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AT2R activation increases in vitro angiogenesis in pregnant human uterine artery endothelial cells

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Abstract

Angiogenesis is vital during pregnancy for remodeling and enhancing vasodilation of maternal uterine arteries, and increasing uterine blood flow. Abnormal angiogenesis is associated with decreased uteroplacental blood flow and development of pregnancy disorders such as gestational hypertension, preeclampsia, fetal growth restriction, preterm delivery, stillbirth, and miscarriage. The mechanisms that contribute to normal angiogenesis remain obscure. Our previous studies demonstrated that expression of the angiotensin type 2 receptor (AT2R) is increased while the angiotensin type 1 receptor (AT1R) is unchanged in the endothelium of uterine arteries, and that AT2R-mediated pregnancy adaptation facilitates enhanced vasodilation and uterine arterial blood flow. However, the role of AT2R in regulating angiogenesis during pregnancy has never been studied. This study examines whether or not AT2R activation induces angiogenesis and, if so, what mechanisms are involved. To this end, we used primary human uterine artery endothelial cells (hUAECs) isolated from pregnant and nonpregnant women undergoing hysterectomy. The present study shows that Compound 21, a selective AT2R agonist, induced proliferation of pregnant-hUAECs, but not nonpregnant-hUAECs, in a concentration-dependent manner, and that this C21-induced mitogenic effect was blocked by PD123319, a selective AT2R antagonist. The mitogenic effects induced by C21 were inhibited by blocking JNK—but not ERK, PI3K, and p38—signaling pathways. In addition, C21 concentration dependently increased cell migration and capillary-like tube formation in pregnant-hUAECs. The membrane-based antibody array showed that C21 increased expression of multiple angiogenic proteins, including EGF, bFGF, leptin, PLGF, IGF-1, and angiopoietins. Our qPCR analysis demonstrates that C21-induced increase in expression of these angiogenic proteins correlates with a proportional increase in mRNA expression, indicating that AT2R activates angiogenic proteins at the transcriptional level. In summary, the present study shows that AT2R activation induces angiogenesis of hUAECs in a pregnancy-specific manner through JNK-mediated pathways with associated transcriptional upregulation of multiple proangiogenic proteins.

Introduction

Angiogenesis (growth of new blood vessels from pre-existing vasculature) and vascular remodelling (including branching, enlargement and network formation) are process is crucial during pregnancy to deliver sufficient oxygen and nutrients to the uterus as well as to the embryo [1]. Specifically, the endothelial cells in the uterine artery undergo proliferation, differentiation, and migration to establish a "low resistance, high capacitance vessel" capable of increasing the uterine blood flow by 53 fold compared to the nonpregnant state [2, 3]. Abnormal angiogenesis is asscoaited with decreased uterine blood flow and development of pregnancy disorders such as gestational hypertension, preeclampsia, fetal growth restriction, preterm delivery, stillbirth, and miscarriage [4, 5]. Therefore, proper establishment and remodeling of blood vessels within the uterine artery is central to fetal growth and survival, and understanding the angiogenic mechanisms may help develop therapeutic strategies for mitigating clinical conditions associated with altered angiogenesis [4].

Angiogenic stimuli during pregnancy are generated through activation of endothelial cell signaling and gene transcription of key molecules, such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) [6]. VEGF promotes angiogenesis through interaction with 2 high-affinity receptors, VEGF receptor-1 (VEGFR-1, also known as Flt-1) and VEGF receptor-2 (VEGFR-2, also known as KDR/Flk-1). The production of antiangiogenic factors increases toward the end of gestation when continued expansion in increased blood flow is no longer needed. One such antiangiogenic factor is a soluble form of VEGFR-1 (sFlt-1) that binds to circulating VEGF and placental growth factors, inhibiting their angiogenic activities. More recently, angiogenesis and vascular remodeling was shown to be induced by angiotensin II (Ang II), a key regulator of blood pressure and the main effector of the renin–angiotensin-aldosterone system [7–9].

Ang II mediates its effect mainly through two types of receptors: the Ang II type 1 receptor (AT1R) and the Ang II type 2 receptor (AT2R). During normal pregnancy, plasma levels of Ang II progressively increase with advancing gestation with a proportional increase in AT2R and unchanged AT1R in the uterine arteries of rodents, sheep, and women [10–12]. For example, in pregnant rats and women, both the expression of AT2R mRNA and protein levels are approximately 4- and 6-fold greater, respectively, during pregnancy than in nonpregnant conditions [10]. This pregnancy-related increase in AT2R expression occurs specifically in endothelial cells of uterine arteries but not in other vascular beds [13]. Coincident with the increased AT2R expression, uterine blood flow increase dramatically during normal pregnancy [14]. Conversely, AT2R expression in placental vessels is decreased during preeclampsia and feto-placental growth restriction [10]. Knockout or blockade of AT2R in pregnant mice and rats induces preeclampsia-like features along with feto-placental growth restriction [14–16]. On the other hand, activation of AT2R with a selective agonist compound 21 (C21) in animal models of preeclampsia increase vasodilation, restore blood pressure, enhance uterine artery blood flow, and improve feto-placental growth [10]. C21 is a highly selective AT2R agonist with a K_i value of 0.4 nM for the AT1R and a $K_i > 10$ mM for the AT1R [17]. Although these studies indicate that the pregnancy-specific increase in AT2R expression is critical for uterine arterial remodeling and blood flow, the direct regulatory role of AT2R in angiogenesis in the uterine arteries is unknown. Herein, we hypothesize that AT2R positively induces angiogenesis. To test this hypothesis, we isolated human uterine artery endothelial cells (hUAECs) from normal nonpregnant and pregnant women, treated with a selective AT2R agonist C21, and examined measures of angiogenesis and underlying mechanisms. Our results show for the first time that AT2R activation promotes proliferation in pregnant-hUAECs but not nonpregnant-hUAECs through JNK pathways but not through ERK, p38, and PI3K pathways. Further,

AT2R-induced angiogenesis in pregnant-hUAECs is associated with transcriptional upregulation of multiple proangiogenic proteins.

