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Effect of oxygen on the expression of renin-angiotensin system components in a human trophoblast cell line

Sarah J. Delforce^{a*}, Yu Wang^{a*}, Meg E. Van-Aalst^a, Celine Corbisier de Meaultsart^a, Brian. J. Morris^b, Fiona Broughton-Pipkin^c, Claire T. Roberts^d, Eugenie R. Lumbers^a, Kirsty G. Pringle^{a†}

^aSchool of Biomedical Sciences and Pharmacy, Mothers and Babies Research Centre, Hunter Medical Research Institute, University of Newcastle, Newcastle, New South Wales, Australia

^bSchool of Medical Sciences and Bosch Institute, University of Sydney, Sydney, New South Wales, Australia

^cDepartment of Obstetrics & Gynaecology, School of Medicine, University of Nottingham, NG5 1PB, UK

^dDiscipline of Obstetrics and Gynaecology, School of Paediatrics and Reproductive Health, Robinson Research Institute, University of Adelaide, Adelaide, South Australia, Australia.

*These authors contributed equally to this work.

[†]Corresponding author. Mothers and Babies Research Centre, Hunter Medical Research Institute, Level 3 East, 1 Kookaburra Circuit, New Lambton Heights, NSW 2305, Australia. Tel: +61-2-4985-5643; fax: +61-2-9421-4394

E-mail address: kirsty.pringle@newcastle.edu.au

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Abbreviations

ACE, angiotensin-converting enzyme; ACE1, angiotensin-converting enzyme 1; ACE2, angiotensin-converting enzyme 2; AGT, angiotensinogen; AT₁R, angiotensin II type 1 receptor; *AGT1R* mRNA, angiotensin II type 1 receptor mRNA; Ang, angiotensin; ATP6AP2, ATPase, H⁺ transporting, lysosomal accessory protein 2/(pro)renin receptor; BCA, bicinchoninic acid; EVT, extravillous trophoblast; HIFs, hypoxia inducible factors; iNOS, inducible nitric oxide synthase; RAS, renin-angiotensin system; *REN* mRNA; (pro)renin mRNA; VEGF, vascular endothelial growth factor.

ABSTRACT

During the first trimester, normal placental development occurs in a low oxygen environment that is known to stimulate angiogenesis via upregulation of vascular endothelial growth factor (VEGF). Expression of the placental renin-angiotensin system (RAS) is highest in early pregnancy. While the RAS and oxygen both stimulate angiogenesis, how they interact within the placenta is unknown. We postulated that low oxygen increases expression of the proangiogenic RAS pathway and that this is associated with increased VEGF in a first trimester human trophoblast cell line (HTR-8/SVneo). HTR-8/SVneo cells were cultured in one of three oxygen tensions (1%, 5% and 20%). RAS and *VEGF* mRNA expression were determined by qPCR. Prorenin, angiotensin converting enzyme (ACE) and VEGF protein levels in the supernatant, as well as prorenin and ACE in cell lysates, were measured using ELISA. Low oxygen significantly increased

the expression of both angiotensin II type 1 receptor (*AGT1R*) and *VEGF* (both $P<0.05$). There was a positive correlation between *AGT1R* and *VEGF* expression at low oxygen ($r=0.64$, $P<0.005$). Corresponding increases in VEGF protein were observed with low oxygen ($P<0.05$). Despite no change in *ACE1* mRNA expression, ACE levels in the supernatant increased with low oxygen (1% and 5%, $P<0.05$). Expression of other RAS components did not change. Low oxygen increased *AGT1R* and *VEGF* expression, as well as ACE and VEGF protein levels, suggesting that the proangiogenic RAS pathway is activated. This highlights a potential role for the placental RAS in mediating the proangiogenic effects of low oxygen in placental development.

