CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Human Placenta Expresses α_2 -Adrenergic Receptors and May Be Implicated in Pathogenesis of Preeclampsia and Fetal Growth Restriction

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α_2 -Adrenergic receptors (α_2 ARs) are G-protein-coupled receptors involved in catecholamine signaling by extracellular regulated protein kinase 1 and 2 (ERK1/2) pathways. We examined placental expression and function of α_2 AR subtypes in women with severe preeclampsia (sPE) with and without intrauterine growth restriction (IUGR). Placental biopsies were analyzed from 52 women with i) sPE ($n = 8$); ii) sPE + IUGR ($n = 9$); iii) idiopathic IUGR ($n = 8$); iv) idiopathic preterm birth ($n = 16$); and v) healthy term controls ($n = 11$). Expression of α_2 AR subtypes (α_{2A} , α_{2B} , α_{2C}) and phospho-ERK1/2 (receptor activation marker) was investigated by immunohistochemistry and/or quantitative real-time RT-PCR. The effects of α_{2C} AR knockdown on syncytialization (syncytin-1 and -2) and β -human chorionic gonadotropin secretion were examined in BeWo cells stimulated with forskolin. The effects of α_2 AR agonist UK 14,304 and specific α_{2C} AR antagonist were tested, using a trophoblast migration assay. All three α_2 ARs were expressed and functionally active in human placenta with site-specific localization. Highest α_{2B} AR and α_{2C} AR mRNA expression was identified in sPE + IUGR. α_{2C} AR knockdown increased expression of syncytin-1 and -2 but decreased secretion of β -human chorionic gonadotropin. UK 14,304 impaired trophoblast migration. The observed α_2 AR expression pattern suggests different function for each subtype. α_{2C} AR modulates trophoblast syncytialization and migration and may carry pathogenic role in sPE + IUGR. (*Am J Pathol* 2018, 188: 2774–2785; <https://doi.org/10.1016/j.ajpath.2018.08.011>)

The α_2 adrenoceptors (α_2 ARs), encoded by *ADRA2* genes, are G-protein-coupled receptors with vital roles in cellular physiological processes and adaptation to stress.¹ Generally speaking, engagement of α_2 ARs by catecholamines (preferentially by norepinephrine) results in negative feedback inhibition of adenylate cyclase by G-protein subunit α , group i, resulting in reduced cAMP synthesis.^{2–4} α_2 AR agonists have been widely used for treatment of various medical conditions, including hypertension, attention-deficit/hyperactivity disorder, opioid withdrawal, and, more recently as, anesthesia adjuvants.⁵ These uses result from the preponderant expression of α_2 ARs in vasculature and in the central nervous system. However, a number of

studies have reported presence of α_2 AR on other cell types with important sympathetic reactive responses such as adipocytes, pancreatic β -cells, and even cancer epithelial cells where it was found that α_2 AR stimulation increases metastatic burden in the context of surgical stress.⁶

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The relevance of the α_2 ARs in reproductive biology was demonstrated through the use of genetically engineered murine models in which the three known receptor subtypes (α_{2A} , α_{2B} , and α_{2C}) were knocked out.⁷ In mice in which all three α_2 ARs were deleted, lethality at embryonic day 10.5 was significantly increased because of defective vascular development.⁷ Furthermore, it was shown that phosphorylation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) in the placenta was lower than in wild-type animals. Of interest, several lines of evidence pointed toward placental α_{2B} AR and not α_{2A} or α_{2C} AR subtypes as critical for embryonic survivability, at least in the mouse.⁷ One proposed explanation was that co-expression of α_{2B} AR and type 1 receptor for vascular endothelial growth factor (VEGF) by spongiotrophoblasts and trophoblast giant cells prevents the generation of soluble type 1 receptor for VEGF, the antagonist of VEGF.⁸ Collectively, the above data provide baseline evidence that perturbation of placental α_2 AR expression and/or activity may contribute to pregnancy-specific pathologies, including fetal loss and defective angiogenesis.

Aberrant placental growth, maturation, angiogenesis, and invasion are histopathologic hallmarks of both preeclampsia (PE) and intrauterine growth restriction (IUGR); however, the underlying molecular mechanisms remain elusive.^{9–11} Trophoblast syncytialization is a highly coordinated cell-to-cell fusion process necessary for formation and function of the syncytiotrophoblast, which produces and secretes many hormones, including β -human chorionic gonadotropin (β -hCG).^{12–15} Several proteins, growth factors, and cytokines can modulate trophoblast syncytialization.^{16,17} Chief among them are syncytin-1 and syncytin-2, which are considered hallmarks of terminal differentiation of placental trophoblast lineage.¹⁸ The synthesis and expression of syncytins depends on the cAMP/protein kinase A pathway as demonstrated through experiments that involve pharmacologic stimulation of cytotrophoblast-like (BeWo) cells with the adenylate cyclase activator and intracellular cAMP-elevating agent forskolin.^{16,19}

From the above experimental data it is tempting to speculate that aberrant expression and activation of α_2 ARs could disrupt placental development, leading to pathologic processes. This concept has biological plausibility because trophoblast syncytialization is defective in both PE and IUGR.^{20–22} These two conditions may occur if heightened expression of α_2 ARs inhibits adenylate cyclase, thereby reducing cAMP production and in turn the synthesis of syncytins.

