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Pringle KG, Lumbers ER, 'The placental renin angiotensin system', The Placenta: Development, Function and Diseases, Nova Science, New York 261-288 (2013)

Available from:
https://www.novapublishers.com/catalog/product_info.php?products_id=40044

Accessed from: http://hdl.handle.net/1959.13/1295580

THE PLACENTAL RENIN ANGIOTENSIN SYSTEM

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Abstract

Intrauterine growth restriction (IUGR) and preeclampsia are major and common complications of human pregnancy. Not only do they represent life threatening events for mothers and babies but they also enhance the susceptibility of the baby to diseases like hypertension, coronary artery disease and diabetes mellitus in adult life. One of the major causes of IUGR and preeclampsia is impaired placentation. Development of the placenta requires a complex interplay between proliferation of trophoblast cells and their invasion of the maternal decidua and maternal spiral arterioles, which they plug so that the early placenta (<12 weeks gestation) normally develops in a hypoxic milieu. The placenta contains its own renin-angiotensin system (RAS). Angiotensin II, the end-product of the renin-renin substrate reaction, acting through its type 1 receptor stimulates placental angiogenesis and trophoblast invasion and migration and is involved in placental development. This chapter outlines the expression and localisation of the placental RAS, the role of the placental RAS in placental development and function throughout pregnancy and how disturbances in the placental renin angiotensin system likely contribute to the pathogenesis of PreE and IUGR.

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Introduction

The formation of the human placenta requires the complex regulation of trophoblast proliferation, differentiation and invasion, as well as the formation of a branching network of fetal vessels (angiogenesis) within the chorionic villi and remodeling of the maternal spiral arterioles so they become spacious conduits opening into the intervillous space [1]. The orderly progress of these processes is essential for the provision of sufficient uteroplacental blood flow to support fetal growth, particularly in late pregnancy. Inadequate placentation has been implicated in several complications of pregnancy including unexplained miscarriage [2],

preeclampsia, intrauterine growth restriction (IUGR) [3], placental abruption [4], pre-term labour with intact membranes [5], premature rupture of the membranes [6] and stillbirth [7].

The renin angiotensin system (RAS) is usually recognised as a circulating endocrine system but it also exists in many tissues including the human intrauterine tissues where it is most likely involved in endometrial remodeling, decidualisation, implantation, placentation and regulation of uteroplacental blood flow. The human placenta contains its own renin-angiotensin system [8-13], which is expressed from at least 6 weeks gestation. Angiotensin II (Ang II), the major end product of the renin-angiotensinogen reaction, acting through its type 1 receptor (AT_1R) stimulates angiogenesis [14-17] and invasion [9, 18]. Thus the RAS may be important in placental development. Importantly disturbance of the placental RAS may, through interactions with both fetus and mother, lead to a variety of pregnancy complications.

The Renin-Angiotensin System

The renin angiotensin system (RAS, Figure 1), first described in 1898 by Tigerstedt and Bergman [19], primarily functions as an endocrine system that regulates blood pressure, and fluid and electrolyte homeostasis. Active renin, an aspartyl protease, is released by the juxtaglomerular cells lining the renal afferent arterioles in response to increases in renal sympathethic nerve activity, changes in renal perfusion pressure or by alterations in the delivery of solute to the distal nephron [20]. Circulating renin cleaves angiotensinogen, which is produced by the liver, to form the decapeptide Angiotensin I (Ang I). Angiotensin converting enzyme (ACE), present in most tissues, including the pulmonary endothelium, removes a further two C-terminal amino acids to generate Ang II, the most well known of the family of Ang peptides (Figure 1). Ang II then acts via one of two angiotensin receptors (AT_1R or AT_2R), which are both 7-transmembrane domain G-coupled protein receptors. Most of the classical actions of Ang II, including its actions within the brain, peripheral vasoconstriction, release of aldosterone from the adrenal cortex and sodium reabsorption by the renal tubules are mediated by the interaction of Ang II with the AT_1R. Ang II via the AT_1R also stimulates angiogenesis [14-17], cell proliferation and invasion [9, 18, 21, 22], which is important when considering the role of the RAS in placentation. In contrast, Ang II acting on AT_2R has mainly opposing effects [23] as it is associated with inhibition of cell growth, apoptosis [24], vasodilatation and fetal tissue development. The AT_2R is predominantly expressed in fetal tissues [25].