Highlights

- The human placenta develops in a low oxygen environment
- The placental renin-angiotensin system (RAS) is highly expressed in early pregnancy
- Culture in 1% O₂ increased *AGT1R* mRNA and ACE protein in HTR-8/SVneo cells
- The latter was associated with increased VEGF mRNA and VEGF protein
- Stimulation of the placental RAS by low oxygen may be proliferative and angiogenic

Introduction

A low oxygen environment during the first trimester of pregnancy is required for optimal placental development that is essential for supplying fetal demand in late gestation. This early gestation low oxygen environment occurs as a result of extravillous

trophoblast (EVT) cell proliferation and subsequent invasion of the decidua and its vasculature, initially occluding maternal spiral arterioles from about two weeks after implantation [1]. Rodesch *et al.* [2] established that the oxygen tension in the intervillous space at 8 weeks is 17.9 mmHg (~2.5%) with a range of 5–30 mmHg (~0.7–4.3%) while the oxygen tension in the endometrium is higher at 39.6 mmHg (~5.7%) with a range of 25–70 mmHg (~3.5–10%). Thus there is an oxygen gradient experienced by first trimester trophoblasts that can range from ~1% (in the placental villi) to up to 10% in the decidua. The low oxygen environment stimulates angiogenesis and vascularization of the placenta. Poor placental development, characterized by insufficient decidual invasion by EVTs, incomplete occlusion of maternal arterioles with inadequate remodeling of maternal spiral arterioles, and early onset of maternal blood flow to the conceptus, ultimately results in poor nutrient and oxygen exchange during the 2nd and 3rd trimesters. These are associated with intrauterine growth restriction and preeclampsia [3, 4].

A low oxygen tension stabilizes hypoxia inducible factors (HIFs) which promote angiogenesis and vascularization by activating pro-angiogenic factors such as vascular endothelial growth factor (VEGF), angiopoietins, factors involved in regulation of vascular tone such as inducible nitric oxide synthase (iNOS) and proteins involved in nutrient transfer such as transferrin and glycolytic enzymes [5-8].

Another system that might regulate placental angiogenesis is the renin-angiotensin system (RAS). Tissue RASs have been shown to be involved in the regulation of angiogenesis, as well as cell proliferation and apoptosis [9]. We have shown that mRNA expression of prorenin (*REN* mRNA), (pro)renin receptor (*ATP6AP2* mRNA),

angiotensinogen (*AGT* mRNA), angiotensin (Ang) II type 1 (*AT₁R*) (*AGTR1* mRNA) and Ang converting enzyme 2 (*ACE2*) (*ACE2* mRNA) are all very high in early gestation placentae compared with term [10]. In addition, we have shown that the mRNA expression of placental *VEGF* is correlated with those of *REN*, *ATP6AP2* and *AGTR1* mRNAs [10]. Thus the placental RAS is most active during the first trimester and therefore could stimulate angiogenesis, as it does in other tissues. The ocular RAS is stimulated by ischemia. The increased activity of the ocular RAS is associated with a potent angiogenic response mediated via the Ang II/*AT₁R* pathway [11]. In addition, early renal development requires activation of the RAS by a low oxygen milieu [12].

While a low oxygen environment regulates placental development [13], the extent to which the RAS is essential for normal placental development has not been established. To investigate interactions between a low oxygen milieu and the placental RAS, we examined the effects of low oxygen on the expression of the RAS and VEGF in a first trimester human trophoblast cell line, HTR-8/SVneo, which we have previously shown expresses mRNAs encoding those RAS pathway components that stimulate angiogenesis in the eye and kidney [14, 15].

Materials and methods

Cell culture

HTR-8/SVneo cells are an immortalized first trimester trophoblast cell line (a kind gift from Prof. Charles Graham, Queens University, Ontario). HTR-8/SVneo trophoblast cells were chosen for the study as they are a transformed first trimester human extravillous trophoblast cell line. Because of this they are an ideal tool for investigating the effect of

low oxygen on the proliferative/angiogenic RAS pathways in placental development and are superior to other cell lines such as choriocarcinoma BeWo cells. We have demonstrated previously that the pro-angiogenic/proliferative pathway of the RAS is expressed in the HTR-8/SVneo cell line [14], as occurs in the first trimester placenta *in vivo* [9], but which is not seen in BeWo cells [14].