At least two α_2 AR subtypes are known to be present in human placenta, specifically α_{2A} AR and α_{2B} AR.²³ This conclusion was reached from radioligand binding experiments and not by immunohistochemistry, which, before the present study, has not been performed. Here, we tested the hypotheses that PE and IUGR are associated with differential expression of placental α_2 ARs and that α_2 ARs are key regulators of trophoblast syncytialization and migration.

Materials and Methods

Patients and Samples

Placental tissues retrieved from 52 women were distributed in the following groups: i) PE with severe features [sPE; $n = 8$; means $\pm$ SEM gestational age (GA), 31 ± 1 weeks]; ii) sPE + IUGR ($n = 9$; mean GA, 30 ± 1 weeks); iii) idiopathic IUGR ($n = 8$; mean GA, 31 ± 1 weeks); iv) idiopathic preterm birth (iPTB; $n = 16$; mean GA, 31 ± 1 weeks), and v) healthy term controls ($n = 11$; mean GA, 39 ± 1 weeks) were analyzed.

GA in all cases was established from last menstrual period and/or early ultrasound (<20 weeks).²⁴ Preterm birth was defined as delivery before 37 weeks GA. For all iPTB cases intraamniotic infection was excluded based on negative results of amniotic fluid biochemical testing, negative bacterial cultures, and absence of histologic chorioamnionitis. iPTB tissues were the best possible control to identify potential GA regulation in the expression of α_2 ARs. This control was important because women with sPE and IUGR are frequently delivered at earlier GAs. Because patient recruitment and sample collection were completed before publication of the 2013 Task Force (May 2005 to July 2012), the definition of PE was based on American College of Obstetricians and Gynecologists criteria valid at the time of sample collections.^{25,26} IUGR was defined ultrasonographically as an estimated fetal growth <10 th percentile for GA.²⁷ A diagnosis of idiopathic IUGR was established in babies without detectable structural anomalies, and when examination of the placenta and the work-up to rule out chromosomal aneuploidy did not provide a cause for lack of growth. Presence of fetal structural anomalies and antenatal viral infections (ie, HIV, hepatitis) were considered exclusion criteria. All women were delivered at Yale-New Haven Hospital and provided signed informed consent under protocols approved by the Human Investigation Committee of Yale University. Within minutes from the time of delivery of the placenta, a full-thickness biopsy was retrieved from the central portion of the placenta. Approximately one-half of the tissue was fixed in formalin. For the second half, decidua basalis was dissected from the villous trophoblast in sterile fashion, rinsed in sterile saline, frozen in liquid nitrogen, and kept at -80°C for RNA studies.

Immunohistochemistry

Purified rabbit polyclonal antibodies were used to stain for α_{2A} AR, α_{2C} AR, and α_{2B} AR (ab91648; Abcam Inc., Cambridge, MA).²⁸ The three polyclonal antisera are specific for their respective α_2 AR subtype and do not cross-react with other α_2 AR subtypes. The specificity of these antibodies has been established in several previously published studies.^{29–32} The anti- α_{2C} AR antibody recognizes the approximately 70-kDa mature receptor form that translocates from the Golgi compartment to the cell surface.³³ A rabbit

monoclonal antibody was used to detect phospho-ERK1/2 (p-ERK1/2) (catalog number 4370; Cell Signaling Technology, Danvers, MA).

Five-micron serial paraffin sections of fetal membranes and placental villous tissue were immunostained for α_2 ARs and p-ERK1/2 as previously described.³⁴ Term human myometrium was used as a positive control. After antigen retrieval with citrate buffer and pretreatment with 1% hydrogen peroxide (15 minutes), sections were incubated overnight (4°C) with affinity-purified rabbit polyclonal antibodies to α_{2A} AR, α_{2B} AR, or α_{2C} AR (dilution 1:100)³² or p-ERK1/2 (dilution 1:100) then at room temperature (60 minutes) with biotinylated donkey anti-rabbit IgG (dilution 1:600; Jackson ImmunoResearch Laboratories, West Grove, PA). Negative controls consisted of tissue samples from the same patient that were processed identically but incubated with non-immune rabbit IgG (Jackson ImmunoResearch Laboratories). Sections were stained by using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) with VECTOR NovaRed chromogen, counterstained with hematoxylin, and subjectively scored, as previously described.³⁴ All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless specified. Specific staining in extravillous trophoblasts, villous cytotrophoblasts, syncytiotrophoblast, and wall of placental vascular structures was evaluated semiquantitatively in a blinded fashion by two independent observers (H.K.B.M. and I.A.B) from three random fields per slide. Staining intensity was scored on a scale from 0 (absent) to 5 (intense).

BeWo Cell Culture and Immunofluorescence

The human choriocarcinoma BeWo cell line (gift from Dr. John M. Robinson, The Ohio State University, Columbus, OH) shares morphologic and biosynthetic features with human invasive trophoblasts and is widely used to study placental trophoblast function.¹⁷ In this study BeWo cells were used for *in vitro* manipulation of α_{2C} AR. BeWo cells were cultured in Ham's F12 medium supplemented with 10% fetal bovine serum and L-glutamine (2 mmol/L), containing 100 U/mL penicillin and 100 μ g/mL streptomycin (Gibco, Gaithersburg, MD), and were incubated under humidified atmosphere of 5% CO₂ in air at 37°C. Cells were seeded at 0.5 to 1 $\times$ 10⁶ in T25 25-cm² flasks and passaged at 60% to 80% confluency by using 0.25% Trypsin (Gibco). For β -hCG measurements and for protein and RNA isolation, cells were seeded at 10⁵ cells per well on 6-well plates.

