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The effects of cyclic AMP, sex steroids and global hypomethylation on the expression of genes controlling the activity of the renin-angiotensin system in placental cell lines

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ABSTRACT (241 words)

The placental renin-angiotensin system (RAS) is involved in placentation. We have shown that prorenin mRNA (*REN*) is expressed in a first trimester trophoblast cell line (HTR-8/SVneo) but not in a choriocarcinoma cell line (BeWo). We attempted to stimulate RAS expression in these cells by cAMP, 5'-aza-2'-deoxycytidine (AZA; an inhibitor of methylation), cAMP and AZA combined, and the sex steroids medroxyprogesterone acetate (MPA) and estradiol-17 β (E₂) with and without cAMP. RAS mRNAs were measured by qPCR and prorenin concentration in supernatants measured by an ELISA. In HTR-8/SVneo cells, all treatments increased *REN* expression compared to controls and cAMP + AZA combined was more effective than either treatment alone. Prorenin levels in supernatants were similarly upregulated. In HTR-8/SVneo cells, angiotensinogen (*AGT*) mRNA expression was increased by MPA + E₂ either with or without cAMP. *AGT* expression was also significantly increased by AZA. BeWo cells did not express *REN* or prorenin and it was not inducible with any treatment. *AGT* expression was significantly increased with AZA, the combination of cAMP + AZA, and MPA + E₂ + cAMP treatments. Since cAMP, AZA, cAMP and AZA combined, or MPA and E₂ with and without cAMP in HTR-8/SVneo cells, a cell line most similar in its RAS expression to the *in vivo* placenta, these factors may affect placental RAS activity. Surprisingly, these treatments also induced *AGT* expression in BeWo cells. Whether they are involved in regulating *AGT* in choriocarcinomas *in vivo* remains to be determined.

1 INTRODUCTION

2 Studies have shown that the renin angiotensin system (RAS) is involved in the
3 regulation of trophoblast invasion [1] and spiral artery remodelling [2] and
4 consequently, may play a role in implantation and placentation.

5 Within the placenta, renin acts on angiotensinogen (AGT) to form angiotensin
6 (Ang) I, which is catalysed by angiotensin converting enzyme (ACE) to form Ang II.
7 Ang II can act either through the angiotensin II type 1 (AT₁R) or type 2 (AT₂R)
8 receptor or it can be converted by ACE2 to Ang 1-7. Potentially, the placental RAS
9 could have a number of actions, some of which may be cooperative and some
10 antagonistic. RAS pathway genes and proteins have been detected in both the early
11 and late gestation human placentae [1, 3, 4].

12 We have studied two established trophoblast cell lines that are commonly used
13 for studying placental function: HTR-8/SVneo (a transformed first trimester
14 extravillous trophoblast cell line developed by Charles Graham, Ontario, Canada [5])
15 and BeWo cells (derived from a choriocarcinoma [6]), postulating that these cell lines
16 would show similar patterns of RAS expression to freshly obtained human placenta.
17 This was not the case however. HTR-8/SVneo cells expressed prorenin (*REN*),
18 angiotensinogen (*AGT*), the prorenin receptor (*ATP6AP2*), ACE (*ACE1*) and AT₁R
19 (*AGTR1*), but not AT₂R (*AGTR2*), ACE2 (*ACE2*) nor the Ang 1-7 receptor, MasR
20 (*MAS1*), while BeWo cells only expressed *AGT*, *ACE1*, *ACE2* and *MAS1* and not *REN*
21 [7]. Although the expression of RAS genes and proteins in the human placenta and
22 these trophoblast cell lines has been established, the mechanisms regulating their
23 expression are unknown.