Materials and methods

hUAEC isolation and culture

We isolated hUAECs from the main uterine arteries of 30- to 45-year-old nonpregnant and pregnant (35–36 weeks of gestation; n = 5/group) women undergoing hysterectomy, as we had described previously [13, 18]. Written consent was obtained from all subjects, and ethical approval (HS#2013–9763) was granted by the Institutional Review Board at the University of California Irvine [18]. After collagenase digestion, endothelial cells were purified, validated, and cultured in endothelial cell medium (ECM; ScienCell, La Jolla, CA) containing 5% fetal bovine serum, endothelial growth supplements, and 1% penicillin/streptomycin and used within 4–5 passages. When cells reached 70% confluence, the medium was changed to M199 medium containing 0.1% BSA, 0.5% fetal bovine serum, 1% antibiotics, and 25 mmol/L HEPES and was used for experimental treatments.

Proliferation assay

To assess the proliferation competency of hUAECs we used the MTS cell proliferation colorimetric assay kit (Biovision, Milpitas, CA) following the manufacturer's instructions. Briefly, nonpregnant-hUAECs and pregnant-hUAECs (5×10^3/well) were seeded in a 96-well plate treated with or without increasing concentrations of C21 (1, 10, and 100 nmol/L; C21 was kindly provided by Vicore Pharma, Gothenburg, Sweden) for 48 h. MTS reagent (20 μL) was added for the last 3 h, and we evaluated the *in vitro* index of proliferation by measuring optical density at 490 nm with a SpectraMax i3x multi-mode plate reader (Molecular Devices, San Jose, CA). We blocked AT2R by pretreating hUAECs for 30 min with 10 μmol/L of the AT2R antagonist PD123319 (Tocris, Minneapolis, MN). To determine the roles of extracellular signal-regulated kinases (ERKs), p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinase (JNK), and phosphatidylinositol 3-kinase (PI3K) pathways, specific inhibitors were used. Cells were treated with C21 in the absence or presence of ERK1/2 inhibitor (PD98059, 10 μmol/L, Tocris), p38 MAPK inhibitor (SB303580, 10 μmol/L, Tocris), JNK inhibitor (SP600125, 10 μmol/L, Cayman, Ann Arbor, MI), and PI3K inhibitor (LY294002, 10 μmol/L, Cell Signaling Technology, Danvers, MA), and were processed for proliferation assay as described above.

Cell-migration assay

We assessed endothelial cell migration by using an 8-μm pore-sized filter transwell (Corning FluoroBlok, Corning, NY) as described previously [19]. The inserts were coated with 20 μg/ml of fibronectin (Sigma-Aldrich, St. Louis, MO) and kept overnight at 4°C. The next day, hUAECs were plated on the upper chamber of the insert membrane, treated with increasing concentrations of C21 (1, 10, and 100 nmol/L) and incubated in 5% CO_2 at 37°C. After 16 h, the number of cells that had migrated through the membrane to the lower chamber was measured with a fluorescent dye, calcein acetoxymethyl ester (Calcein-AM, 0.2 μg/ml; Invitrogen, Waltham, MA). The cells in the lower chamber were visualized with a BZ-X710 inverted microscope (Keyence, Osaka, Japan) and quantified with a bottom-reading SpectraMax i3x fluorescence plate reader (Molecular Devices).

Tube-formation assay

"Growth factor"-reduced Matrigel (Corning FluoroBlok, Corning, NY) was added to a 48-well plate and incubated at 37˚C for 30 min to allow for solidification. hUAECs were seeded (1 X 10^5 cells per well) onto Matrigel-coated plates and incubated in the presence or absence of increasing concentrations of C21 (1, 10, and 100 nmol/L) and incubated for 8 h at 37˚C. Images were obtained with a MicroPublisher 5.0 camera mounted on an Olympus SZX-10 stereomicroscope (Tokyo, Japan). Images of five randomly chosen fields of each well were captured. Then, with an ImageJ "Angiogenesis Analyzer" tool, we quantified tube formation by counting the total number of tube branches and averaged the values.

Angiogenesis arrays

With antibody-based human angiogenesis array (AAH-ANG-1000; RayBiotech, Peachtree Corners, GA), we simultaneously detected 43 proteins, comprising growth and differentiation factors, membrane-bound receptors, extracellular matrix components, and intracellular signaling molecules (Table 1).

The assay was performed as per the manufacturer's instructions (RayBiotech) and as previously described [20]. Briefly, hUAECs were treated with C21 (10 nmol/L) for 24 h and then lysed with ice-cold radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Danvers, MA) containing protease and phosphatase inhibitors (Sigma). Lysates were cleared by centrifugation at 14,000 g at 4˚C for 10 min, and protein concentration was determined with the BCA assay kit (Pierce; Thermo Scientific, Waltham, MA). The human angiogenesis array membranes were blocked with 2-ml blocking buffer for 30 min at room temperature. The membranes were incubated with 1 ml of 1:10 diluted cell lysates overnight at 4˚C. The membranes were washed and incubated with primary antibody for 2 h at room temperature. After the membranes were washed again with a washing buffer, they were incubated with horseradish peroxidase-labeled anti-rabbit secondary antibody for 2 h. Membrane-bound proteins were detected with electrochemiluminescent detection reagents (Pierce; Thermo Scientific, Waltham, MA), and normalized band intensities were quantified with ImageJ and RayBiotech software tools.