Cells were incubated overnight in culture media replaced daily with fresh media that contained 20 μ mol/L forskolin or dimethyl sulfoxide (DMSO; vehicle control), as appropriate for the planned experiments. α_{2C} AR was visualized with polyclonal rabbit antibody,³² followed by goat anti-rabbit secondary antibody conjugated to Alexa Fluor 594 (A11037; Life Technologies, Carlsbad, CA). E-cadherin (marker of progenitor cytotrophoblasts) was visualized with

mouse monoclonal anti-E-cadherin monoclonal antibody (HECD-1; dilution 1:200; Invitrogen, Carlsbad, CA), followed by goat anti-mouse IgG secondary antibody conjugated to Alexa Fluor 488 (A11001; Invitrogen).

Quantitative Real-Time RT-PCR

For gene expression studies, placental tissue was flash frozen in liquid nitrogen immediately after delivery and maintained at -80°C. BeWo cells were washed with ice-cold phosphate-buffered saline, 1 mL TRIzol was added to each well, and plates were stored at -80°C. Placental and BeWo cell RNA was extracted with TRIzol and quantitated and reverse transcribed into cDNA with random hexamer primers by using standard procedures. RT-PCR was performed by using TaqMan (Applied Biosystems, Carlsbad, CA) chemistry in 20- μ L reactions composed of 10 μ L mastermix (TaqMan Fast Advanced Master Mix), 8 μ L water, 1 μ L cDNA template, and 1 μ L PCR probe set (TaqMan Gene Expression Assays). The following probes (Applied Biosystems) were used: α_{2A} AR (*ADRA2A*; Hs01099503_s1; reference sequence NM_000681.3), α_{2B} AR (*ADRA2B*; Hs00265090_s1; reference sequence NM_000682.6), α_{2C} AR (*ADRA2C*; Hs03044628_s1; reference sequence NM_000683.3), and for the housekeeping genes ribosomal protein L30 (*RPL30*; Hs00265497_m1; reference sequence NM_000989.3) and β -2-microglobulin (*B2M*; Hs99999907_m1; reference sequence NM_004048.2). Unique probes for experiments with BeWo cells were syncytin-1 (*ERVW-1*; Hs00205893_m1; reference sequence NM_001130925.1) and syncytin-2 (*ERVFRD-1*; Hs01652148_m1; reference sequence NM_207582.2) with *RPL30* as housekeeping gene.

Placental mRNA expression of α_2 ARs was normalized to *RPL30* and *B2M* by using cDNA derived from pools of RNA extracted from iPTB and term control placenta tissue, and BeWo expression was normalized to *RPL30*. Amplification was performed in duplicate reactions for each target in a two-step cycle (denaturation, 95°C for 15 seconds; annealing/extension at 62°C for 60 seconds) for 40 cycles in the StepOnePlus Real-Time PCR System (Applied Biosystems).

Post-processing calculations were performed by using StepOnePlus Software version 2.1 (Applied Biosystems, Foster City, CA). C_t values for α_2 AR were normalized to the geometric mean of the endogenous control RNAs by using calculations of dC_t (C_t of the target - C_t mean of endogenous controls). Calculation of ddC_t (dC_t of individual sample - C_t of same target in a reference sample) used as reference sample a cDNA pool of all tissues. For BeWo cell experiments, RNA from cells transduced with the non-human target control vector was used as reference.

Western Blot Analysis

α_{2C} AR expression in BeWo cells was detected with affinity-purified rabbit polyclonal α_{2C} AR antisera (dilution 1:500)

from total cellular protein prepared as previously described.³² Cells were lysed in buffer that contained protease inhibitor cocktail (Complete; Roche Applied Sciences, Indianapolis, IN), and 20 μ g of total protein was boiled in reducing sample buffer and separated on 4% to 20% Mini Protean TGX gels (Bio-Rad, Hercules, CA). Intensity of the band that corresponded to α_{2C} AR was quantified by using ImageQuant TL software version 8.1 (GE Healthcare, Pittsburgh, PA) and normalized to glyceraldehyde-3-phosphate dehydrogenase as loading control.

Pharmacologic Targeting of α_2 ARs

Pharmacologic studies used the nonsubtype selective α_2 AR receptor agonist UK 14,304 [5-bromo-N-(4,5-dihydro-1H-imidazole-2-yl)-6-quinoxalinamine] or the antagonist MK912 that is more selective for α_{2C} AR.³⁵ After 48 hours, cells were treated in a dose-dependent fashion with UK 14,304 (0.01 to 10 nmol/L) or DMSO for 15 minutes. In another set of experiments, cells were treated with UK 14,304 for 15 minutes after a 30-minute pretreatment with 1 nmol/L MK912 or corresponding volume of water (vehicle control for MK912). β -hCG secretion in cell culture media was assayed by enzyme-linked immunosorbent assay (BioVendor Research and Diagnostic Products, Asheville, NC).

α_{2C} AR shRNA Knockdown Experiments

BeWo cells were seeded 10^5 per well and incubated overnight. In the morning, culture medium was removed and replaced with fresh solution. BeWo cells were transduced with lentiviral particles at a multiplicity of infection = 1 and were subjected to puromycin selection according to the supplier's protocol (Sigma-Aldrich). Transduced cells underwent three consecutive passages under puromycin selection (9 days total) before use in knockdown assays.

