Pregnancy Hypertension xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Pregnancy Hypertension

journal homepage: www.elsevier.com/locate/preghy

Short communication

Decidual ACVR2A regulates extravillous trophoblast functions of adhesion, proliferation, migration and invasion *in vitro*

Hannah E.J. Yong*, Padma Murthi[1], Bill Kalionis, Rosemary J. Keogh[2], Shaun P. Brennecke[2]

The University of Melbourne, Department of Obstetrics and Gynaecology and Department of Maternal-Fetal Medicine, Pregnancy Research Centre, The Royal Women's Hospital, Locked Bag 300, Corner Grattan Street and Flemington Road, Parkville 3052, Victoria, Australia

ARTICLE INFO

Keywords:
ACVR2A
Decidual stromal cells
Extravillous trophoblast

ABSTRACT

Decidual stromal cells form the largest proportion of maternal cells at the maternal-fetal interface. Our aim was to investigate the role of the pre-eclampsia associated decidual activin receptor, ACVR2A, in regulating trophoblast functions at this interface. St-T1b and HTR-8/SVneo cell lines were used to model decidual stromal and trophoblast cells respectively. St-T1b conditioned medium inhibited HTR-8/SVneo adhesion, proliferation, migration and invasion; all effects that were attenuated by decidual *ACVR2A* siRNA transfection. These findings suggest that altered decidual ACVR2A expression perturbs the maternal-fetal crosstalk involved in regulating trophoblast function at the interface, which may affect placentation and lead to pre-eclampsia.

1. Introduction

In pregnancy, decidual stromal cells form the largest proportion of maternal cells at the maternal-fetal interface, where they regulate multiple functions of fetal extravillous trophoblast cells. The decidualised stromal cells produce and release secretory factors [1,2], which can inhibit trophoblast gelatinolytic activity [3] by modifying expression of metalloproteinases [4], integrins and tissue inhibitors of metalloproteinases [5] that are necessary for invading the decidual matrix. Past studies show that activin A, a TGFβ family member, is actively involved in inducing and amplifying the decidualisation process, which prepares the uterine endometrium for pregnancy [6,7]. Expression of the activin A βA subunit is significantly upregulated in uterine stromal cells during the secretory phase of the menstrual cycle, when decidualisation begins *in vivo* and in early pregnancy [8,9]. A similar temporal expression pattern is also observed for the activin A binding receptor, ACVR2A [10]. Nevertheless, while the role of activin A in preparing and supporting a healthy pregnancy at the maternal-fetal interface is well-established, the role of its receptor ACVR2A in mediating its effects is currently unknown. Additionally, genetic variants of *ACVR2A* show associations with the hypertensive disorder – pre-eclampsia [11–15], where ACVR2A expression is significantly reduced at the maternal-fetal interface [13,16]. Therefore, our study aim was to explore the functional effects of altered decidual ACVR2A at this interface using an *in vitro* cell culture model.

2. Materials & methods

2.1. Cell culture

St-T1b cells were maintained in DMEM medium and Ham's F12 medium in a 1:1 ratio, with 10% charcoal-stripped fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin under humidified conditions (5% CO_2/95% air) at 37 °C; and decidualised *in vitro* with 0.5 mM 8-Br-cAMP (Sigma-Aldrich Corp.) to model the decidual stromal cells [17]. HTR-8/SVneo cells were maintained in RPMI-1640 medium with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin under humidified conditions (5% CO_2/95% air) at 37 °C; and used to model the extravillous trophoblast cells [18].

2.2. Decidualisation of St-T1b cells

St-T1b cells were seeded 4×10^5 cells per wells in a 6-well plate in normal growth medium. When confluent, new medium containing 0.5 mM 8-Br-cAMP in DMEM medium and Ham's F12 medium in a 1:1

* Corresponding author at: Centre for Trophoblast Research, Department of Physiology, Development and Neuroscience, University of Cambridge, Downing Street, Cambridge CB2 3EG, UK.
E-mail address: hy324@cam.ac.uk (H.E.J. Yong).
[1] Present address: Department of Medicine and Department of Obstetrics and Gynaecology, School of Clinical Sciences, The Ritchie Centre, Monash University, Monash Medical Centre, Clayton 3168, Victoria, Australia.
[2] Joint senior authors.

https://doi.org/10.1016/j.preghy.2017.11.002
Received 30 May 2017; Received in revised form 11 August 2017; Accepted 13 November 2017

ratio with 2% (v/v) charcoal-stripped FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin was used to decidualise the cells for 72 h. Concurrently, a separate batch of St-T1b cells from the same passage received the same treatment with the exception of no 8-Br-cAMP addition to determine the effects of decidualisation.

2.3. Immunocytochemistry

Immunocytochemistry was performed as described previously [19]. Rabbit polyclonal activin receptor type IIA ab71521 antibody (5 µg/ml, Abcam) was used to detect ACVR2A.

2.4. Western immunoblotting

Western immunoblotting was performed as described previously with either ACVR2A antibody (1.7 µg/ml) or rabbit anti-GAPDH Polyclonal IgG (0.5 µg/ml, Imgenex) [20].

2.5. RNA extraction, cDNA synthesis and real-time polymerase chain reaction

RNA extraction, cDNA synthesis and real-time PCR were performed as described previously [19,21]. *PRL* and *ACVR2A* mRNA transcripts were probed using inventoried FAM-labelled TaqMan® Gene Expression Assays (Applied Biosystems) Hs00168730_m1 and Hs00155658_m1 respectively. Relative mRNA expression levels were then calculated using the $2^{-\Delta\Delta CT}$ method [22].