Quantitative real-time PCR

Cells were treated with C21 (10 nmol/L) for 24 h, and total RNA was isolated with an RNeasy mini kit (QIAGEN, Valencia, CA) as per the manufacturer's instructions. The RNA integrity and concentrations were determined with a DS-11 spectrophotometer (DeNovix, Wilmington, DE), and cDNA synthesis was done with 1 μg of total RNA using an iScript cDNA synthesis

Table 1. Human angiogenesis antibody array proteins.

Angiogenin	PDGF-BB	Angiostatin	MCP-3
EGF	PlGF	Endostatin	MCP-4
ENA-78	RANTES	G-CSF	MMP-1
bFGF	TGF-beta 1	GM-CSF	MMP-9
GRO	TIMP-1	I-309	PECAM-1
IFN-gamma	TIMP-2	IL-10	Tie-2
IGF-I	Thrombopoietin	IL-1 alpha	TNF alpha
IL-6	VEGF	IL-1 beta	u PAR
IL-8	VEGF-D	IL-2	VEGF R2
Leptin	Angiopoietin-1	IL-4	VEGF R3
MCP-1	Angiopoietin-2	I-TAC	

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Table 2. Quantitative real-time PCR primer sequence.

Gene	Forward	Reverse
EGF	GTGCAGCTTCAGGACCACAA	AAATGCATGTGTCGAATATCTTGAG
ENA-78	GCCCGTGTCCCCGGTCCTTCGAG	CTGGATCAAGACAAATTTCCTTC
bFGF	CAAGCGGCTGTACTGCAA	CCCAGGTCCTGTTTTGGA
IGF-1	CTTCAGTTCGTGTGTGGAGACAG	CGCCCTCCGACTGCTG
Leptin	TCCCCTCTTGACCCATCTC	GGGAACCTTGTTCTGGTCAT
PLGF	CAGAGGTGGAAGTGGTACCCTTCC	CGGATCTTTAGGAGCTGCATGGTGAC
TIMP-1	TCTGGCATCCTGTTGTTGCT	CGCTGGTATAAGGTGGTCTGG
TIMP-2	ATGCAGATGTAGTGATCAGG	CGTTGATGTTCTTCTCTGTG
Angiopoetin-1	GGGGGAGGTTGGACTGTAAT	AGGGCACATTTGCACATACA
Angiopoetin-2	TGGACAATTATTCAGCGACGTG	GCTGGTCGGATCATCATGGTTG

kit (Bio-Rad, Hercules, CA). After dilution, we amplified cDNA corresponding to 20 ng of RNA by using qRT-PCR, a CFX96 real-time thermal cycler (Bio-Rad, Hercules, CA), and an SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). Primer sequences are shown in Table 2. To analyze the data, we used the $2^{-\Delta\Delta CT}$ method, and expressed the results as the fold change of the gene of interest in treated versus control samples. All reactions were performed in duplicate, and GAPDH was used as an internal control.

Statistical analysis

Data were analyzed with GraphPad Prism (GraphPad Software, San Diego, CA) presented as mean ± SD. Experiments were performed in triplicates and all experiments were repeated at least three to five times using cells from different subjects. Two groups were compared with unpaired Student t-tests. Multiple group comparisons were performed using ANOVA, followed by Newman–Keuls tests. Statistically significant differences were reported when $p < 0.05$.

Results

AT2R activation increased cell proliferation of pregnant-hUAECs, but not nonpregnant-hUAECs

In our effort to assess the effects of AT2R activation on cell proliferation, hUAECs isolated from pregnant and nonpregnant women were exposed to varying concentrations of C21 for 48 h. As shown in Fig 1, C21 induced proliferation of pregnant-hUAECs, but not nonpregnant-hUAECs, in a concentration-dependent manner. Increasing concentrations of C21 (1, 10, 100 nmol/L) resulted in increased cell proliferation by 14%, 84%, and 104%, respectively. We next assessed whether the effect of C21 was mediated via binding to its cognate receptor AT2R. As shown in Fig 1, AT2R selective antagonist-PD123319 blocked cell proliferation induced by C21 in pregnant-hUAECs. Overall, these findings indicate that C21 induces hUAEC proliferation through AT2R in a pregnancy-specific manner.