α_{2C} AR expression was knocked down in BeWo cells by using a lentiviral shRNA construct that targeted *ADRA2C* (TRCN0000008077; 5'-CCGGCAACGACGAGACCTGGTACATCTCGAGATGTACCAGGTCTCGTCGTTGTTTTT-3'; Sigma-Aldrich). BeWo cells transduced with a non-human target control, pLKO.1-puro (Sigma-Aldrich), served as negative control. Cells were incubated in the presence or absence of 20 μ mol/L forskolin to induce differentiation. RNA was extracted, and expression of α_{2C} AR and of syncytin-1 and syncytin-2 mRNA was measured by quantitative real-time RT-PCR. Culture media were collected before and at 24, 48, and 72 hours after 15-minute exposure to UK 14,304 or DMSO (vehicle control). Media were stored at -80°C until β -hCG measurement.

Trophoblast Migration Analysis

The migration potential of BeWo cells was assessed by wound healing assay as previously described.³⁶ A single wound gap was induced on each well by scratching the cell

monolayer with a pipette tip. BeWo cells were pretreated with 1 nmol/L MK912 or H₂O for 15 minutes, followed by incubation with 10 mmol/L UK 14,304 or DMSO for 60 minutes. Images of five wounds for each treatment condition were analyzed at 0 and 24 hours after treatment, and percentage of migration at 24 hours was calculated from three points per wound. ImageJ software version 1.46r (NIH, Bethesda, MD; <https://imagej.nih.gov/ij>) was used for image analysis.

Statistical Analysis

Statistical analyses were performed with SigmaPlot statistical software version 12.5 (Systat Software, San Jose, CA). After distribution testing by using Shapiro-Wilk test, data were compared with *t*-tests, one-, two-, or three-way analysis of variance or Kruskal-Wallis analysis of variance on ranks as appropriate. Proportions were compared with χ^2 test. *P* < 0.05 was considered statistically significant.

Results

Demographic and Clinical Characteristics

The demographic, clinical, and outcome characteristics of the patients enrolled in this study are presented in [Supplemental Table S1](#). Women with sPE with and without IUGR had the highest blood pressure levels, proteinuria, and frequency of neurologic manifestations (ie, headache, visual disturbances, etc). Patients diagnosed antenatally with IUGR delivered babies with the lowest birthweights.

Immunolocalization of α_2 AR Subtypes in Preterm and Term Placenta

Immunoreactivity for all three α_2 AR subtypes was identified in preterm and term placenta but each with a differing pattern of cellular localization ([Supplemental Figure S1](#)). The signal for α_{2A} AR dominated in vascular structures of placental stem villi. This was in contrast to α_{2B} and α_{2C} , which were primarily expressed by trophoblast cells. Term placental specimens showed decreased immunostaining for all α_2 AR subtypes ([Supplemental Figure S1](#)).

Immunolocalization of α_2 ARs Subtypes in Placenta Affected by sPE and/or IUGR

[Figure 1](#) shows representative micrographs of placental immunostaining for α_{2A} AR, α_{2B} AR, α_{2C} AR, and p-ERK1/2 in patients with iPTB, sPE, sPE + IUGR, and IUGR. α_{2A} AR staining ([Figure 1A](#)) predominated in blood vessels of the placental stem and intermediate villi. Terminal villi were almost always free of α_{2A} AR staining. Semi-quantitative scoring appreciated no difference in staining with respect to sPE, but significantly weaker staining was seen in blood vessels of placentas affected by IUGR

Figure 1 Immunohistochemical staining of α_2 -adrenergic receptor subtype A (α_{2A} AR), α_{2B} AR, α_{2C} AR, and phospho-extracellular signal-regulated protein kinase (p-ERK)1/2 in human placental tissue. **A:** Representative micrographs stained for α_{2A} AR from pregnancies complicated by idiopathic preterm birth (iPTB), severe preeclampsia (sPE), sPE with intrauterine growth restriction (sPE + IUGR), and IUGR alone. The imaged zones are representative for the areas scored semiquantitatively. **B:** α_{2A} AR staining was much stronger in the media of blood vessels in SV and IV than in TV. **C:** Staining for α_{2B} AR predominated in villous cytotrophoblasts (CYTs) and extravillous trophoblasts (EVTs). There was an irregular pattern of stromal staining in sPE + IUGR. **D:** Overall no significant differences were observed in signal intensity among groups. **E:** Staining for α_{2C} AR was most prominent in EVT, CYT, and syncytiotrophoblast (SCT). **F:** Results of histologic scoring indicated significantly higher α_{2C} AR staining of EVT and SCT associated with sPE + IUGR. **Insets** display regions of interest at higher magnification. Representative p-ERK1/2 staining from a patient with sPE + IUGR indicated activation in placental vasculature and EVT. Data are expressed as means $\pm$ SEM. $n = 6$ to 15 patients in each group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, determined by three-way analysis of variance. Scale bars: 100 μ m (**A**, **C**, and **E**); 25 μ m (**insets**). SV, stem villi; IV, intermediate villi; TV, terminal villi; VS, villous stroma.

irrespective of sPE status (three-way analysis of variance, $P < 0.001$ for villous grade, $P = 0.007$ for IUGR, and $P = 0.913$ for sPE) (Figure 1B).