24 Another difference between BeWo and HTR-8/SVneo cells is in their response
25 to cAMP. When stimulated with cAMP, *REN* expression and prorenin protein

synthesis was induced in HTR-8/SVneo cells, but not in BeWo cells [7]. This was interesting, given that cAMP is a classical stimulator of *REN* expression, as seen in juxtaglomerular [8] and primary decidual cells [9]. We postulated that BeWo cells lack the *REN* response to cAMP stimulation, as they are derived from a choriocarcinoma and have a high degree of polyploidy and therefore could be heavily methylated in order to be viable. Therefore, in BeWo cells, hypermethylation of DNA may prevent cAMP from binding to the cAMP response element (CRE), which is responsible for the regulation of *REN* gene transcription [10, 11]. We postulated that a genome wide inhibitor of methylation such as 5'-aza-2'-deoxycytidine (AZA) might therefore initiate *REN* expression and prorenin secretion in BeWo cells, particularly in response to cAMP. AZA inhibits DNA methyltransferases, resulting in global hypomethylation as cells continue to divide. Hypomethylation could increase transcriptional protein binding at the CRE [12].

Furthermore, if as we proposed, AZA did stimulate *REN* expression through global hypomethylation of BeWo DNA, then AZA might also have a similar effect in HTR-8/SVneo cells, so that not only do cAMP (as previously shown, [7]) and AZA stimulate *REN* expression and prorenin secretion but the combination of cAMP and AZA together would be additive, having a greater effect on *REN* expression in HTR-8/SVneo cells than that seen with either agent alone.

In normal pregnancy, the developing blastocyst secretes human chorionic gonadotropin (hCG). hCG levels are maximal at about 6 weeks gestation, at which time maternal plasma prorenin levels are also at a maximum. hCG administration is associated with increased plasma prorenin levels. hCG maintains the corpus luteum, and maintains production of both estrogen and progesterone [13, 14]. The hCG induced increase in plasma prorenin in early pregnancy also comes from the corpus

luteum [15]. In addition, hCG stimulates placental production of prorenin, as does progesterone [13, 16]. Therefore, we proposed that the combined administration of estrogen and progesterone, hormones essential for the establishment and maintenance of human pregnancy, could stimulate renin expression by both HTR-8/SVneo and BeWo cells.

In this study, therefore, we compared the effects of cAMP with the global hypomethylating agent, AZA, and their combined effects on expression of RAS genes and on prorenin secretion by the placentally derived cell lines, HTR-8/SVneo and BeWo. We also investigated the effects of cAMP, estradiol and progesterone, in combination, on *REN* expression and prorenin production and on the expression of other RAS genes.

MATERIALS AND METHODS

Trophoblast Cell Culture

HTR-8/SVneo and BeWo cells were cultured in phenol red-free RPMI-1640 or DMEM/F-12, respectively, supplemented with 15 mM HEPES, 1.2 g/L NaHCO₃, 1 mg/mL L-glutathione reduced, 0.1 g/L albumin fraction V, 0.65 µg/mL aprotinin, 10% fetal bovine serum and 40 µg/mL gentamicin. Cells were seeded at a density of 200,000 cells, in each well of a 6 well plate with 2 mL of incubation medium. Cells were allowed to settle for 24 h, after which the media was changed, cells were then treated with one of the following:

- 1) 150 µM 8-bromo-cAMP (cAMP, Sigma-Aldrich, St. Louis, MO, USA);
- 2) 7.5 µM 5'-aza-2'-deoxycytidine (AZA, Sigma-Aldrich);
- 3) 150 µM cAMP with 7.5 µM AZA;

- 4) 1 μ M medroxyprogesterone acetate (MPA, Sigma-Aldrich) with 10 nM estradiol-17 β (E₂, Sigma-Aldrich);
- 5) 1 μ M MPA with 10 nM estradiol-17 β and 150 μ M cAMP; or
- 6) incubation medium alone (vehicle).

In a previous study we used 300 μ M 8-bromo cAMP [7] to induce prorenin release. To ensure that a significant increase in prorenin mRNA and protein levels would be seen when cAMP treatment was combined with AZA or E₂ and MPA compared with cAMP treatment alone, we dropped the cAMP dose by half, and treated the cells with 150 μ M cAMP. The concentration of AZA is within the range normally used in cell culture experiments. E₂ and MPA concentrations were selected based on previous studies examining the effects of these hormones in human endometrial stromal cells [17] and are similar to what trophoblast cells would be exposed to *in vivo* during normal human pregnancy [18]. Since placental estrogen and progesterone levels increase in pregnancy in a coordinated fashion [18] we chose to only assess the effects of these hormones in combination, to better mimic the *in vivo* environment. Incubation media were collected at 24 and 48 h and cells were harvested. Cells and media were snap frozen in liquid nitrogen for subsequent protein and mRNA analyses. There were 3 independent experiments and in each experiment the treatments were in triplicate. Cell viability was assessed by an LDH cytotoxicity assay for all treatments as well as by measuring RNA stability and quality (data not shown). None of the treatments used in this study affected cell viability at 48 h.