JNK inhibitor blocked C21-stimulated cell proliferation

ERK, PI3K, p38, and JNK pathways have been demonstrated to play a crucial role in cell proliferation [21, 22]. Pretreatment with PD98059 (ERK1/2), SB303580 (p38), and LY294002 (PI3K) inhibitors did not have any effect, but JNK inhibitor SP600125 blocked C21-mediated proliferation of pregnant-hUAECs (Fig 2). Incubation with inhibitors alone did not affect cell proliferation (Fig 2).
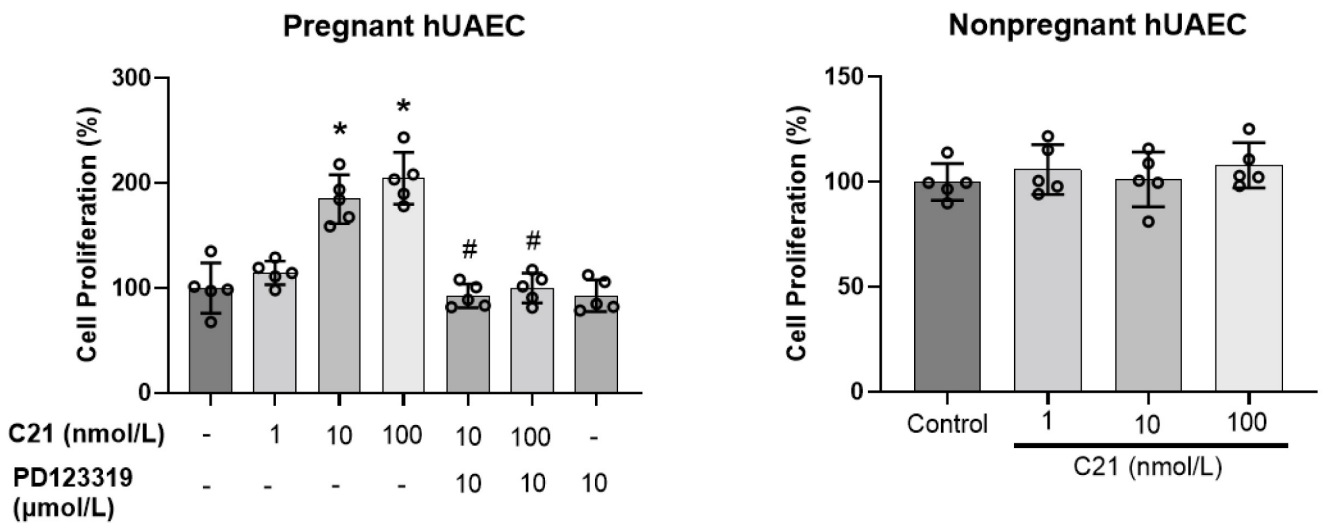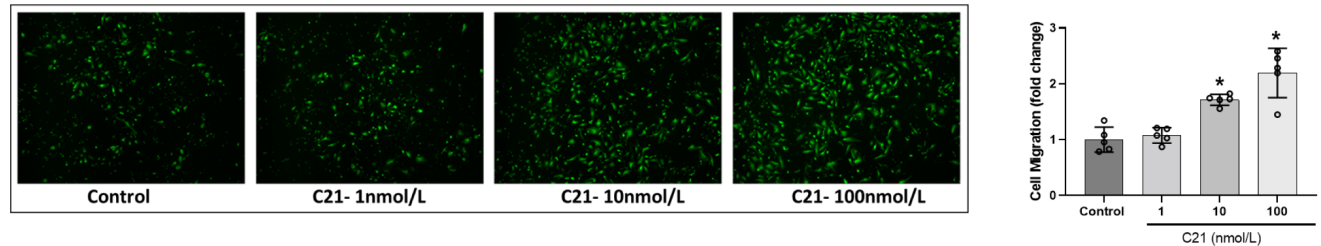
Fig 1. Effect of AT2R agonist C21 on proliferation of pregnant- and nonpregnant-hUAECs. Cells were treated with increasing concentrations of C21 (1–100 nmol/L) for 48 h with or without AT2R blocker PD123319 (10 μmol/L). Cell proliferation was measured with the MTS Cell Proliferation Assay kit. Data were expressed as mean ± SD from five independent experiments using hUAECs from five donors. *P < 0.05 vs control. #P < 0.05 vs respective C21 concentration without PD123319.

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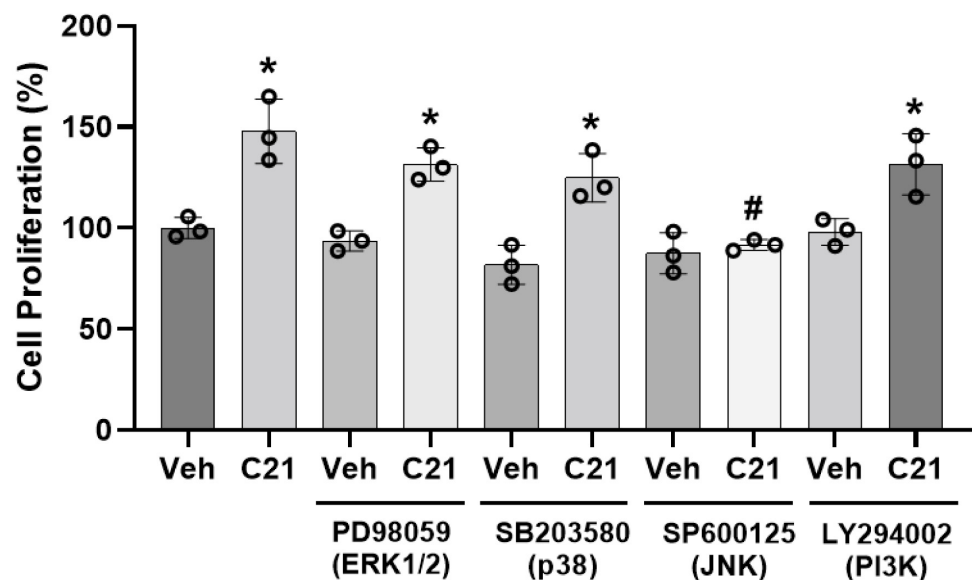
Fig 2. Effect of blockade of ERK1/2, p38, JNK, and PI3K on C21-induced proliferation of pregnant-hUAECs. Cells were pretreated with specific inhibitors for 30 min followed by treatment with C21 (10 nmol/L) for another 48 h. Cell proliferation was measured with an MTS Cell Proliferation Assay kit. Data were expressed as mean ± SD from three independent experiments using hUAECs from three donors and calculated as fold of control. *P < 0.05 vs control.