2.6. siRNA transfection

ACVR2A expression was silenced over 72 h using a pool of *ACVR2A*-specific siRNA (Santa Cruz Biotechnology Inc.). Non-targeting siRNA (Santa Cruz Biotechnology Inc.) was used as the negative control (NC). St-T1b cells were seeded at 4×10^5 cells per well in 6-well plates and allowed to reach confluence prior to siRNA transfection. HiPerFect transfection reagent (Qiagen) was then used to transfect 5 nM siRNA into St-T1b cells according to the manufacturer's protocol. Wells also contained 0.5 mM 8-Br-cAMP to decidualise the cells during the transfection period.

2.7. Conditioned medium collection and treatment

Conditioned medium was aspirated from St-T1b culture plates after 72 h of either 8-Br-cAMP, siRNA or control treatment and centrifuged at $600 \times g$ for 5 min at room temperature. The supernatant was then aliquoted and stored at −80 °C. For *in vitro* functional assays, HTR-8/SVneo cells were treated with 25% conditioned medium pooled from a minimum of n = 3 batches collected under sterile conditions.

2.8. Functional assays

Adhesion, proliferation, migration and invasion assays were performed over 24 h using the xCELLigence RTCA DP Analyzer (ACEA Biosciences Inc.) with E-16 plates and CIM-16 plates for adhesion/proliferation and migration/invasion respectively. Invasion assays were performed with wells pre-coated with Matrigel™ Basement Membrane Matrix (Becton Dickinson). xCELLigence results were then analysed as described previously [19].

2.9. Statistical analyses

Paired Student's t test and 2 X 2 contingency tables with Fisher's Exact Test were used as appropriate. All data are expressed as mean ± SEM unless stated otherwise. GraphPad Prism 5 (GraphPad Software Inc.) was used for statistical analyses. A value of $p < .05$ was considered statistically significant.

3. Results

The ACVR2A receptor was expressed by the St-T1b cell line and was localised primarily to the cytoplasm in both non-decidualised and decidualised cells (Fig. 1A). Decidualisation of St-T1b cells with 8-Br-cAMP was verified by significant induction of prolactin *PRL* mRNA by over 1,000 fold (Fig. 1B). Decidualisation with 8-Br-cAMP significantly increased *ACVR2A* mRNA expression by 2.2 fold (Fig. 1C). The representative immunoblot demonstrates an increase of immunoreactive ACVR2A protein after decidualisation of St-T1b with 8-Br-cAMP (Fig. 1D). After correcting for protein loading with the GAPDH control, a significant increase of 1.2 fold was observed at the protein level (Fig. 1E). Trophoblast functions of adhesion, proliferation, migration and invasion were determined by xCELLigence assays. Treatment with conditioned medium from decidualised St-T1b cells significantly inhibited HTR-8/SVneo adhesion at 1 hour, and proliferation, migration and invasion at 24 h by 45%, 62%, 58% and 72% respectively (Fig. 1F–I).

To model decreased decidual ACVR2A expression in PE, siRNA transfection was used to silence ACVR2A expression of decidualised St-T1b cells. *ACVR2A* mRNA expression was significantly reduced by 75% in decidualised St-T1b cells transfected with *ACVR2A* siRNA compared with the negative control after 72 h (Fig. 2A). The representative immunoblot demonstrates an evident knockdown of immunoreactive ACVR2A protein (Fig. 2B). A knockdown of approximately 31% was achieved at the protein level (Fig. 2C). Additionally, prolactin expression was compared to examine the effect of ACVR2A receptor knockdown on decidualisation. Transfection of *ACVR2A* siRNA into decidualised St-T1b cells significantly lowered *PRL* mRNA induction by 83% (Fig. 2D). Conditioned medium from decidualised *ACVR2A* siRNA-transfected St-T1b cells significantly increased HTR-8/SVneo trophoblast adhesion at 1 hour and proliferation, migration and invasion at 24 h by 14%, 17%, 41% and 28% respectively (Fig. 2E–H).

4. Discussion

To test the suitability of the St-T1b model for *in vitro* functional analyses, expression of the ACVR2A receptor in St-T1b cells was first verified by immunocytochemistry (Fig. 1A). Decidualisation was then confirmed by significantly increased mRNA expression of prolactin (Fig. 1B), which is a marker of decidualisation levels in pregnancy [23]. The significant increase in ACVR2A expression with *in vitro* decidualisation (Fig. 1C–E) was consistent with a previous report of increased receptor expression during the secretory phase of the menstrual cycle, when decidualisation occurs *in vivo* [10]. Conditioned medium collected from decidualised St-T1b cells significantly inhibited HTR-8/SVneo trophoblast adhesion, proliferation, migration and invasion (Fig. 1F–I), which is also consistent with past studies [2,3]. Having established the suitability of the model, further functional analyses were performed to examine the effects of altered decidual ACVR2A expression observed in pre-eclampsia on decidualisation and its ability to regulate trophoblast functions.

Silencing of decidual ACVR2A by siRNA transfection significantly reduced production of the decidualisation marker prolactin (Fig. 2D), suggesting that altered ACVR2A expression can impair decidualisation of stromal cells. A recent microarray study showed that insufficient decidualisation or endometrial maturation in the first trimester is implicated in the development of pre-eclampsia [24]. Another study demonstrated that pre-eclamptic decidua produced significantly less prolactin [25]. This study also found that serum from pre-eclamptic women did not affect normotensive decidua explant prolactin production, suggesting an inherent genetic defect in pre-eclamptic decidua rather than the influence of a circulatory factor [25]. Hence, these observations suggest that adequate ACVR2A expression plays a role in the optimal conditioning of the decidua for pregnancy. Conversely, the genetic dysregulation of *ACVR2A* expression may lead to suboptimal