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

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Each sample was examined for RNA

integrity, DNase treated (Qiagen N.V., Hilden, Germany), spiked with a known amount of Alien RNA (Agilent Technologies, La Jolla, CA, USA; 10^7 copies per microgram of total RNA) and reverse transcribed using Superscript III RT kit (Invitrogen). The alien qRT PCR inhibitor alert system (Agilent Technologies) served as a reference for internal standardization [19]. RAS primers for qPCR have been described previously [3, 4, 20]. Messenger RNA abundance was calculated using the $\Delta\Delta CT$ method and relative abundance was calculated relative to Alien mRNA and a calibrator sample (term placenta).

Measurement of prorenin protein by ELISA

Prorenin levels in culture media were measured using the Human Prorenin ELISA kit (Molecular Innovations Inc; Novi, MI) according to the manufacturer's instructions. The methods and reagents used have been previously described [3, 4, 20]. Samples were assayed in duplicate and on the same ELISA plate. Therefore there was no inter-assay variability. Intra-assay coefficient of variation was 10.8%.

Data Analyses

A one-way ANOVA with Bonferroni post hoc analysis was used to determine if there were any differences in RAS mRNA abundance and prorenin protein concentration in the supernatant between each treatment. GraphPad Prism (Prism version 6.0) was used for all analyses. Significance was set at $P < 0.05$.

RESULTS

cAMP stimulates prorenin in HTR-8/SVneo but not BeWo cells

In this study, we used 150 μ M 8-bromo-cAMP as compared to the 300 μ M used in our previous study [7]. Despite this, cAMP significantly increased *REN* mRNA expression in HTR-8/SVneo cells at both 24 and 48 h incubation (both $P<0.001$, Figure 1A). In supernatants from cultures of HTR-8/SVneo treated cells, there was a corresponding increase in prorenin protein concentrations at 48 h compared to vehicle ($P<0.001$, Figure 1B).

In BeWo cells, cAMP was not able to induce *REN* expression or prorenin in the supernatant.

AZA alone, or in combination stimulates *REN* in HTR-8/SVneo cells

In order to determine whether *REN* expression could be stimulated in BeWo cells by inhibiting DNA methylation, the two trophoblast cell lines were treated with AZA. In HTR-8/SVneo cells, *REN* expression was significantly increased by treatment with AZA compared to vehicle at both 24 and 48 h incubation (both $P<0.001$, Figure 1A) and AZA significantly increased the levels of prorenin in the supernatant ($P<0.001$, Figure 1B).

To test our hypothesis that cAMP and AZA act through different pathways to stimulate *REN* expression, we compared *REN* expression in cells treated with the combination of cAMP and AZA, to either treatment alone. We found that the combination of cAMP and AZA was more effective at stimulating *REN* expression in HTR-8/SVneo cells compared to vehicle or to cAMP or AZA treatment alone at both 24 and 48 h incubation ($P<0.001$, $P<0.001$, $P<0.001$, $P<0.001$, $P<0.05$ and $P<0.001$ respectively, Figure 1A). There were corresponding increases in prorenin concentrations in the supernatants from the treated HTR-8/SVneo cells, ($P<0.001$,

Figure 1B). Prorenin levels however were no greater than levels measured in supernatants treated with either cAMP or AZA alone.

In contrast, AZA, alone or in combination with cAMP failed to induce *REN* expression in BeWo cells and prorenin was not detected in the supernatant.

Effects of MPA and E₂, alone or in combination with cAMP on prorenin mRNA and protein levels

The combination of MPA & E₂ significantly increased HTR-8/SVneo cell expression of *REN* compared to vehicle at both 24 and 48 h ($P<0.001$) and was more effective at stimulating *REN* expression compared with cAMP treatment ($P<0.01$ and $P<0.001$, respectively, Figure 2A). Prorenin concentration in the supernatants of the MPA and E₂ treated cells was greater than vehicle ($P<0.001$, Figure 2B), however there was no significant difference between MPA, E₂ or cAMP treated cells.