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Fig 3. Effect of AT2R agonist C21 on migration of pregnant-hUAECs. Cells were treated with increasing concentrations of C21 (1–100 nmol/L) for 24 h and chemotactic mobility was measured with FluoroBlok Assay. The migrated cells on the bottom of the insert were stained with calcein AM (0.2 µg/ml), and fluorescence images were captured with an inverted microscope (shown at left). The migrated cells were quantified with a fluorescence microplate reader (shown at right). Data were expressed as mean ± SD from five independent experiments using pregnant-hUAECs from five donors and presented as fold of control. *$P < 0.05$ vs control.

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AT2R activation increased migration and tube formation in pregnant-hUAECs

Since C21 induced proliferation only in pregnant-hUAECs, we further focused on these cells to determine if AT2R activation induced cell migration and capillary-like tube formation. Assessment of chemotactic motility using FluoroBlok transwell assay showed that C21 induced cell migration in a concentration-dependent manner with 1, 10, and 100 nmol/L inducing 1.2-, 1.7-, and 2.2-fold increases in cell migration, respectively, in pregnant-hUAECs (Fig 3).

Capillary-like tube formation was assessed with Matrigel matrix. As shown in Fig 3, increasing concentrations of C21 (1, 10, 100 nmol/L) resulted in an increased amount of tubular structures in pregnant-hUAECs. Capillary tube length was 2370 ± 119.3 µm in controls, and C21 increased mean tube length to 4050 ± 319.3 µm at 1 nmol/L C21, 6550 ± 476.7 µm at 10 nmol/L C21, and 7245 ± 308.7 µm at 100 nmol/L C21. Similarly, increasing the concentration of C21 increased the total number of branches (control: 13.0 ± 1.9; 1 nmol/L: 16.4 ± 2.1; 10 nmol/L: 25.6 ± 2.7; 100 nmol/L: 32.3 ± 6.8) and total number of junctions (control: 25.2 ± 2.6; 1 nmol/L: 31.0 ± 3.5; 10 nmol/L: 61.0 ± 10.9; 100 nmol/L: 72.3 ± 11.5) in pregnant-hUAECs (Fig 4).

AT2R activation-increased expression of angiogenic proteins in pregnant-hUAECs

Lacking any clues about which angiogenic factors might possibly be involved in the above phenomenon, we resolved to screen the differential expression of angiogenesis-related protein targets in pregnant-hUAECs after the 10 nmol/L C21 treatment. We used a membrane-based antibody array to quantify 43 proteins representing growth and differentiation factors, membrane-bound receptors, extracellular matrix components, and intracellular signaling molecules. Of the 43 array proteins tested, 24 were detectable in pregnant-hUAECs. Among these, C21 significantly increased the expression of 10 angiogenic proteins: growth factors (EGF, ENA-78, bFGF, IGF-1, PLGF, angiopoietin-1, angiopoietin-2), TIMPs (TIMP-1, TIMP-2), and adipokines (leptin) (Fig 5). The respective positions of these proteins on the membrane are shown in the top panel, and representative blots are shown in the middle panel (Fig 5).

AT2R activation induced expression of angiogenic proteins at the transcriptional level

We next used qPCR analysis to examine whether C21-induced increase in expression of angiogenic proteins occurs at the transcriptional level. C21 (10 nmol/L) increased the mRNA
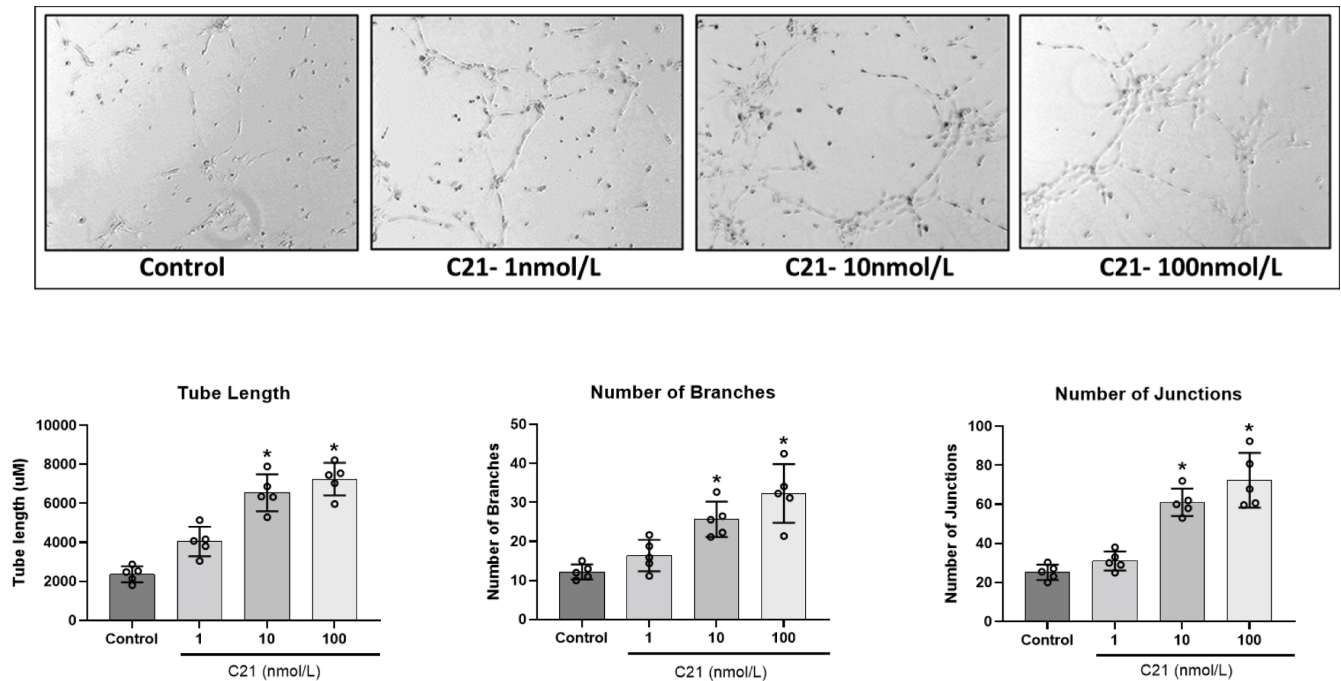
Fig 4. Effect of AT2R agonist C21 on tube formation in pregnant-hUAECs. Cells were seeded in 48-well plates on a semi-solid Matrigel matrix and treated with an increasing concentration of C21 (1–100 nmol/L) for 8 h. Images were captured with a MicroPublisher 5.0 camera and quantification was performed with an ImageJ Angiogenesis Analyzer tool. Representative images are shown at the top and quantified tube length, number of branches, and number of junctions are shown at the bottom. Data were expressed as mean ± SD from five independent experiments using hUAECs from five donors. $^*P < 0.05$ vs control.