HTR-8/SVneo cells were cultured in RPMI-1640 medium (HyClone), supplemented with 10% fetal bovine serum (SAFC Biosciences), 1 mg/ml antibiotic-antimycotic (Gibco) and 1% L-glutamine in 5% CO₂ in room air at 37°C (cells were between passages 10–20). Cells were seeded at a density of 200,000 or 400,000 cells per well for 24 h or 48 h incubation, respectively. They were seeded in 6 well plates with 2 ml of incubation medium per well and allowed to settle for 24 h, after which time the medium was changed. Cells were then transferred to sealed oxygen chambers containing either 1%, 5% or 20% O₂ and 5% CO₂ in N₂ and cultured for 24 or 48 h, with chambers flushed every 24 h. Cells were harvested and the incubation medium was collected at 24 and 48 h, then snap frozen in liquid nitrogen at –80°C for subsequent protein and mRNA analyses. Three experiments were conducted in triplicate. RNA quantity was used as an indicator of cell viability and was assessed using the Nanodrop spectrophotometer, no differences in RNA quantity were detected between the treatment groups (data not shown). RNA quality was determined by agarose gel electrophoresis.

Semi-quantitative real-time reverse transcriptase polymerase chain reaction (qPCR)

Total RNA was isolated using the RNeasy mini kit according to the manufacturer's instructions (Qiagen). In addition, we examined the integrity of the total RNA in each

sample using gel electrophoresis. RNA samples were DNase treated (Qiagen) and total RNA was spiked with a known amount of Alien RNA (Stratagene), 10^7 copies per μg of total RNA, before the RNA was reverse transcribed using a Superscript III RT kit with random hexamers (Invitrogen). The Alien qRT PCR inhibitor alert system serves as a reference for internal standardization [16]. qPCR was performed in an Applied Biosystems 7500 Real Time PCR System using SYBR Green for detection. Each reaction contained 5 μl of SYBR Green PCR master mix (Applied Biosystems), RAS and VEGF primers that we have described previously [10, 17], cDNA reversed transcribed from 10 ng total RNA, and water to 10 μl . Messenger RNA abundance was calculated as described previously, using the $2^{-\Delta\Delta\text{CT}}$ method and expressed relative to Alien mRNA and a calibrator sample (a term placental sample collected at elective Cesarean section) [10].

Extraction and quantification of total protein from cells

Protein was extracted from cells using a radioimmunoprecipitation assay (RIPA) lysis and extraction buffer. One milliliter of RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 100 nM sodium orthovanadate and Complete Mini Protease Inhibitor Cocktail tablets (Roche Diagnostics Australia) and 10 μL of 100 nM PMSF were added to each sample. Samples were incubated on ice for 30 min then centrifuged at 13,000 rpm at 4°C for 10 min. Supernatants were collected. Protein was quantified using the Pierce BCA Protein assay kit (Life Technologies) according to the manufacturer's instructions.

Measurement of prorenin, VEGF and ACE by ELISA

Prorenin, VEGF and ACE concentrations in culture medium and cell lysates (at both 24 and 48 h) were measured using the Human Prorenin ELISA kit (Molecular Innovations), Human VEGF DuoSet ELISA kit (R&D systems) and the Human ACE DuoSet ELISA kit (R&D Systems), respectively, according to the manufacturers' instructions, as described previously [18]. For prorenin, VEGF and ACE proteins in culture medium and prorenin in cell lysates, all samples were assayed in duplicate on one ELISA plate. Therefore there was no inter-assay variability. For ACE protein in cell lysates, samples were assayed in duplicate over two plates and inter-assay variability was 9.4%. Intra-assay coefficients of variation were 13.1% and 3.2% for prorenin culture medium and cell lysate, 6.8% for VEGF culture medium and 14.2% and 2.9% for ACE culture medium and cell lysate, respectively.