The MPA + E₂ + cAMP mix significantly increased *REN* expression in HTR-8/SVneo cells compared to vehicle at 24 and 48 h (both $P<0.001$, Figure 2A) and was more effective at stimulating *REN* expression than cAMP treatment alone ($P<0.001$, Figure 2A) but not more effective than the combined treatment of MPA and E₂ alone. After 48 h incubation, prorenin levels in the supernatants of HTR-8/SVneo cells treated with MPA + E₂ + cAMP was significantly greater than vehicle ($P<0.001$, Figure 2B), which corresponds with their greater *REN* gene expression levels. There was no difference however, between prorenin levels in MPA and E₂ treated, MPA + E₂ + cAMP treated, and cAMP treated cells.

In BeWo cells, MPA + E₂, or in combination with cAMP, failed to induce prorenin gene expression or prorenin protein production.

Effects of cAMP and AZA on the expression of other genes in the placental RAS pathways of HTR-8/SVneo and BeWo cells

cAMP treatment alone did not alter the expression of any of the other RAS genes studied in HTR-8/SVneo cells and had no effect on any of the RAS genes expressed by BeWo cells.

In contrast, AZA treatment of HTR-8/SVneo cells increased *AGT* mRNA abundance compared to vehicle at 24 h ($P<0.01$, Figure 3A). There were no significant effects however, of AZA on *ATP6AP2*, *ACE1* and *AGTR1* expression. In HTR-8/SVneo cells, combined cAMP and AZA treatment was more effective at stimulating *AGT* expression compared to vehicle ($P<0.001$) and cAMP treatment at 24 h ($P<0.05$, Figure 3A). There were no significant effects of the combined cAMP and AZA treatment on *ATP6AP2*, *ACE1* and *AGTR1* expression.

In BeWo cells, treatment with AZA increased *AGT* mRNA abundance at 24 h incubation compared to vehicle ($P<0.01$, Figure 4A). This effect was reversed by the combination of cAMP and AZA treatment ($P<0.05$, Figure 4A) and was no longer different from controls. Neither AZA nor cAMP had any effect on *ACE1*, *ACE2* or *MAS1* mRNA abundances in BeWo cells.

Effects of MPA and E₂, alone or in combination with cAMP the expression of other genes in the placental RAS pathways of HTR-8/SVneo cells and BeWo.

Combined MPA and E₂ treatment significantly increased expression of *AGT* at 24 h incubation ($P<0.05$) in HTR-8/SVneo cells compared to vehicle (Figure 3B). Like MPA and E₂ treatment alone, MPA + E₂ + cAMP combined significantly increased HTR-8/SVneo cell expression of *AGT* compared to vehicle at 24 h incubation ($P<0.05$, Figure 3B) but this was not seen at 48 h.

In BeWo cells, there was no effect of MPA and E₂ treatment alone on RAS gene expression however, the combined MPA + E₂ + cAMP treatment significantly increased *AGT* expression at 24 h incubation compared to vehicle ($P<0.05$, Figure 4B). This effect was no longer observed at 48 h.

DISCUSSION

Our previous work showed that cAMP stimulated prorenin mRNA and protein levels in HTR-8/SVneo cells and supernatants but not BeWo cells, in which *REN* expression could not be detected [7]. HTR-8/SVneo cells express *REN* and produce prorenin as well as expressing other RAS genes likely to regulate Ang II/AT1R interactions and respond to cAMP, like renal renin-secreting cells. They are more similar to early gestation placentae and are therefore useful for studying effects of renin/ACE/Ang II/AT1R on cell function [7]. BeWo cells express the ACE2/Ang1-7/Mas pathway, and therefore are useful for studying the effects of this pathway on trophoblast function.

In the present study, we wanted to find out if *REN* expression could be induced in BeWo cells and further stimulated in HTR-8/SVneo cells by reducing gene silencing through global hypomethylation using AZA, or by combined treatment with the two major hormones of pregnancy, estrogen and progesterone. We stimulated cells with cAMP alone to confirm our previous findings and to quantitate the effects of these novel treatments (relative to the cAMP induced effects) on *REN* and other RAS genes by both cell lines.