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expression of EGF (2.3-fold), ENA-78 (2.2-fold), bFGF (1.7-fold), IGF-1 (3.8-fold), leptin (1.7-fold), PLGF (1.3-fold), TIMP-1 (2.7-fold), TIMP-2 (1.8-fold), angiopoietin-1 (2.1-fold), and angiopoietin-2 (2.1-fold) (Fig 6), indicating that AT2R activation upregulates angiogenic proteins at the mRNA level.

Discussion

The main aim of this study has been to investigate the angiogenic potential of AT2R activation in hUAECs isolated from normal nonpregnant and pregnant women. Our results indicate that AT2R agonist C21 significantly increased proliferation *in vitro* in pregnant-hUAECs, but not nonpregnant-hUAECs. In addition, JNK appears to transduce intracellular signals that regulate AT2R-stimulated mitogenic responses in pregnant-hUAECs. Finally, AT2R activation induced both migration and tube formation in pregnant-hUAECs, with associated increases in the mRNA and protein levels of ten proangiogenic proteins: EGF, ENA-78, bFGF, IGF-1, leptin, PLGF, TIMP-1, TIMP-2, and angiopoietin-1 and -2. These results indicate for the first time that AT2R agonist C21 induces pregnancy-dependent JNK-mediated angiogenesis in hUAECs with an associated transcriptional upregulation of multiple angiogenic proteins.