Data analysis

Two-way ANOVA with Tukey's multiple comparisons test was used to determine the effects of differing oxygen tensions on the relative abundance of RAS mRNAs, prorenin, ACE and VEGF protein levels. A Kruskal-Wallis non-parametric test was used to determine the effects of differing oxygen tensions on prorenin concentration in both the culture medium and cell lysate at 48 h. Pearson correlation coefficients were calculated to determine any relationships between the various mRNA levels. GraphPad Prism (Prism version 6.0) was used for all graphs and correlation analyses and the SPSS statistical package (SPSS for Windows, Release 17.0.0. Chicago) was used for all other analyses. Significance was set at $P < 0.05$.

Results

Effect of O₂ on RAS and VEGF mRNA expression

At all oxygen tensions, HTR-8/SVneo cells expressed genes of the pro-angiogenic pathway of the RAS, i.e., *AGT*, *REN*, *ATP6AP2*, *ACE1* and *AGTR1*, as well as *VEGF*, but expression of genes of the anti-angiogenic RAS pathway, i.e., *ACE2*, *AGT2R*, *MAS1* was not detected (Figure 1); this is consistent with previously published data on the expression of RAS genes in this cell line [14].

Expression of *AGT*, *REN*, *ATP6AP2* and *ACE1* mRNAs was not affected by the prevailing oxygen tension, but *AGTR1* and *VEGF* mRNA expression were significantly higher in HTR-8/SVneo cells cultured in low oxygen (1% O₂) compared with 5% or 20% O₂ at 48 h (all $P < 0.01$). A similar trend was seen in 24 h incubates. This did not, however, reach statistical significance (Fig. 1).

There was a significant effect of incubation time on the expression of *AGT*, *REN*, *ATP6AP2*, *ACE1* and *AGTR1* mRNAs, in that expression of these mRNAs was significantly higher at 48 h compared with 24 h (all $P < 0.05$; Fig. 1).

A significant correlation was found between *AGTR1* and *VEGF* mRNA and between *AGTR1* and *ATP6AP2* mRNA ($r = 0.6378$ and 0.6500 respectively, $R^2 = 0.4067$ and 0.4733 respectively, both $P < 0.01$; Fig. 2).

Effect of O₂ on the concentration on prorenin and VEGF protein concentration in the culture medium

Although measurable levels of *REN* mRNA were found (Fig. 1), prorenin protein was low in both the culture medium and cell lysate of HTR-8/SVneo cells at 24 and 48 h incubation irrespective of the prevailing O₂ (only 48 h data shown; Fig. 3).

After 48 h VEGF levels in the culture medium of HTR-8/SVneo cells grown in 1% O₂ were much higher than levels in medium from samples cultured in 5% and 20% O₂ ($P<0.05$ and $P<0.01$, respectively; Fig. 4). No effect of oxygen tension on VEGF protein was observed at 24 h incubation (data not shown).

Effect of O₂ on the concentration of ACE in the culture medium

After 48 h culture, HTR-8/SVneo cells cultured in 1% and 5% O₂ had significantly higher levels of ACE protein in the supernatant than cells cultured in 20% O₂ ($P<0.05$; Fig. 5). ACE levels in the cell lysate were not affected by O₂. No effect of oxygen tension on ACE protein in culture medium or cell lysates was observed at 24 h incubation (data not shown).

Discussion

Incubation of primary HTR-8/SVneo trophoblast cells at a low (1%) oxygen tension increased the expression of *AGTR1* and *VEGF* mRNA and stimulated the production of ACE and VEGF proteins. We propose, therefore, that a low oxygen milieu activates the pro-angiogenic pathway of the RAS by increasing AT₁R and ACE protein levels and that these changes are associated with increases in VEGF, an important angiogenic factor.