Immunostaining for α_{2B} AR was identified primarily in villous cytotrophoblasts (Figure 1C). Overall, no significant changes were found with either sPE or IUGR state for the three scored zones (extravillous trophoblasts, villous cytotrophoblasts, and villous stroma; $P = 0.046$ for zone, $P = 0.329$ for sPE, and $P = 0.778$ for IUGR) (Figure 1D). An interesting irregular granular pattern of staining was seen in stroma of many terminal villi in the sPE + IUGR group.

The most conspicuous staining for α_{2C} AR (Figure 1E) was localized to extravillous trophoblasts, villous cytotrophoblasts, and syncytiotrophoblast of placenta from women with sPE + IUGR (three-way analysis of variance, $P = 0.001$ for trophoblast types, and $P = 0.047$ for interaction between sPE and IUGR) (Figure 1F). To support the notion of α_2 AR functional engagement, adjacent sections were stained for p-ERK1/2. Representative images from a patient with sPE + IUGR are presented in Figure 1, A, C, and E. p-ERK1/2 immunoreactivity was present in the wall of blood vessels of stem villi and in extravillous trophoblasts scattered in the wall of the anchoring villi and abundantly infiltrating within placental islands. The pattern of p-ERK1/2 in groups with sPE and/or IUGR paralleled the cell types stained by α_{2A} AR and α_{2C} AR but not α_{2B} AR.

mRNA Expression of α_2 AR Subtypes in Human Placenta

To learn if placenta was a site of α_2 AR synthesis and if that varied with GA, tissues retrieved from women with iPTB and healthy term deliveries were first examined. Quantitative real-time RT-PCR showed that transcripts for α_{2A} AR, α_{2B} AR, and α_{2C} AR were present in both preterm and term tissues (Figure 2A). Transcripts for α_{2A} AR were the most abundant, whereas those for α_{2B} AR were the least

represented. For each α_2 AR subtype, mRNA levels were similar in iPTB and term placenta (two-way analysis of variance, $P < 0.001$ for receptor subtype and $P = 0.327$ for GA periods).

Next, the placental expression of α_{2A} AR, α_{2B} AR, and α_{2C} AR were compared in sPE, sPE + IUGR, and IUGR by using ddCt. The iPTB and term groups were used for baseline. No difference was found in α_{2A} AR transcript levels across groups ($P = 0.137$) (Figure 2B). Of interest, placenta of women with sPE, sPE + IUGR, and IUGR had significantly higher expression of α_{2B} AR than with iPTB ($P = 0.015$ for IUGR and $P = 0.012$ for sPE) (Figure 2C) but not compared with term tissues. sPE + IUGR had significantly higher α_{2C} AR placental mRNA levels than both the iPTB ($P = 0.019$) (Figure 2D) and term ($P = 0.031$) groups.

To the best of our knowledge, this is the first report of α_{2C} AR expression in human placenta; with the use of human trophoblasts, the possible function of this receptor were studied.

Validation of α_{2C} AR Expression by BeWo Trophoblast Cell Line

BeWo cells were chosen as model because of their well-characterized ability to differentiate *in vitro* in response to adenylate cyclase activation by forskolin.¹⁷ By Western blot analysis, it was determined that BeWo cells express constitutively α_{2C} AR as the approximately 50- to 55-kDa band (Supplemental Figure S2A), which was previously reported to represent the core glycosylated form of the receptor.³⁷ When fusion was induced through activation of the cAMP/protein kinase A pathway by forskolin, an increased level of expression of the same band as quantified by densitometric analysis was detected ($P < 0.001$) (Supplemental Figure S2B), consistent with the previous report of receptor

Figure 2 Relative abundance of α_2 -adrenergic receptor subtype A (α_{2A} AR), α_{2B} AR, and α_{2C} AR mRNA in placenta. **A:** Relative expression of α_{2A} AR, α_{2B} AR, and α_{2C} AR mRNA in placental tissue of idiopathic preterm birth (iPTB) and term placenta are displayed. Relative quantitation (RQ) of different receptor subtypes was reported relative to expression of housekeeping genes β 2-microglobulin and ribosomal protein L30 by using dCt. **B–D:** Estimate of relative placental mRNA abundance (ddCt) for α_{2A} AR (**B**), α_{2B} AR (**C**), and α_{2C} AR (**D**) for pregnancies complicated by iPTB, severe preeclampsia (sPE), sPE with intrauterine growth restriction (sPE + IUGR), and IUGR, and term controls. ddCt RQ values are reported relative to a reference RNA pool of all tissues. Data are expressed as means $\pm$ SEM. $n = 4$ to 6 samples in each group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

inducibility by cAMP.³² Double immunofluorescence of DMSO-treated BeWo cells confirmed constitutive $\alpha_2\text{cAR}$ expression, albeit at low levels, whereas expression of E-cadherin, a marker of nonfused trophoblasts, was identified at cellular boundaries (Supplemental Figure S2C). As expected, E-cadherin fluorescence diminished on treatment with forskolin, indicating trophoblast differentiation toward syncytialization. This concurred with increased fluorescence for $\alpha_2\text{cAR}$ (Supplemental Figure S2D).