Over the last decade or so our understanding of the roles of the RAS has been increased with the description of two additional RAS pathways. ACE2, a homolog of ACE [26], terminates the action of Ang II by converting it to Ang 1-7 (Figure 1). Ang 1-7, acting via a Mas G-coupled protein receptor, opposes many of the actions of Ang II mediated via the AT_1R [27-29].

As well, a (pro)renin receptor ((P)RR) has been identified which can bind both active renin and its inactive precursor, prorenin (Figure 1, [30]). Until this receptor was discovered it was assumed that prorenin was an inactive precursor of renin, i.e. a zymogen. Prorenin bound to the (P)RR is non-proteolytically activated, the prosegment is unfolded, exposing the catalytic site and allowing removal of the decapeptide Ang I from angiotensinogen (Figure 2).

(P)RR can also exist in a soluble form [31, 32], so that prorenin, as well as active renin could generate Ang I from angiotensinogen in the circulation. Bound to this receptor,

prorenin/renin can also stimulate intracellular signaling pathways (e.g. via MAP kinases) through non-angiotensin dependent pathways. Ang II independent targets for the (P)RR include cell signaling via ERK1/2 [33] or HSP27/p38 [34] and interaction with the Wnt signaling pathway through the action of (P)RR as an adaptor [35, 36]. Therefore prorenin has biological activity in its own right.

Although the RAS is still widely considered to be mainly an endocrine system, it is now evident that the various organ and tissue-based renin angiotensin systems (RASs) can act as paracrine or possibly autocrine systems. Tissue renin angiotensin systems have been identified in the brain, ovary, testis, adrenal, adipose, kidney, heart and vasculature. They are however different from the circulating RAS as only the kidney can secrete active renin in response to specific stimulation. Other tissue RASs lack the intracellular processing pathways that result in the formation and storage of active renin in juxtaglomerular cells. Active renin stored within the juxtaglomerular cells can be released rapidly in response to life threatening events such as hemorrhage and its secretion is exquisitely regulated. While in other cells, prorenin is secreted as it is synthesized and its release in response to stimulation is slow (Figure 3, [37]).

The activity of the RAS in extrarenal tissues depends on spontaneous activation of prorenin to active renin (about 1%, [38]) and on the presence of the (P)RR or proteases that can convert prorenin to active renin at neutral pH.

In the female reproductive tract, the placenta and other intrauterine tissues (decidua, chorion, amnion and myometrium), the amniotic fluid and the ovaries [39, 40] all contain prorenin. Lumbers [41] and Morris and Lumbers [42] showed that amniotic fluid prorenin could be activated by exposure to a low pH milieu and cleavage of the prosegment by endogenous proteases or by exposure *in vitro* to endopeptidases (Figure 2). Since that early work, numerous proteases have been shown to activate prorenin, including plasmin, kallikrein and cathepsins [43-47]. Therefore, provided angiotensinogen, ACE, ACE2 and the various angiotensin receptors are present in the placenta, the placental RASs could act independent of the circulating fetal and maternal RASs. As well, the presence of various RAS components within the placenta can interact with both circulating RASs (fetal and maternal) and affect the circulatory and renal function of both mother and baby.

Identification of placental RAS gene expression

Most research into the expression and localization of the placental RAS has investigated term placentae and compared them with placentae from pathological pregnancies (i.e. preeclampsia) and very few studies have examined the levels of the expression of RAS genes in the first and second trimester compared to expression at term. This comparison, however clearly demonstrates that the expression of the placental RAS changes across gestation.

The human placenta has been shown to express all components of the RAS that lead to the generation of Ang II and Ang 1-7 (Figure 1). Renin (*REN*) mRNA is found in both early and late gestation placentae [8, 12, 13, 48, 49]. Its abundance is highest between 6-9 weeks gestation compared to placentae collected between 10-16 weeks and is lowest at term [13]. Angiotensinogen (*AGT*) mRNA levels are also highest in early gestation (6-16 weeks) compared to term placentae, as is *ACE2* [13]. On the other hand, *ACE* expression is highest in term placentae [13].