We demonstrated that cAMP, AZA, MPA and E₂ all increased *REN* expression and prorenin levels in HTR-8/SVneo cells but not in BeWo cells. On the other hand, treatment of HTR-8/SVneo cells with either AZA or MPA and E₂ had

effects on renin that were independent of any effects of cAMP and in fact AZA treatment amplified the stimulatory effect of cAMP on *REN* expression. This amplified cAMP action on *REN* mRNA however, did not translate into increased levels of prorenin protein. The additive effect of the combined AZA and cAMP treatment was minimal, suggesting that AZA is likely acting through the cAMP/CRE pathway, possibly by allowing better access for cAMP and the associated binding factors to bind to the CRE of the *REN* gene, as opposed to AZA acting independently of cAMP [12].

This is the first report that global hypomethylation of a placental cell line is associated with increased *REN* expression and prorenin production. It is also the first report that the combination of E₂ and MPA stimulates *REN* mRNA and prorenin production by trophoblast cells.

We showed, as postulated, that E₂ and MPA in combination stimulate placental *REN* expression and prorenin secretion in HTR-8/SVneo cells. While a role for estrogen and progesterone in regulating the placental RAS has been suggested, there are no conclusive studies to support this. In this study we have been able to successfully demonstrate that estrogen and progesterone are able to stimulate *REN* and *AGT* mRNA as well as prorenin protein by trophoblast cells *in vitro*. Further studies are necessary to determine whether the human placental RAS *in vivo* responds to sex steroids in a similar manner.

The addition of cAMP to the MPA and E₂ mixture had no additive effect on *REN* mRNA abundance; that is, there was no significant difference between treatments after addition of cAMP. Furthermore, MPA and E₂ together, with or without the addition of cAMP, were more effective at stimulating *REN* expression than cAMP alone. This suggests that MPA and E₂ most likely act by stimulating

cAMP and, as such, additional cAMP did not further stimulate renin expression in HTR-8/SVneo cells.

All of the treatments used in this study stimulated prorenin mRNA and protein levels but only in HTR-8/SVneo cells. We propose that the reason we are unable to induce renin in BeWo cells is due to the *REN* gene in these cells not being transcribable or, that the region of the mRNA our primers are designed against is mutated or missing. In addition, we were unable to detect *AGTR1* or *AGTR2* mRNAs in BeWo cells, which may suggest that this cancer cell line has undergone significant physiological and molecular changes.

It is important to note that the lack of translation of differences in *REN* mRNA abundance to differences in prorenin protein in the media may be because all treatments resulted in maximal prorenin production. On the other hand, active renin production in these supernatants was not measured and it is possible that prorenin in these cells may have undergone activation to renin, through the release of proteases by global hypomethylation, or through stimulation by cAMP and steroid hormones, which could act on the constitutively expressed prorenin [21, 22]. The ELISA we use is targeted specifically to the pro-segment of prorenin and does not detect active renin.

The treatments used in this study were selected primarily to examine their potential effects on placental *REN* and prorenin protein however we did observe significant increases in *AGT* expression in both cell lines in response to both AZA and the sex steroids. Interestingly, these increases were only transient and were no longer evident after 48 h. Whether this transient effect is due to negative feedback from the concomitant production of high levels of prorenin in HTR-8/SVneo cells, as seen in other cell types [23], is unknown. It seems more likely however, since we see similar affects in BeWo cells which do not express renin, that it is more likely due to the

rapid turnover of *AGT* mRNA to protein or that the treatments have a different mechanism of action to that against renin and require additional or prolonged stimuli to have a sustained affect.

Interestingly, both the *AGT* and *REN* gene lie on chromosome 1, however analysis has shown that there is a great genetic distance between the two genes [24]. Nevertheless, AZA administration results in global hypomethylation and could cause activation of both *AGT* and *REN*. Indeed, we have demonstrated increased *AGT* and *REN* expression in HTR-8/SVneo but only increased *AGT* expression in BeWo cell lines. The combination of AZA and cAMP also had a small additive effect on *AGT* mRNA abundance in HTR-8/SVneo cells, similar to that seen in *REN*, but it blocked the effects of AZA on *AGT* in BeWo cells (Figures 3 & 4). This also supports our hypothesis that BeWo cells are physiologically abnormal.