Endothelial cell proliferation is important for the promotion of angiogenesis. We demonstrated that C21 induced the proliferation of hUAECs isolated from pregnant—but not nonpregnant—women, indicating that pregnancy augments AT2R-mediated responses in hUAECs. The finding that pretreatment with the AT2R antagonist inhibited C21-induced cell proliferation in pregnant-hUAECs confirms that C21 mediates its effects through AT2R as reported previously [23, 24]. The inability of AT2R activation to induce proliferative responses
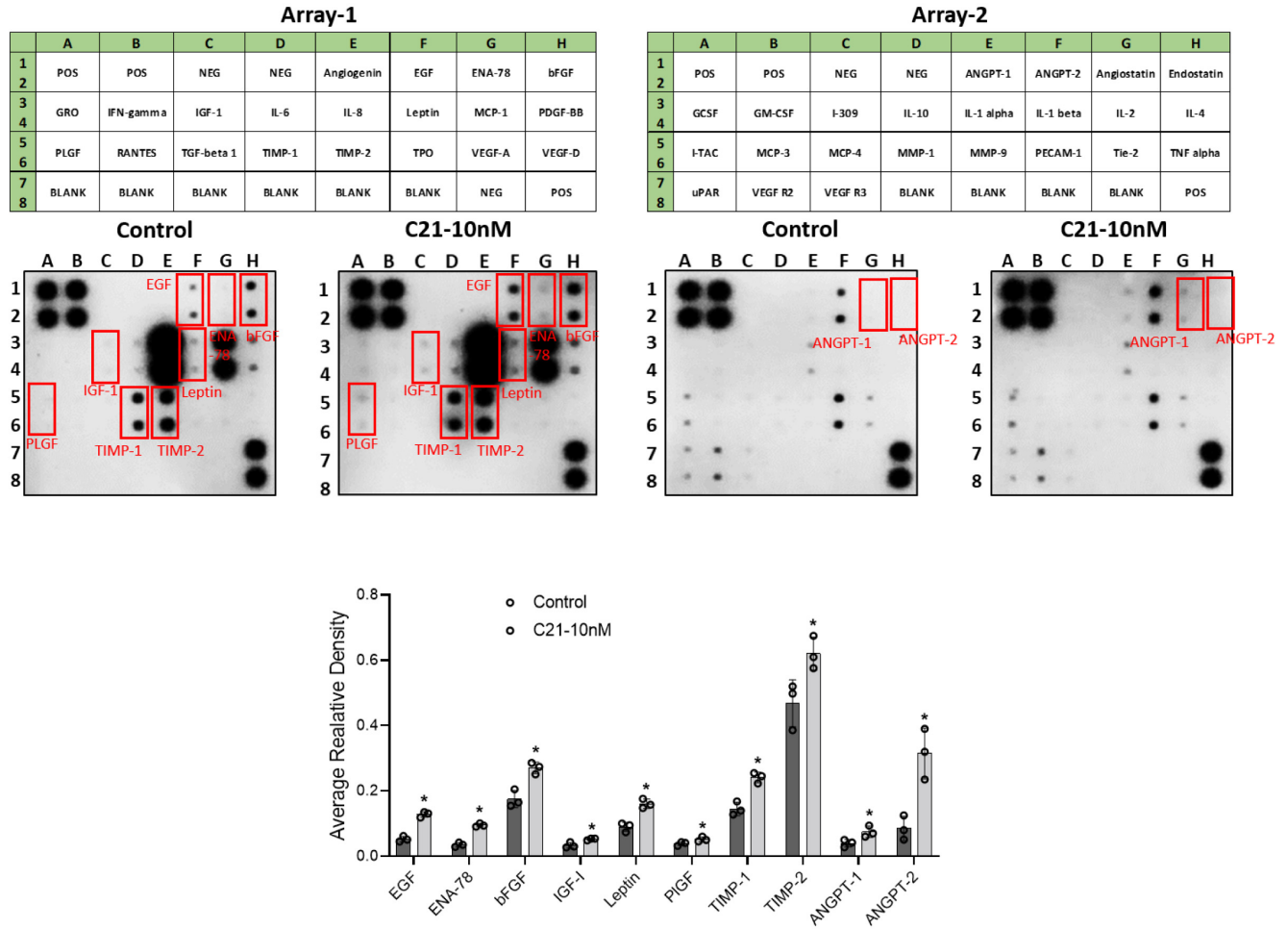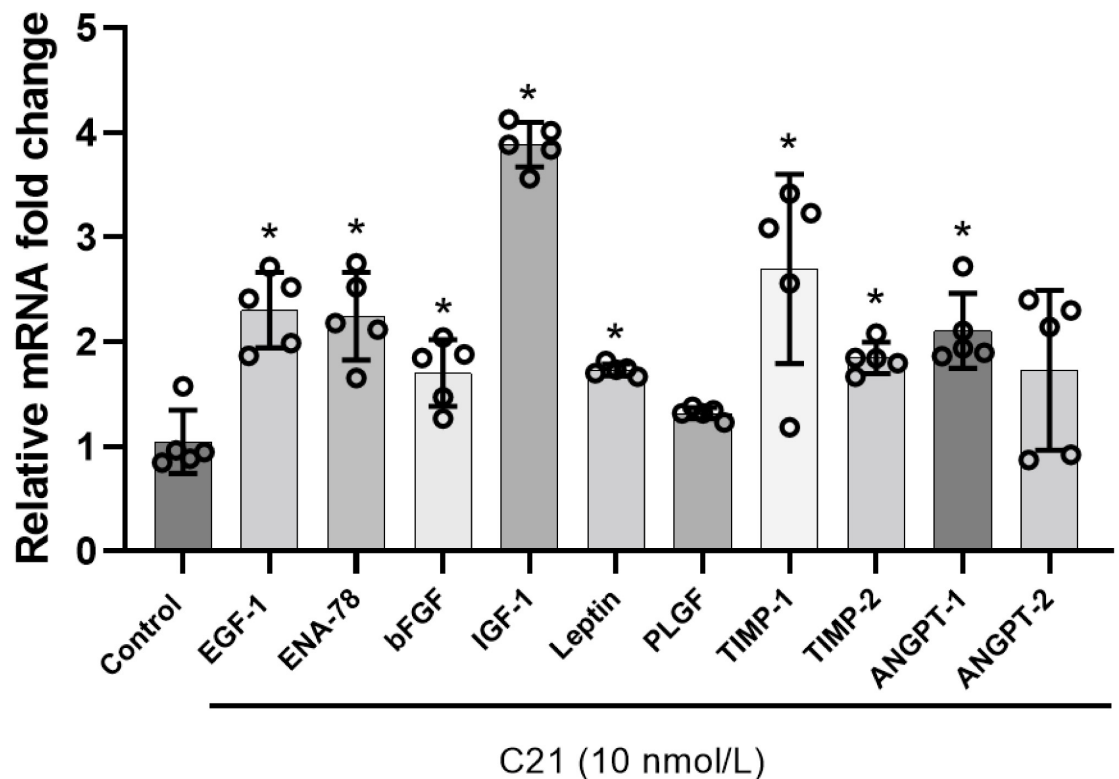
Fig 5. Effect of AT2R agonist C21 on the expression of angiogenic proteins in pregnant-hUAECs. The top panel presents the layout of the Human Antibody Angiogenesis Array (RayBiotech, AAH-ANG-1 and 2), including the positions of each protein. Representative images of human angiogenesis array membranes are shown in the middle panel. Normalized densitometric quantification of the arrays is shown in the bottom panel. Data were expressed as mean ± SD from three independent experiments using hUAECs from three donors. *P < 0.05 vs control.

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in cells from the nonpregnant state is in line with observations regarding CGP42112A (another AT2R agonist) stimulation of primary bovine retinal endothelial cells and rat coronary endothelial cells [25, 26]. Whether the higher AT2R expression in pregnant-hUAECs relative to nonpregnant-hUAECs [13, 14] contributes to differential mitogenic responses is unclear. However, such a contribution seems unlikely because previous studies has shown that *in vitro* overexpression of AT2R in hepatocellular carcinoma cells induce marked anti-proliferative and apoptotic responses [27]. The observation that high (≥5%) serum induces proliferation in both pregnant- and nonpregnant-hUAECs [18] suggests that nonpregnant-hUAECs have proliferative capacity, but that pregnancy may reprogram the hUAECs in a way that intensifies their response to certain mitogenic agonists and related factors. This suggestion is supported by the observation that estradiol and VEGF induce mitogenic responses only in pregnant-UAECs, but not nonpregnant-UAECs [18, 28]. The mechanistic significance of pregnancy-inducing endothelial-cell adaptation favoring enhanced proliferative responses to certain agonists/factors remains to be elucidated.