Studies examining the expression of AT_1 and AT_2 receptor mRNAs (*AGTR1* and *AGTR2*) have reported conflicting results. *AGTR1* mRNA levels in human placentae were first detected in 1999 by Cooper and colleagues who examined placentae collected from the first, second and third trimester using non-quantitative techniques [8]. Using quantitative real time PCR, Williams *et. al.* [9] examined placentae collected at 8-10 weeks, 12-14 weeks and at term and found that *AGTR1* mRNA levels did not change throughout gestation. In contrast, we have recently found that *AGTR1* mRNA abundance was highest in 10-13 week placentae compared with those collected at term and tends to be higher overall in early gestation (6-16 weeks) compared to term placentae [13]. *AGTR2* mRNA has been found to be low to undetectable in early and late gestation placentae in several independent studies [8, 48, 50]. We have recently reported that *AGTR2* mRNA abundance in placentae is very low throughout gestation and does not change with gestational age [13], however a previous study has reported that *AGTR2* mRNA levels are decreased in term placentae compared to early and mid gestation placental samples [9]. Mas (*MAS*) mRNA is usually undetectable in human placentae [10, 13] with only one study reporting measurable levels in chorionic villi [48].

mRNA for the placental (pro)renin receptor (*ATP6AP2*) was first detected in 2002 by Nguyen *et. al.* [30] who found abundant placental expression of *ATP6AP2* compared to other human tissues (liver, pancreas, kidney, lung and skeletal muscle). Subsequently, we showed that *ATP6AP2* mRNA is more abundant in the term placenta than other term intrauterine tissues (amnion, chorion and decidua, [12]) and that its levels were higher in early gestation placentae compared to term placentae [13].

Expression and localization of RAS proteins in the placenta

(Pro)renin and the (pro)renin receptor

In early pregnancy, prorenin protein was found in syncytiotrophoblasts, villous and extravillous cytotrophoblasts of the human placenta (Figure 4, [13]). Like prorenin, immunostaining for the (P)RR was also found in syncytiotrophoblasts and extravillous cytotrophoblasts in early gestation placentae (8-14 weeks), however it was not found in villous cytotrophoblasts (Figure 4, [13]). At term, prorenin, renin and the (P)RR are found mainly in syncytiotrophoblasts (Figure 4, [10, 30]) and, to a lesser extent in fetal vascular endothelium [30]. To date there have been no studies to determine if levels of prorenin, active renin or the (P)RR protein in the placenta change with gestation. The highest levels of enzymatically determined prorenin are found in gestational fluids collected in early pregnancy [51]; they are 1000 times normal maternal plasma levels. In both maternal plasma and gestational fluid levels are at a peak at ~6 weeks of gestation [51, 52]. Since *REN* mRNA is highest in the first trimester, we postulate that placental prorenin production is also highest early in pregnancy. Furthermore the placenta as well as the ovaries [53] may contribute to the peak in maternal plasma prorenin levels, although it should be noted that the ovary is the major source of the elevated maternal plasma prorenin levels seen at this time [54].

The co-localisation of (P)RR and prorenin in the placenta coupled with their high mRNA expression in very early gestation, when placental invasion is maximal, means that prorenin

could act via the placental RAS and regulate trophoblast migration and vascular remodeling. In addition, Ang-II independent pathways may be essential for early placental growth and trophoblast invasion as the (P)RR knockout is embryo lethal [55].

Other components of the placental RAS

AGT mRNA levels are low in the placenta [13] and the protein is weakly expressed in early gestation in syncytiotrophoblasts, cytotrophoblasts and villous stroma [13]. Abundant amounts of angiotensinogen are however found in term placenta (Figure 4, [10]). Since the placental villi are bathed by maternal blood entering the intervillous space, it is possible that the syncytiotrophoblasts take up angiotensinogen from maternal blood perfusing the intervillous space.

