2.4. Outgrowth assay

Trophoblast outgrowth from villous tips (in quadruplicate) cultured in control (media alone) or HESC CM was assessed as previously published [17].

2.5. Cell Invasion assay

EVT invasion was assessed using the QCM™ Chemotaxis 96-well Cell Invasion assay (Millipore). EVT (1.8×10^5 cells) were plated (in triplicate) into the upper chamber containing 1part HESC CM or control (media alone) and 1part DMEM/F12 + 10%FBS. Trophoblast invasion into the bottom chamber (DMEM/F12 + 20%FBS) was measured after 36 h by fluorescence (480/520 nm, Envison).

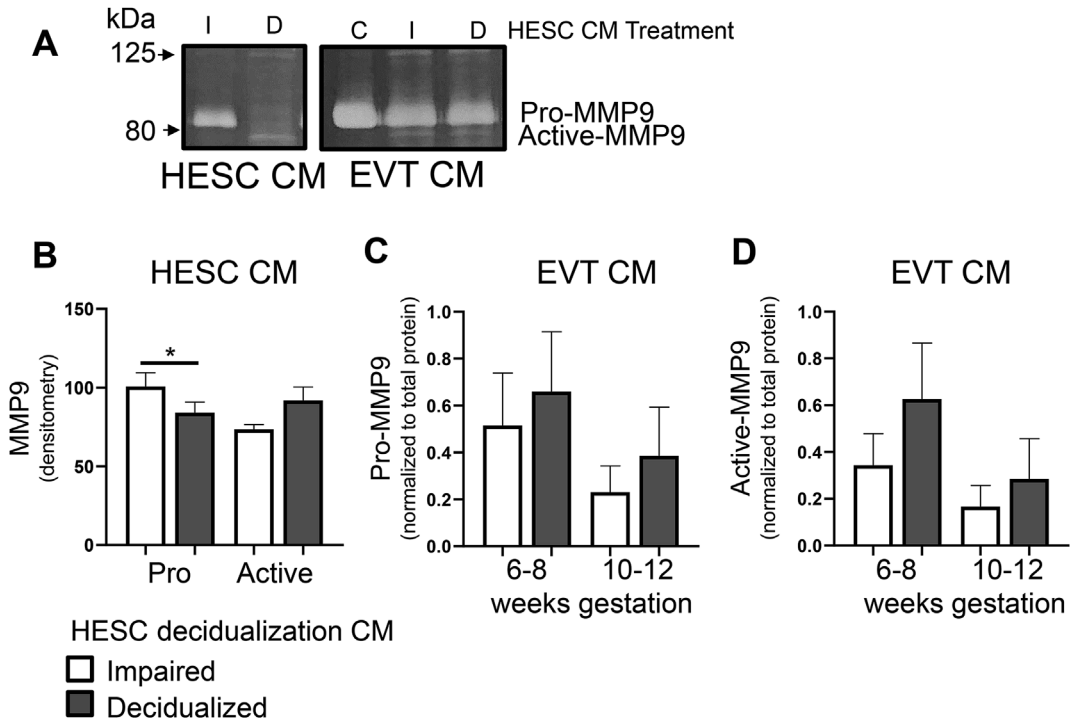

Fig. 2. HESC CM did not alter EVT MMP9 production.
A. Representative image of gelatin zymography showing pro- and active-MMP9 in HESC CM and isolated EVT CM. Active-MMP9 was not identified in EVT CM under control (C; media only, no HESC CM) conditions, it was only detected in EVT treated with HESC CM.
B. Pro-MMP9 was significantly reduced in decidualized HESC CM compared to impaired decidualization HESC CM (n = 3/group). Statistical test: paired *t*-test.
C. Isolated EVT production of pro-MMP9 was not altered by treatment with HESC CM (6–8, n = 4/group; 10–12, n = 3/group). Statistical test: paired *t*-test.
D. Isolated EVT production of active-MMP9 was not altered by treatment with HESC CM (6–8, n = 4/group; 10–12, n = 3/group). Statistical test: paired *t*-test.
CM, conditioned media; C, control; D, decidualized; EVT, extravillous trophoblast; HESC, Human endometrial stromal cells; I, impaired; MMP9, matrix metalloproteinase 9; Data shows mean ± SEM; *, $p < 0.05$.