Impact of Pharmacologic Modulation of $\alpha_2\text{cAR}$ on β -hCG Secretion by BeWo Cells

Forskolin-stimulated BeWo cells were treated with increasing concentrations of UK 14,304 (nonselective $\alpha_2\text{AR}$ receptor agonist) to determine whether general engagement of $\alpha_2\text{ARs}$ affects β -hCG secretion, an attribute of differentiated trophoblast. Despite the observed lowering trend, only the highest concentration of UK 14,304 produced a statistically significant reduction in β -hCG secretion ($P < 0.05$) (Figure 3A). This supported some general involvement of $\alpha_2\text{ARs}$ in the negative regulation of adenylate cyclase. More definitive evidence to implicate $\alpha_2\text{cAR}$ was the ability to block the UK 14,304-mediated reduction of β -hCG secretion by pretreatment with the $\alpha_2\text{cAR}$ antagonist

MK912 (Figure 3B). These results suggested that stimulation of $\alpha_2\text{cAR}$ may result in decreased β -hCG secretion and/or decreased syncytialization.

Impact of $\alpha_2\text{cAR}$ Pharmacologic Modulation on Invasive Potential of BeWo Cells

To examine the potential involvement of $\alpha_2\text{cAR}$ in modulating processes required for trophoblast cell migration, wounds produced in monolayers of BeWo cells were subjected to $\alpha_2\text{AR}$ agonist UK 14,304 alone or after pretreatment with MK912. Figure 3C contains images of identical fields in representative wound closure assays photographed at 0 and 24 hours after wounding. BeWo cells treated with the agonist UK 14,304 showed significantly less migration compared with nontreated control cells ($P = 0.029$) (Figure 3D). Pretreatment of BeWo cells with MK912 blocked the inhibition of cell migration mediated by UK 14,304, thus enhancing wound closure ($P = 0.031$).

Impact of $\alpha_2\text{cAR}$ Knockdown on Syncytin mRNA and β -hCG Secretion by BeWo Cells

Because none of the available pharmacologic agents are entirely specific nor have they been previously studied for

Figure 3 Effect of α_2 -adrenergic receptor ($\alpha_2\text{AR}$) agonist activation on secretory and migratory function of BeWo cells. **A:** Levels of β -human chorionic gonadotropin (β -hCG) secreted from BeWo cells stimulated with forskolin (FSK; 20 $\mu\text{mol/L}$) and exposed to increasing concentrations of $\alpha_2\text{AR}$ agonist UK 14,304. **B:** β -hCG secreted from FSK-stimulated BeWo cells treated with UK 14,304 for 15 minutes after pretreatment with the $\alpha_2\text{cAR}$ antagonist MKI912. **C:** Representative images of *in vitro* wound healing scratch assay assessed BeWo cell migration at 24 hours after treatment with $\alpha_2\text{AR}$ agonist UK 14,304 alone or after pretreatment with $\alpha_2\text{cAR}$ antagonist MK912. Control wells (CRL) were treated with corresponding volume of vehicle (water and dimethyl sulfoxide). **D:** Quantification of the gap after 24 hours showed that $\alpha_2\text{AR}$ activation with UK 14,304 resulted in a significant reduction in cell migration and that pretreatment with $\alpha_2\text{cAR}$ antagonist MK912 blocked the UK 14,304-mediated inhibition. **Dashed lines** mark baseline level. Data are expressed as means $\pm$ SEM. $n = 3$ independent experiments expressed as percentage of baseline level before agonist (**A**); $n = 3$ independent experiments (**B**); $n = 3$ independent experiments (**D**). * $P < 0.05$ versus baseline (one-way repeated measures analysis of variance); $^{\dagger}P < 0.05$ (one-way analysis of variance).

affinity for trophoblast α_2 CAR, the gene knockdown approach was used. Lentiviral targeting of *ADRA2C* resulted in 67% reduction in α_2 CAR mRNA levels compared with the non-human target control (paired *t*-test $P = 0.019$) (Figure 4A). With forskolin stimulation, an increase in mRNA transcripts for syncytin-1 (104-fold) (Figure 4B) and syncytin-2 (280-fold) (Figure 4C) were observed relative to non-human target control.

Compared with the non-human target control, *ADRA2C* knockdown significantly decreased β -hCG secretion under basal conditions (DMSO control) at 24 (two-way repeated measures analysis of variance, $P = 0.012$) (Figure 4D), 48 ($P = 0.024$), and 72 ($P = 0.017$) hours. Forskolin stimulation increased β -hCG concentration in the medium

approximately eightfold at 24 hours. *ADRA2C*-knockdown cells secreted significantly less β -hCG than control cells on forskolin stimulation at 24 hours ($P = 0.046$) (Figure 4E). At 48 hours, the difference between knockdown and control cells did not reach significance ($P = 0.086$) (Figure 4E). At 72 hours, β -hCG secretion was unsustainable and decreased in both *ADRA2C* knockdown and control with no difference among these two groups ($P = 0.276$).