ACE protein is specifically localized to the fetal vascular endothelium of the placental villi in early and late gestation (Figure 4, [10, 13, 56]). The absence of ACE protein in trophoblasts does not mean that production of Ang II is limited to fetal placental vascular endothelium. ACE is also abundant in maternal blood bathing the placental villi. Alternatively, Ang I may be converted to Ang II through the action of chymase, by an ACE independent pathway [57]. Chymase is expressed in the human placenta and its activity is increased in placentae from women with preeclampsia [58], so it may increase Ang II production [59].

Both Ang I and Ang II peptides are present in the placental bed and chorionic villi at term [48, 49]. AT_1R is the predominant angiotensin receptor in receptor binding studies [48], suggesting that Ang II-AT_1 receptor mediated activity is the dominant RAS pathway within the human placenta [8]. Immunostaining for the AT_1R occurs throughout the chorionic villi in both early and late gestation, being found in syncytiotrophoblasts, villous and extravillous cytotrophoblasts, Hofbauer cells and the fetal endothelium [8-10, 13, 50]. It is significant that AT_1R immunoreactivity in trophoblasts is greater in first and second trimester placentae compared to term [8]. This is in line with the increased *AGTR1* mRNA expression seen in early gestation placentae [13].

The AT_2R is down regulated in maternal tissues in pregnancy (e.g. myometrium and endometrium), where it is the dominant Ang II receptor in the non-pregnant state [60] and its levels in the placenta are low [50]. When AT_2R protein has been detected, it has been localized to the villous and extravillous trophoblasts and Hofbauer cells [9, 13, 50]. Like the expression of *AGTR2,* levels of AT_2R protein also decreased with advancing gestation [9].

The Ang 1-7 – Mas axis

Unlike ACE protein, the localization of ACE2 in chorionic villi is much more widespread. In early gestation placentae (6-16 weeks), ACE2 is abundant in the syncytiotrophoblast, villous stroma and, to a lesser extent, cytotrophoblasts (Figure 4, [13]). At term ACE2 is co-localised with Ang 1-7 in syncytiotrophoblast, cytotrophoblast and fetal endothelium [10, 48] as well as the invading and intravascular trophoblasts in the maternal decidua [49]. The localisation of ACE2 in syncytiotrophoblasts of placental villi means that ACE2 could regulate the release of Ang 1-7 into maternal blood perfusing the intervillous

space. Since this peptide causes vasodilatation through enhanced production of nitric oxide (NO, [61]), placentally produced Ang 1-7 could lower peripheral vascular resistance in other maternal vascular beds. Thus, placental production of Ang 1-7, through its antagonism of Ang II-AT_1 receptor mediated actions [27], may be important in the control of maternal blood pressure and salt and water balance.

Regulation of the expression and activity of the placental RAS

Since the placental RAS is involved in the regulation of trophoblast invasion [9] and spiral artery remodeling [11], it likely plays important roles in implantation and placentation. Although the expression of RAS genes and proteins in the human placenta has been described in detail (see above), the regulation of expression of the placental RAS requires further investigation.

Regulation of placental (pro)renin

Cyclic adenosine monophosphate (cAMP) is a potent positive regulator for *REN* gene expression in the juxtaglomerular cells [62]. Modulation of *REN* expression by cAMP occurs through the binding of cAMP to both the cAMP response element (CRE) and the pituitary-specific positive transcription factor 1 (Pit-1) motif. The CRE and the Pit-1 motif are held in close interaction by the cAMP response element-binding (CREB) protein [62, 63]. cAMP has also been shown to increase prorenin release from primary decidual cell cultures in a dose dependent manner [64] and increases prorenin synthesis and release in a first trimester trophoblast cell line (HTR-8/SVneo cells) [65] as well as by cultured villous placenta [66, 67].