2.6. Zymography

Gelatinase activity in CM from isolated EVT treated with HESC CM for 16 h [14] was determined by zymography [18]. MMP9 was identified by comparison with molecular weight marker on each gel.

2.7. Statistical analysis

GraphPad Prism 8.01 was used for all statistical analysis. Data was tested for normality (Shapiro-Wilk test). Paired t-tests (Fig. 1D&E, Fig. 2B–D) or Wilcoxon matched pairs sign rank tests (Fig. 1A&B) were used. All data is presented as mean ± SEM. $p < 0.05$ was considered statistically significant.

3. Results

3.1. HESC regulation of EVT function was gestational age dependent

Decidualization did not affect HESC viability (LDH production; no decidualization treatment control 0.23 + 0.01; impaired 0.18 ± 0.04; decidualized 0.14 ± 0.01) or isolated EVT viability following HESC CM treatment (Fig 1A; n = 3/group).

6–8-week isolated EVTs adhered more strongly to decidualized HESC compared to non-decidualized HESC, however there was no significant effect in 10–12-week isolated EVTs (n = 5–6/group; Fig. 1B).

EVT outgrowth from villous tips (Fig. 1C) was not different between 6 and 8 (2442 ± 1035 n = 3) and 10–12-weeks (1905 ± 900, n = 5; $p > 0.05$) in media-only controls. Decidualized CM significantly enhanced 6–8-week EVT outgrowth compared to impaired decidualization CM (Fig 1D; n = 3/group). Conversely, decidualized CM significantly reduced 10–12-week EVT outgrowth compared to impaired decidualization CM (Fig 1D; n = 5/group).

Isolated EVT invasion was not different between 6 and 8 ($7.67 \times 10^5 \pm 1.01 \times 10^5$; n = 6) and 10–12-weeks ($1.00 \times 10^6 \pm 3.15 \times 10^5$; n = 4; $p > 0.05$) in media-only controls. Decidualized CM significantly enhanced 6–8-week isolated EVT invasion (n = 6/group) but had no effect at 10–12-weeks (n = 4/group; Fig. 1E).

3.2. HESC CM did not alter EVT MMP9 production

uNK promote EVT invasion via increased EVT MMP9 production [19], therefore we determined the effect of HESC CM on isolated EVT MMP9 production (Fig. 2A).

Decidualization significantly decreased HESC pro-MMP9 secretion (n = 3/group; Fig. 2B).

Isolated EVT secreted active-MMP9 only following treatment with HESC CM (n = 3–4/group; Fig. 2A). HESC CM treatment did not alter isolated EVT pro-/active-MMP9 production (Fig. 2C and D). There was no effect on MMP2 production (data not shown).

4. Discussion

This is the first study to utilize primary first-trimester trophoblast to determine the effect of the extent of decidualization on trophoblast function. EVT function was altered by decidual cues in a gestational-dependent manner. 'Younger' EVTs exposed to impaired decidualization cues showed reduced adhesion, outgrowth and invasion compared to complete decidualization. Impaired decidualization occurs in pregnancy disorders which are associated with reduced EVT invasion [10,11,13]. Our data suggests impaired decidualization may restrict EVT invasion early in gestation, possibly initiating the cascade of events

which result in pregnancy pathologies including recurrent miscarriage and preeclampsia. This study identifies the importance of using first-trimester trophoblast to investigate early placentation and highlights the importance of trophoblast-decidual synchrony to facilitate appropriate EVT-HESC crosstalk and EVT invasion during implantation/placentation.

Authors' contributions

EM – conceived, experimental design, primary cultures, viability, adhesion, outgrowth & invasion experiments, zymography, wrote manuscript.

MVS – primary cultures, adhesion and invasion experiments.

JC – zymography.

ED – conceived, experimental design and edited manuscript.

Declarations of interest

None to declare.

Conflicts of interest

The authors declare no conflict of interest.

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