The study also showed that even small reductions in O₂ have substantial effects on the expression of the AT₁R and pro-angiogenic factors such as VEGF. This was based on

our finding that culture in 1% oxygen had a much more powerful effect than culture in 5% oxygen. A low oxygen environment was not associated with increased expression of prorenin mRNA or its protein. This suggests that a low oxygen tension alone is not responsible for the high levels of *REN* mRNA found in first trimester placentae [10]. It is more likely that increased *REN* mRNA expression in the placenta early in pregnancy is the result of hormonal activation and/or activation by cAMP of *REN* mRNA expression. Human chorionic gonadotropin (hCG) has been recognized as a stimulus for prorenin production by villous placenta [19], as have β -adrenoceptor agonists [19]. We have shown previously that cAMP increases *REN* mRNA expression and prorenin protein secretion by HTR-8/SVneo cells [14]. Expression of *AGT* and *ATP6AP2* mRNAs were not affected by oxygen. This suggests that their high expression in early gestation placentae [10] is not due to the low oxygen milieu.

Although the expression of *ACE1* was not stimulated by culturing cells in a low oxygen environment, we did observe increased ACE protein in medium from incubations carried out in low oxygen. Goyal *et al.* have observed a similar effect [20]. In animal studies they showed that maternal hypoxia was associated with upregulation of placental ACE protein without a corresponding increase in *ACE1* mRNA levels [20]. They concluded that this was because hypoxia-regulated miR-27, which regulates ACE translation, was down-regulated [20]. It is possible that a hypoxia-regulated miRNA in the HTR-8/SVneo cell cultures could be similarly affected by oxygen.

In a previous publication we reviewed evidence suggesting that the low oxygen milieu of early placental development activated the placental RAS, stimulating angiogenesis and cell proliferation [15]. The RAS pathway most likely to be involved in inducing these

effects is the prorenin/AGT/ACE/Ang II/AT₁R pathway because the Ang II/AT₁R blocking drug, candesartan, blocks Ang II-induced endothelial proliferation [21]. Hypoxia inducible factor (HIF-1 α) is essential for normal trophoblast differentiation, since both HIF-1 α and AT₁R gene ablation in mice impairs placental vascularization [22, 23]. Ang II acting via the AT₁R causes cell proliferation [24].

We were surprised that a low oxygen milieu had no effect on prorenin expression and secretion. There may, however, be a number of factors acting on the placental renin/AGT/ACE/Ang II/AT₁R pathway that upregulate different components, so ensuring increased placental Ang II production. As mentioned above, hCG and cAMP are potent stimuli for placental prorenin release. cAMP also stimulates *AGT* mRNA expression [25]. MicroRNAs (miRNAs) may affect the stability or translation of *ACE1* mRNA as has been observed in animal experiments [20]. *AGTR1* mRNA expression in HTR8/SVneo cells is, moreover, increased by low oxygen.

We found that *AGTR1* mRNA abundance was highly correlated with levels of both *VEGF* and *ATP6AP2* mRNA. The association between *AGTR1* and *ATP6AP2* mRNA suggests that increased levels of the (pro)renin receptor (ATP6AP2) may stimulate the activation of the placental RAS cascade and in turn increase AT₁R, while the association between *AGTR1* and *VEGF* mRNA suggests that increased AT₁R in first trimester placenta may increase expression of *VEGF* mRNA and protein, and thereby stimulate angiogenesis within the developing placenta.

In conclusion, we have shown that a low oxygen environment stimulates the expression of *AGTR1* and *VEGF* mRNAs in HTR-8/SVneo cells *in vitro*. Both AT₁R and *VEGF* are involved in angiogenesis and proliferation, which are key properties of first trimester

EVTs. We showed that incubation in a low oxygen environment increased production of ACE and VEGF protein by HTR-8/SVneo cells and increased expression of the Ang II/AT₁R pathway. We propose that the placental RAS is under the influence of a number of endocrine, molecular and environmental factors.