Interestingly, cAMP not only increases the expression of *REN* by HTR-8/SVneo cells but also increases their expression of *ATP6AP2* and *AGTR1*. By contrast, a choriocarcinoma cell line (BeWo), which is also used to study human placental cellular activity, *REN*, *ATP6AP2*, *AGTR1* and *AGTR2* were not expressed and treatment with cAMP failed to induce their expression [65]. In BeWo cells however, the expression of *ACE2* and *MAS* mRNAs did increase in response to cAMP [65]. Whether these effects were due to direct interactions of cAMP with these RAS genes has yet to be determined, however a similar upregulation of *AGTR1* expression has been reported in smooth muscle cells [68].

Human chorionic gonadotrophin (hCG) is released very early in pregnancy from trophoblast. It is important in maintaining the corpus luteum until such time that the fetoplacental unit can produce sufficient levels of oestrogen and progesterone to maintain the developing conceptus. HCG also stimulates placental *REN* expression [69], whilst in the decidua, endothelin-1 [70] and relaxin [71] have been demonstrated to increase decidual renin synthesis.

Epigenetic regulation of the placental RAS

Methylation of CpG islets near the promoter region of genes generally silences gene expression. We found that 3 genes of the RAS pathway have a high density of CpG islets in their promoter region, *ATP6AP2*, *ACE* and *AGTR1* (unpublished observations). In 4 term and 4 early (up to 17 weeks gestation) placentae the *ACE* gene was hypomethylated, thus any gestational changes in the expression of the *ACE* gene cannot be due to epigenetic changes. As noted above *ACE* mRNA abundance increases towards term, presumably because the fetal vascular endothelium is much more abundant in term placenta relative to other cells and ACE is only expressed in the fetal vascular endothelium. Interestingly the *ATP6AP2* gene showed intermediate methylation in 2 early placentae and hypomethylation in the other two early placentae and in term placentae while *AGTR1* showed intermediate methylation in one early placenta and was hypomethylated in 3 early and the 4 term placentae. Therefore we don't think that the gestational changes in expression of these genes as described above is related to changes in their methylation profiles but must be due to other factors, as yet not determined.

On the other hand, when we inhibited DNA methylation using a drug that blocks DNA methyltransferase (DNMT), 5-aza-2'-deoxycytidine (AZA), the expression of *REN* and production of prorenin by HTR-8/SVneo cells increased (unpublished observations), yet *REN* lacks differentially methylated regions in its promoter region. AZA may be inhibiting methylation of other genes that ultimately affects *REN* expression, perhaps other transcription factors involved in its regulation, or inhibiting methylation of other sequences within *REN* itself such as a palindromic sequence that acts as an enhancer and is located within intron 1 [72].

Hypoxia and the Placental RAS

Extravillous trophoblast cells invade the maternal decidua and the spiral arterioles, plugging them, so that the early placenta (<12 weeks gestation) normally develops in a low oxygen milieu [73]. This 'hypoxic' phase of placental development occurs early in pregnancy, is essential for normal placentation and, therefore, for a normal pregnancy outcome. Under low oxygen conditions the transcription factor Hypoxia Inducible Factor (HIF)-1α, is stabilised. HIF-1α is the primary mediator of the cellular response to hypoxia [74], it up regulates those placental genes involved in angiogenesis, proliferation, glucose transport and invasion, including genes of the placental RAS.

HIF-1α is expressed by the human placentae and its expression is highest in the first trimester [75-78], when (as described above) placental expression of most RAS [13] genes is also at a maximum. At this time the placenta is developing in a low oxygen environment.

ACE mRNA levels and ACE activity are increased by hypoxia in human umbilical vein endothelial cells (HUVECS) [56] as well as several various pulmonary artery smooth muscle and fibroblast cell lines [79, 80]. ACE was stimulated by HIF-1α binding and transactivating the ACE promotor directly [79] and was associated with increased cell cycle progression and proliferation and with decreased apoptosis [80]. The AT_1R has also been shown to be upregulated by hypoxia or over expression of HIF-1α [80] suggesting that Ang II-AT_1R mediated activity is upregulated by hypoxia. Therefore HIF-1α activates key components of tissue RASs. Interestingly, if this leads to Ang II accumulation then ACE2 is down regulated

[79] resulting in an imbalance in the ACE/ACE2 ratio and a change in the balance of the tissue RASs towards a proangiogenic/proliferative profile.