Estrogen has also been shown to induce angiotensinogen release in rats [25]. In humans, oral contraceptives, which contain synthetic estrogen, cause a dose dependent increase in plasma angiotensinogen levels [26]. In addition, estrogen replacement therapy also increases angiotensinogen levels [27] due to upregulation of hepatic *AGT*. Thus it is perhaps not surprising that *AGT* expression was greater in MPA and E₂ treated cells compared to vehicle in HTR-8/SVneo cells.

In conclusion, we have shown that the two trophoblast cell lines respond very differently to a wide range of treatments, and despite the effectiveness of these agents at stimulating prorenin expression in HTR-8/SVneo cells, they were unable to induce *REN* expression in BeWo cells. As a result, we propose that AZA, progesterone and estradiol stimulate *REN* expression and prorenin protein in trophoblast cells through a cAMP-dependent pathway, although they target different aspects of this pathway. AZA, likely acting through hypomethylation of the region around the CRE, probably

allows for better binding of cAMP and other associated binding proteins, whereas progesterone and estradiol probably act by stimulating cAMP release.

Additive effects of these treatments on the placental RAS pathway could be proposed to further enhance the activity of the placental RAS through provision of more prorenin binding sites, stimulation of placental *AGT* expression and upregulation of *AGTRI*. The role(s) of epigenetic factors such as gene methylation, and the sex steroids on the activity of the placental RAS *in vivo* remain to be determined, but we have demonstrated that the activity of the placental RAS, which is thought to play a role in placentation, is regulated by sex steroids and is subject to epigenetic modification.

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REFERENCES

- [1] Williams P. J., Mistry H. D., Innes B. A., Bulmer J. N. and Pipkin F. B. (2010). Expression of AT1R, AT2R and AT4R and their roles in extravillous trophoblast invasion in the human. *Placenta*, **31**, 448-55.
- [2] Morgan T., Craven C. and Ward K. (1998). Human spiral artery renin-angiotensin system. *Hypertension*, **32**, 683-7.
- [3] Marques F. Z., Pringle K. G., Conquest A., Hirst J. J., Markus M. A., Sarris M., *et al.* (2011). Molecular characterization of renin-angiotensin system components in human intrauterine tissues and fetal membranes from vaginal delivery and cesarean section. *Placenta*, **32**, 214-21.
- [4] Pringle K. G., Tadros M. A., Callister R. J. and Lumbers E. R. (2011). The expression and localization of the human placental prorenin/renin-angiotensin system throughout pregnancy: Roles in trophoblast invasion and angiogenesis? *Placenta*, **32**, 956-62.
- [5] Graham C. H., Hawley T. S., MacDougall R. C., Kerbel R. S., Khoo N. and Lala P. K. (1993). Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Experimental Cell Research*, **206**, 204-11.