Author Contributions

E.R. Lumbers and K.G. Pringle were responsible for designing the experiments. C.T. Roberts, B.J. Morris and F. Broughton-Pipkin assisted in developing the experimental concepts that were the basis of the experiments. Y. Wang, S.J. Delforce, M.E. Van-Aalst and C. Corbisier de Meaultsart carried out the experiments. Wang and Delforce analysed the data. Delforce, Wang, Lumbers, Morris and Pringle interpreted the data and drafted the manuscript.

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References

- [1] Jaffe R, Jauniaux E, Hustin J. Maternal circulation in the first-trimester human placenta – myth or reality? *Am J Obstet Gynecol.* 1997;176:695-705.
- [2] Rodesch F, Simon P, Donner C, Jauniaux E. Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. *Obstet Gynecol.* 1992;80:283-5.
- [3] Salafia CM, Charles AK, Maas EM. Placenta and fetal growth restriction. *Clin Obstet Gynecol.* 2006;49:236-56.
- [4] Silasi M, Cohen B, Karumanchi SA, Rana S. Abnormal placentation, angiogenic factors, and the pathogenesis of preeclampsia. *Obstet Gynecol Clin North Am.* 2010;37:239-53.
- [5] O'Donnell JL, Joyce MR, Shannon AM, Harmey J, Geraghty J, Bouchier-Hayes D. Oncological implications of hypoxia inducible factor-1alpha (HIF-1alpha) expression. *Cancer Treat Rev.* 2006;32:407-16.
- [6] Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med.* 2003;9:677-84.
- [7] Schaffer L, Vogel J, Breymann C, Gassmann M, Marti HH. Preserved placental oxygenation and development during severe systemic hypoxia. *Am J Physiol Regul Integr Comp Physiol.* 2006;290:R844-51.
- [8] Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci USA.* 1995;92:5510-4.
- [9] Paul M, Poyan Mehr A, Kreutz R. Physiology of local renin-angiotensin systems. *Physiol Rev.* 2006;86:747-803.
- [10] Pringle KG, Tadros MA, Callister RJ, Lumbers ER. The expression and localization of the human placental prorenin/renin-angiotensin system throughout pregnancy: roles in trophoblast invasion and angiogenesis? *Placenta.* 2011;32:956-62.
- [11] Wilkinson-Berka JL. Diabetes and retinal vascular disorders: role of the renin-angiotensin system. *Expert Rev Mol Med.* 2004;6:1-18.
- [12] Tufro-McReddie A, Norwood VF, Aylor KW, Botkin SJ, Carey RM, Gomez RA. Oxygen regulates vascular endothelial growth factor-mediated vasculogenesis and tubulogenesis. *Dev Biol.* 1997;183:139-49.
- [13] Genbacev O, Zhou Y, Ludlow JW, Fisher SJ. Regulation of human placental development by oxygen tension. *Science.* 1997;277:1669-72.
- [14] Wang Y, Pringle KG, Chen YX, Zakar T, Lumbers ER. Regulation of the renin-angiotensin system (RAS) in BeWo and HTR-8/SVneo trophoblast cell lines. *Placenta.* 2012;33:634-9.
- [15] Lumbers ER, Pringle KG, Wang Y, Gibson KJ. The renin-angiotensin system from conception to old age: the good, the bad and the ugly. *Clin Exp Pharmacol Physiol.* 2013;40:743-52.
- [16] Gilsbach R, Kouta M, Bonisch H, Bruss M. Comparison of in vitro and in vivo reference genes for internal standardization of real-time PCR data. *Biotechniques.* 2006;40:173-7.
- [17] Marques FZ, Pringle KG, Conquest A, Hirst JJ, Markus MA, Sarris M, et al. Molecular characterization of renin-angiotensin system components in human