As well, Ang II regulates HIF-1α levels even in non-hypoxic tissues. Ang II not only stabilizes HIF-1α [81, 82] but also increases HIF-1α translation and transcription [83-85]. Therefore, hypoxia and Ang II work in conjunction to sustain HIF-1α protein levels and so may regulate placental growth and function [86, 87]. These interactions have not yet been demonstrated in human placental tissue/cell lines, but the data strongly support the hypothesis that the expression of the placental RAS could be affected by the ambient pO_2 so that together with HIF-1α, the placental RAS influences the activity of key genes involved in placental development, in particular those that stimulate angiogenesis. We suggest that in the early placenta the local RAS, like the intraocular RAS, is responsible for neovascularisation. In the eye, the local RAS is responsible for blindness in neonates in the retinopathy of prematurity syndrome. Briefly, exposure of preterm neonatal animals to very high oxygen levels causes cessation of vessel growth in the inner retina of the eye. When the animals are returned to room air, the retina becomes hypoxic and neovascularisation occurs. Not only is the ocular RAS upregulated by this ischaemia but so is vascular endothelial growth factor (VEGF), a key angiogenic factor. VEGF expression and the associated ocular neovascularisation are blocked by drugs that antagonise the RAS [88, 89]. Thus hypoxia/ischaemia induces the ocular RAS, which induces angiogenesis. It is likely that the same occurs in the human placenta. That is, in the physiological normal 'hypoxic' phase of early placental development, the RAS is activated, Ang II is formed, and Ang II together with a low oxygen environment stabilize HIF-1α. HIF-1α acts as a transcription factor increasing expression of genes controlling angiogenesis as well as trophoblast growth, invasion and transformation to an endovascular phenotype [86, 87]. We have shown that there is in fact a very strong correlation between the levels of expression of *VEGF* and *REN*, between *VEGF* and *ATP6AP2* and between *VEGF* and *AGTR1* in human placental tissue (Figure 5, [13]). If, as we postulate, this hypoxic induction of the placental RAS is inhibited in early pregnancy, then placentation will still occur, but it will be abnormal and may result in pathological pregnancy outcomes, as described below.

Regulation of the placental RAS by MicroRNAs

How could hypoxia regulate expression of placental RAS genes apart from its effects on levels of HIF-1α?

One possible mechanism is through alterations in the expression of microRNAs (miRNAs) that regulate translation of RAS genes. MicroRNAs are small non-coding nucleotides that bind to specific sites in the 3' untranslated region (UTR) of mRNA, destabilizing the mRNA or impeding its translation, so that less encoded protein is synthesized. Measurement of the transgestational changes in miRNA expression in fetal membranes [90] and evidence for differences in placental expression of miRNAs in preeclampsia [91] show that these newly discovered mechanisms controlling gene expression do have highly significant implications for understanding the biology of normal and abnormal pregnancy.

There are a large number of miRNAs that could target the RAS. miR-155, for example, binds to the 3'UTR of *AGTR1* mRNA and lowers the expression of AT_1R protein in human

primary lung fibroblasts [92]. miR-155 is expressed in very low levels in the human placenta [92]. Interestingly, expression of AT_1R protein in HUVECs isolated from women with preeclampsia is increased compared to expression in HUVECs obtained from women who have healthy pregnancies. The expression of AT_1Rs was knocked down when cells were transfected with miR-155 [93]. This suggests that miR-155 may be a key regulator of AT_1R expression in normal and preeclamptic placentae. To date research on other miRNAs that target the RAS in human pregnancy is lacking but animal studies described below clearly indicate that they could play a key role in regulation of the placental RAS.

MicroRNAs, the placental RAS and hypoxia

Expression of some miRNAs that target key genes of the RAS are suppressed by hypoxia, leading to increased protein expression [94, 95]. MicroRNAs that target the placental RAS could therefore affect placental development and might contribute to the abnormal placentation characteristic of preeclampsia and IUGR.