Fig 6. Effect of AT2R agonist C21 on the mRNA expression of angiogenic genes in pregnant-hUAECs. Cells were cultured in an endothelial cell medium containing vehicle or C21 (10 nmol/L) for 24 h, real-time PCR was used to assess gene expression, and data were normalized with GAPDH. Values are expressed as mean ± SD from five independent experiments. *P < 0.05 vs control.

Inhibition of ERK1/2, p38, and PI3K failed to reverse the C21-mediated proliferation of pregnant-hUAECs, thereby ruling out the potential role of these pathways. Another interesting finding herein was the observation that activation of JNK is needed for AT2R-mediated proliferation of pregnant-hUAECs, as treatment with SP600125 completely inhibited the proliferation induced by C21. Our results are not consistent with previous reports, since AT2R signaling is known to be mediated by activation of multiple pathways, including p38 MAPK in rat lumbar dorsal root ganglia [29], ERK1/2 in human fibroblasts [30], and PI3K in porcine proximal tubule cells [31]. In addition, AT2R activation inhibited JNK signaling in mice vascular smooth muscle cells [32]. Interestingly, all these studies used cells from the nonpregnant state, in which the expression profiles of kinases and their signaling are very different from those present in the pregnant state [33–35]. Consistent with our finding, other studies have reported that activation of JNK is required for proliferative responses in UAEC isolated from pregnant sheep [21, 22]. Thus, JNK signaling pathways—but not ERK1/2, p38, and PI3K signaling pathways—are involved in pregnant-hUAEC proliferation mediated by AT2R. Future studies should examine how AT2R couples with and activates JNK signaling in pregnant-hUAECs.

Cell migration is another essential feature of vascular endothelial cells during angiogenesis. To detect migration and angiogenesis ability *in vitro*, we performed transwell migration and tube-formation assay. The results confirm that AT2R activation with C21 can enhance cell

migration and the tube-forming ability in pregnant-hUAECs. In trying to better understand the mechanisms of angiogenesis orchestrated by AT2R activation, we examined angiogenic proteins affected by C21 treatment. According to the results of the antibody-array analysis, pregnant-hUAECs treated with C21 exhibited a significant increase in the levels of proangiogenic cytokines and chemokines (EGF, ENA-78, bFGF, IGF-1, PLGF, angiopoietin-1, angiopoietin-2), TIMPs (TIMP-1, TIMP-2), and adipokines (leptin). Previous research has shown that EGF signaling plays a role in the maturation of endothelial tubes and the recruitment of smooth muscle cells [36]. Moreover, other research has shown that bFGF is a positive regulator of angiogenesis [37]. In addition to its mitogenic effect on endothelial cells *in vitro* and *in vivo*, bFGF signaling is critically important for the formation and maintenance of vessels at the uteroplacental interface [38]. Decreased bFGF levels were observed in pregnant women who subsequently developed preeclampsia [39]. PlGF is a member of the VEGF sub-family and is a potent angiogenic factor [40]. In its vital contribution to pregnancy, PlGF specifically promotes the induction of vessel growth and the proliferation, migration, and survival of endothelial cells in various tissues [41, 42]. Reduced levels of PIGF have been reported in preeclampsia [43]. IGF1 increases amino-acid uptake, glucose uptake, and migration and tube formation in endothelial cells [44, 45]. Angiopoietins are critical for vessel homeostasis and angiogenesis. They not only induce vessel maturation, sprouting angiogenesis, and the stability of newly formed vessels, but inhibit pathological vascular permeability, as well [46]. TIMPs are a key regulator of the extracellular matrix that is important in maintaining the connections between endothelial cells [47]. The C21-induced increase in expression of these key angiogenic proteins suggests that AT2R activation may help in the formation of new blood vessels. The qPCR finding that C21 upregulates mRNA levels of these angiogenic proteins suggests that transcriptional mechanisms are involved in the upregulation of the corresponding proangiogenic proteins. Further studies are needed to determine how AT2R induces transcription of the angiogenic genes. In the present study, using angiogenic transcriptomic and proteomic analyses, we have shown that C21 commonly regulates a family of genes associated with angiogenesis, suggesting that AT2R plays a cooperative role in orchestrating an important proangiogenic transcriptional program in pregnant-hUAECs.

In conclusion, in the present study, we have demonstrated that AT2R activation induces proliferation of hUAECs in a pregnancy-specific manner through JNK-mediated signaling with associated transcriptional upregulation of multiple proangiogenic proteins. Vascular AT2R expression is decreased in pregnancy disorders like preeclampsia and fetal growth restriction [48, 49]. The findings of this study showing that AT2R promotes angiogenesis, together with previous reports that AT2R stimulation upregulates expression of its own receptors and enhances uterine blood flow [14], suggest that the AT2R agonist could be a new class of therapeutic agents capable of curtailing pregnancy disorders that involve impaired angiogenesis.

Author Contributions

Conceptualization: Sathish Kumar.

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Investigation: Jay S. Mishra.

Resources: Dong-Bao Chen.

Supervision: Sathish Kumar.

Writing – review & editing: Sathish Kumar.

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