Forskolin-stimulated BeWo cells infected with lentivirus that carried the non-human target gene responded to UK 14,304 with decreased secretion of β -hCG (Figure 4F). The direction of this response was similar to that shown in Figure 3A, although the infection itself appeared to render

Figure 4 Effect of *ADRA2C* knockdown on expression of syncytins and secretory function of BeWo cells. **A:** mRNA levels of α_2 -adrenergic receptor subtype C (α_2 CAR) after lentiviral knockdown of *ADRA2C* (KD) or of a non-human target (CRL). **B and C:** Syncytin-1 (**B**) and syncytin-2 (**C**) mRNA after stimulation with forskolin (FSK). **D and E:** Time course of levels of secreted β -human chorionic gonadotropin (β -hCG) from stably transduced *ADRA2C* knockdown cells (black bars) and non-human target knockdown cells (control; gray bars) exposed to dimethyl sulfoxide (DMSO; **D**) as vehicle control or FSK (20 μ mol/L; **E**) to induce syncytialization. **F:** Levels of β -hCG secreted from FSK-stimulated (20 μ mol/L for 48 hours) *ADRA2C* knockdown cells exposed to increasing concentrations of α_2 AR agonist UK 14,304. **G:** Levels of β -hCG secreted from FSK (20 μ mol/L) stimulated non-human target knockdown cells treated with UK 14,304. **Dashed lines** mark baseline level. Data are expressed as means $\pm$ SEM. $n = 3$ stably transduced *ADRA2C* knockdown cells (**D** and **E**); $n = 3$ non-human target knockdown cells (**D** and **E**); $n = 3$ experiments (**F**); $n = 3$ independent experiments (**G**). * $P < 0.05$ (*t*-test); † $P < 0.05$ (two-way repeated measures analysis of variance); ‡ $P < 0.05$ versus baseline (one-way repeated measures analysis of variance).

the cells sensitive to lower agonist concentrations. *ADRA2C* knockdown resulted in loss of this response to UK 14,304 (Figure 4G). This would be consistent with involvement of α_{2C} AR on trophoblast cells in negative regulation of adenylyl cyclase. Taken together, these results suggested that BeWo cells expressed α_{2C} ARs, which were involved in trophoblast differentiation and/or secretory function through processes that at a minimum included inhibition of syncytin-1 and syncytin-2 synthesis and promotion of β -hCG secretion.

Discussion

In this study, a systematic description has been provided for the pattern of α_2 AR expression and localization in human placenta. The overlap in p-ERK1/2 staining pattern with that of at least two α_2 AR subtypes (α_{2A} AR and α_{2C} AR) suggests that α_2 ARs may be implicated in modulation of multiple regulatory processes essential for fetal and placental development.⁸ By PCR, transcripts for all α_2 ARs were identified. The *in vitro* experiments were designed to provide additional mechanistic evidence that α_{2C} ARs, which are primarily known for their critical role in regulating neurotransmitter release from sympathetic nerves and central noradrenergic neurons,³⁸ function in the placenta as local regulators of trophoblast syncytialization and migration. Furthermore, α_{2B} AR and α_{2C} AR were found to be significantly up-regulated at the mRNA and/or protein level in women with sPE and/or IUGR. Our original hypothesis that PE and IUGR associate with altered expression of placental α_2 ARs was thus confirmed.

Before the present study, the role of α_2 ARs in reproduction was implied from genetically engineered murine models that showed defective implantation and fetal development.⁷ Radio-ligand binding and PCR experiments provided evidence that α_{2A} and α_{2B} subtypes are expressed in human placenta.^{23,39–41} In the present study, with the use of PCR and immunohistochemistry, the first systematic description of tissue localization for all three known α_2 AR subtypes in human placenta is presented. The presence of α_{2A} AR and α_{2B} AR was confirmed, and it was further shown that they are preferentially localized to different structures within the placenta: vascular wall for α_{2A} and trophoblast for α_{2B} and α_{2C} AR subtypes. To our knowledge, identification of α_{2C} ARs in the human placenta and description of their localization within the extravillous trophoblast and syncytiotrophoblast are novel findings.

By both PCR and immunohistochemistry, α_{2B} AR was identified in the placenta predominantly in cytotrophoblasts and extravillous trophoblast. If the functional relevance of the α_{2B} AR would be similar to that of mouse placenta, one would expect this AR subtype to be involved in angiogenesis.⁸ It was suggested that the number of placental α_2 ARs decline with advancing GA.²³ The PCR experiments demonstrated that the relative mRNA levels for each α_2 AR

subtype were similar between iPTB and term placenta, yet the immunohistochemical signal appeared diminished according to the receptor localization (stem, intermediate, and term villi). Collectively, these results imply that involvement of α_2 ARs in regulation of placental vascular tone and activity of extravillous trophoblast and syncytiotrophoblast is functional from early gestation.

The α_2 ARs have both presynaptic and postsynaptic functions and are differentially expressed in a variety of tissues, including adrenal gland, vascular endothelium, brain, and kidney.^{1,2,4} In specialized contractile smooth muscle cells in the blood vessel wall, α_2 ARs mediate constriction of arterioles (referred to as resistance vessels) and veins but not large arteries; α_2 AR density is inversely related to vessel size.^{1,31,42} The remarkable differences in function of the blood vessels that comprise the vascular bed are due in part to differential α_2 AR subtype expression within the vascular wall.³¹ For example, although both α_{2A} AR and α_{2C} AR were localized in human aortas, α_{2C} ARs were immunohistochemically localized in the vascular smooth muscle of adventitial arterioles and not aortic media.³¹ The above-mentioned data confirm the existence of site- and tissue-specific α_2 AR expression, which enables physiological modulation of specific peripheral vascular resistance (blood flow and pressure). Consistent with other human vascular territories, placenta also displays site-specific α_2 AR distribution. Our observed immunolocalization of α_{2A} AR within anchoring and terminal placental blood vessels was consistent with an earlier proposed vascular location that was based on the mere existence of smooth muscle cells.⁴¹ Remarkably, studies have categorized α_{2C} ARs as stress-receptors of the vascular sympathetic system.³¹ However, α_{2C} ARs did not have any vascular association in placenta and were localized in extravillous trophoblast and syncytiotrophoblast, potentially signifying different function(s).