- 337 intrauterine tissues and fetal membranes from vaginal delivery and cesarean section.
338 Placenta. 2011;32:214-21.
- 339 [18] Wang Y, Pringle KG, Sykes SD, Marques FZ, Morris BJ, Zakar T, et al. Fetal sex
340 affects expression of renin-angiotensin system components in term human decidua.
341 Endocrinology. 2011;153:462-8.
- 342 [19] Poisner AM. Regulation of utero-placental prorenin. Adv Exp Med Biol.
343 1995;377:411-26.
- 344 [20] Goyal R, Lister R, Leitzke A, Goyal D, Gheorghe CP, Longo LD. Antenatal maternal
345 hypoxic stress: adaptations of the placental renin-angiotensin system in the mouse.
346 Placenta. 2011;32:134-9.
- 347 [21] Herr D, Rodewald M, Fraser H, Hack G, Konrad R, Kreienberg R, et al. Regulation
348 of endothelial proliferation by the renin-angiotensin system in human umbilical vein
349 endothelial cells. Reproduction. 2008;136:125-30.
- 350 [22] Cowden Dahl KD, Fryer BH, Mack FA, Compennolle V, Maltepe E, Adelman DM,
351 et al. Hypoxia-inducible factors 1 α and 2 α regulate trophoblast differentiation. Mol Cell
352 Biol. 2005;25:10479-91.
- 353 [23] Walther T, Jank A, Heringer-Walther S, Horn LC, Stepan H. Angiotensin II type 1
354 receptor has impact on murine placentation. Placenta. 2008;29:905-9.
- 355 [24] George AJ, Thomas WG, Hannan RD. The renin-angiotensin system and cancer:
356 old dog, new tricks. Nat Rev Cancer. 2010;10:745-59.
- 357 [25] Wang Y, Pringle KG, Lumbers ER. The effects of cyclic AMP, sex steroids and
358 global hypomethylation on the expression of genes controlling the activity of the renin-
359 angiotensin system in placental cell lines. Placenta. 2013;34:275-80.
- 360

Figure Legends

Fig. 1. Effect of O₂ on expression of RAS components in HTR-8/SVneo cells at 24 and 48 h. (A) *AGTR1* and (B) *VEGF* mRNA were significantly upregulated by low oxygen (1% O₂) at 48 h when compared with 5% and 20% O₂ (*, both $P < 0.01$). (B) *VEGF* mRNA was significantly increased at 48 h compared with 24 h by 1% O₂ (#, $P < 0.05$). (A) *AGTR1*, (C) *ATP6AP2*, (D) *REN*, (E) *ACE1* and (F) *AGT* mRNA expression significantly increased with incubation time (□, all $P < 0.05$). Data are presented as mean $\pm$ SEM.

Fig. 2. The correlations between *VEGF* and *AGTR1* mRNA abundance and between *AGTR1* and *ATP6AP2* mRNA abundance in HTR-8/SVneo cells cultured at 1% O₂. (A) *VEGF* and *AGTR1* ($r=0.638$, $R^2=0.407$ and $P=0.0044$); (B) *AGTR1* and *ATP6AP2* ($r=0.650$, $R^2=0.473$ and $P=0.0035$).

Fig. 3. Levels of prorenin protein in culture medium and cell lysates of HTR-8/SVneo cells cultured in varying oxygen tensions for 48 h. Neither culture medium (A) nor cell lysate (B) prorenin protein levels were affected by oxygen tension. Data are presented as mean $\pm$ SEM.

Figure 4. The effect of O₂ on VEGF protein concentration in the culture medium of HTR-8/SVneo cells at 48 h. Culture in 1% O₂ for 48 h significantly increased the concentration of VEGF compared with culture in either 5% or 20% O₂ (*, $P < 0.05$ and $P < 0.01$ respectively). Data are presented as mean $\pm$ SEM.

Fig. 5. The effect of O₂ on ACE protein concentration in the culture medium and cell lysate of HTR-8/SVneo cells at 48 h. (A) The concentration of ACE in the culture medium of HTR-8/SVneo cells was significantly increased after 48 h of culture in both 1% and 5% O₂ when compared with the concentration measured in medium cultured in 20% O₂ (*, both $P < 0.05$). (B) ACE protein levels in cell lysates were unaffected by oxygen. Data are presented as mean $\pm$ SEM.