- [6] Pattillo R. A., Gey G. O., Delfs E. and Mattingly R. F. (1968). Human hormone production in vitro. *Science*, **159**, 1467-9.
- [7] Wang Y., Pringle K. G., Chen Y. X., Zakar T. and Lumbers E. R. (2012). Regulation of the renin-angiotensin system (RAS) in BeWo and HTR-8/SVneo trophoblast cell lines. *Placenta*, **33**, 634-9.
- [8] Klar J., Sandner P., Muller M. W. and Kurtz A. (2002). Cyclic AMP stimulates renin gene transcription in juxtaglomerular cells. *Pflugers Arch*, **444**, 335-44.
- [9] Poisner A. M., Thrailkill K., Poisner R. and Handwerger S. (1991). Cyclic AMP as a second messenger for prorenin release from human decidual cells. *Placenta*, **12**, 263-7.
- [10] Pinet F., Mizrahi J., Menard J. and Corvol P. (1986). Role of cyclic AMP in renin secretion by human transfected juxtaglomerular cells. *J Hypertens Suppl*, **4**, S421-3.
- [11] Pinet F., Mizrahi J., Laboulandine I., Menard J. and Corvol P. (1987). Regulation of prorenin secretion in cultured human transfected juxtaglomerular cells. *J Clin Invest*, **80**, 724-31.
- [12] Sunahori K., Juang Y. T., Kyttaris V. C. and Tsokos G. C. (2011). Promoter hypomethylation results in increased expression of protein phosphatase 2A in T cells from patients with systemic lupus erythematosus. *J Immunol*, **186**, 4508-17.
- [13] Itskovitz J., Sealey J. E., Glorioso N. and Rosenwaks Z. (1987). Plasma prorenin response to human chorionic gonadotropin in ovarian-hyperstimulated women: correlation with the number of ovarian follicles and steroid hormone concentrations. *Proc Natl Acad Sci U S A*, **84**, 7285-9.
- [14] Hunter M. G. and Baker T. G. (1981). Effect of hCG, cAMP and FSH on steroidogenesis by human corpora lutea in vitro. *J Reprod Fertil*, **63**, 285-8.
- [15] Lumbers E. R. (1993). The Ovary. In Nicholls MG and Robertson JIS (Ed.), *The Renin-Angiotensin System* (pp. 46.1-.12). London, England: Gower Medical Publishing Co.
- [16] Sealey J. E., Itskovitz-Eldor J., Rubattu S., James G. D., August P., Thaler I., *et al.* (1994). Estradiol- and progesterone-related increases in the renin-aldosterone system: studies during ovarian stimulation and early pregnancy. *J Clin Endocrinol Metab*, **79**, 258-64.
- [17] Logan P. C., Ponnampalam A. P., Rahnama F., Lobie P. E. and Mitchell M. D. (2010). The effect of DNA methylation inhibitor 5-Aza-2'-deoxycytidine on human endometrial stromal cells. *Human Reproduction*, **25**, 2859-69.
- [18] Chen J. Z. J., Sheehan P. M., Brennecke S. P. and Keogh R. J. (2012). Vessel remodelling, pregnancy hormones and extravillous trophoblast function. *Molecular and Cellular Endocrinology*, **349**, 138-44.
- [19] Gilsbach R., Kouta M., Bonisch H. and Bruss M. (2006). Comparison of in vitro and in vivo reference genes for internal standardization of real-time PCR data. *Biotechniques*, **40**, 173-7.
- [20] Pringle K. G., Zakar T., Yates D., Mitchell C. M., Hirst J. J. and Lumbers E. R. (2011). Molecular evidence of a (pro)renin/(pro)renin receptor system in human intrauterine tissues in pregnancy and its association with PGHS-2. *J Renin Angiotensin Aldosterone Syst*, **12** 304-10.
- [21] Derkx F. H., Schalekamp M. P. and Schalekamp M. A. (1987). Two-step prorenin-renin conversion. Isolation of an intermediary form of activated prorenin. *J Biol Chem*, **262**, 2472-7.

- [22] Derkx F. H., Schalekamp M. P., Bouma B., Kluft C. and Schalekamp M. A. (1982). Plasma kallikrein-mediated activation of the renin-angiotensin system does not require prior acidification of prorenin. *J Clin Endocrinol Metab*, **54**, 343-8.
- [23] Scheffe J. H., Unger T. and Funke-Kaiser H. (2008). PLZF and the (pro)renin receptor. *J Mol Med (Berl)*, **86**, 623-7.
- [24] Lifton R. P. and Jeunemaitre X. (1993). Finding genes that cause human hypertension. *J Hypertens*, **11**, 231-6.
- [25] Gordon M. S., Chin W. W. and Shupnik M. A. (1992). Regulation of angiotensinogen gene expression by estrogen. *J Hypertens*, **10**, 361-6.
- [26] Hollenberg N. K., Williams G. H., Burger B., Chenitz W., Hoosmand I. and Adams D. F. (1976). Renal blood flow and its response to angiotensin II. An interaction between oral contraceptive agents, sodium intake, and the renin-angiotensin system in healthy young women. *Circ Res*, **38**, 35-40.
- [27] Schunkert H., Danser A. H., Hense H. W., Derkx F. H., Kurzinger S. and Riegger G. A. (1997). Effects of estrogen replacement therapy on the renin-angiotensin system in postmenopausal women. *Circulation*, **95**, 39-45.