Both mRNA levels and immunohistochemistry localization studies showed fundamental differences between human and mouse α_2 AR placental expression. The relative mRNA levels in human placenta were $\alpha_{2A} > \alpha_{2C} > \alpha_{2B}$, and in mouse placenta $\alpha_{2B} > \alpha_{2A} > \alpha_{2C}$.⁸ The newly identified human placental α_{2C} AR was the predominant subtype localized to human syncytiotrophoblast and extravillous trophoblast, whereas α_{2B} ARs, the predominant subtype expressed in mouse placenta at embryo day 9.5 to 10.5 localizes to spongiotrophoblasts and giant cells.^{2,7} Collectively, this distribution pattern implies a broad spectrum of functions of the placental α_2 ARs that in addition to vascular growth, development, and blood flow regulation may include roles in implantation, placentation, and endocrine adaptation to pregnancy.

Trophoblast fusion is an essential process for placental development and maturation. Prior studies highlighted forskolin stimulation of cAMP, α_2 AR negative regulation of adenylyl cyclase, and ability of cAMP to mediate downstream events leading to syncytialization.^{43,44} Despite this

large body of research, our study is first to demonstrate a regulatory connection between α_2 ARs and syncytin-1 and syncytin-2, which are essential proteins for trophoblast fusion. Furthermore, we provided a link between α_2 ARs and cell fusion through experimentation with pharmacologic agents with agonist and antagonist effect. In the cell culture system UK 14,304 reduced secretion of β -hCG, indicating α_2 ARs are inhibitory on the secretory function of the syncytiotrophoblast. However, an interpretation based entirely on inhibitory G-protein subunit α group i signaling is insufficient to explain α_{2C} AR modulation of trophoblast fusion. The gene knockdown experiments provided additional proof. First, in *ADRA2C* knockdown cells under both basal and forskolin-stimulated conditions, β -hCG secretion was significantly decreased. This observation is at odds with the experiments demonstrating increased expression of syncytin-1 and syncytin-2. The simplest explanation for these results is that syncytialization and β -hCG are independent processes as previously shown.¹⁷ As described, it was shown that, in addition to multinucleated syncytia, β -hCG can also be secreted by mononucleated trophoblast experimentally hindered to undergo fusion. β -hCG may also have a protein-kinase A-independent component.¹⁷

We hypothesized that α_2 ARs may have implications for pathogenesis of PE and IUGR. By PCR significant differences were observed in expression of both α_{2B} AR and α_{2C} AR mRNAs and proteins for both conditions. Significantly elevated α_{2B} AR and α_{2C} AR mRNA levels were associated only with sPE complicated by IUGR, but interestingly, with neither pathologic condition alone. This aspect is remarkable because of our data linking the functional relevance of placental α_2 ARs and β -hCG secretion. Human data generated from part of the first trimester screening for aneuploidy reported that maternal serum β -hCG is significantly reduced in patients who developed preeclampsia and IUGR later in gestation.⁴⁵

These data provide new circumstantial evidence of a link between α_2 AR dysregulation and pathogenesis of PE with IUGR.⁴⁶ First, the *in vitro* experiments demonstrated that engagement of the α_2 AR led to poor syncytialization which is known to be defective in PE.^{10,11} Second, the predominant expression of α_{2C} ARs in the extravillous trophoblast coupled with the experiments demonstrating less migratory potential in the presence of UK 14,304 agonist suggest that activation of α_{2C} ARs may affect extravillous trophoblast migration and remodeling of uterine spiral arteries, which is a pathophysiologic hallmark of PE.⁴⁷ Finally, the predominant mouse subtype, α_{2B} AR, has a role in placental angiogenesis; thus, it is reasonable to expect that α_{2B} AR may have analogous functions.^{7,8} The reason α_{2B} AR and α_{2C} AR are significantly up-regulated only in sPE complicated by IUGR remains unknown. Certainly, the human data are at odds with the observation that embryos of α_{2B} AR-deficient mice were smaller in size compared with wild-type animals, inferring that not only deletion but also overexpression of α_{2B} AR and α_{2C} AR may affect fetal

growth.⁸ Many studies agree on the pivotal role of α_2 AR in mediating sympathetic tone in response to environmental stress. Genetic association studies between common α_2 AR variants and phenotypic variability in blood pressure and heart rate responses among individuals have started to emerge.⁴⁸ Considering the previously reported link between α_2 AR signaling and regulation of VEGF and soluble type 1 receptor for VEGF,⁸ these data should stimulate research to investigate a potential link between α_{2C} AR genetic variants and clinical subphenotypes in women with PE. This study demonstrates that the placenta possesses α_2 ARs and brings into consideration a possible local placental effect of α_2 AR pharmacologic modulators. Blocking the activity of α_2 ARs in pregnancies complicated by sPE and IUGR may improve placental blood flow. More studies should be conducted to interrogate the safety and efficacy of α AR blockade in human gestation.

Conclusions

This study provides evidence for differential expression and localization of α_2 ARs in human placental tissue and found a compelling association between elevated α_{2C} AR levels and the pregnancy-related pathologies of sPE and IUGR.

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Supplemental Data

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