MicroRNAs that target the RAS have been found in mouse placenta and lung [94, 95]. Their targets include placental *REN* mRNA (miR-199b), placental *ACE* mRNA (miR-27a) and *AGTR1* mRNA (miR-468), all key components of the RAS pathway. These miRNAs are suppressed by maternal hypoxia [94, 95], thus the low oxygen tension that occurs during the hypoxic phase of human placental development may also suppress those miRNAs that target placental RAS mRNAs and so activate Ang II production. At the end of the first trimester when maternal blood flow to the placenta commences and the oxygen tension rises steeply from <20 mmHg at 8 weeks of gestation to >50 mmHg at 12 weeks [96], the expression of these miRNAs could be stimulated and so the activity of the placental RAS would be suppressed after this time.

If these putative human placental miRNAs that target the RAS are not adequately suppressed in early gestation, Ang II production and Ang II-AT_1 receptor activated functions will be suppressed leading to impaired placental development. Searching the miRanda database we have found a number of miRNAs that have *REN* mRNA as their target. Research is lacking on which of them are expressed in the human placenta but they have been implicated in tumorigenesis, as their down regulation is associated with enhanced cell growth [97, 98]. If the placenta, in view of its growth and invasion of the maternal decidua and angiogenesis, is considered a 'controlled cancer', it is also reasonable to postulate that miRNAs are involved in regulating its growth and development.

Functions of the placental RAS

Ang II via binding to AT_1R causes vasoconstriction, proliferation and angiogenesis. In view of the often opposing actions of Ang II by AT_1R and AT_2R and the actions of Ang 1–7 at the MasR it is likely that dysregulation of expression of components of the placental RAS could affect placental development and lead to placental insufficiency.

The RAS may stimulate placental angiogenesis

Tissue RASs play important roles in vasculogenesis [15] and in diseases associated with neovascularisation [88]. Ang II is predominantly pro-angiogenic and stimulates angiogenesis in the chorioallantoic membrane of the chick embryo [14]. Activation of the AT_1R by Ang II leads to induction of vascular endothelial growth factor (VEGF) [17, 99, 100], which is involved in establishing the fetoplacental circulation [101]. As stated above, Ang II stabilizes HIF-1α [81, 82], which is also implicated in angiogenesis and tumour progression and enhances VEGF production (see below). Watanabe *et. al*. [16] found that the endometrial cancer cell line HEC-1A increased VEGF secretion when exposed to Ang II (10^{-9}–10^{-7} M). This effect was abolished by 10^{-5} M CV11974 (an AT_1R antagonist). When HEC-1A cells over expressed adipocyte-derived leucine aminopeptidase (ALAP), Ang II treatment failed to stimulate VEGF production because Ang II was rapidly destroyed. Furthermore, culture medium from Ang II-stimulated HEC-1A cells induced endothelial migration of human umbilical vein endothelial cells (HUVEC), whilst culture medium from HEC-1A cells over expressing ALAP did not [16]. We have shown strong correlations between *VEGF* expression and that of *REN*, *ATP6AP2* and *AGTR1* (Figure 5, [13]). Together with evidence that the Ang II/AT_1R pathway regulates the VEGF system [17, 99, 100], it is probable that the placental RAS regulates placental angiogenesis.

Ang II can also inhibit Ang II angiogenesis. Ang II acting via AT_2R inhibits VEGF production, an effect blocked by the AT_2R antagonist PD123,319 and mimicked by the AT_2R agonist CGP-42112A [102]. Ang 1–7 acting via the Mas receptor, has also been shown to inhibit tumour angiogenesis by decreasing VEGF mRNA and protein levels [103]. Furthermore, Ang II also induces expression of a soluble VEGF receptor (sFlt-1) [104], which binds and inhibits the actions of VEGF and placental growth factor (PlGF).

The RAS regulates trophoblast proliferation and invasion/migration

The Ang II/AT_1R interaction stimulates cell proliferation [105], while Ang II acting via the AT_2R inhibits cell growth and promotes apoptosis [106]. Therefore the actions of Ang II via AT_1R and AT_2R are antagonistic when it comes to cell growth. The Ang 1-7/Mas receptor axis has also been shown to inhibit proliferation of cultured tumour cells [107], suggesting that local production of ACE2 also has an important role in determining whether RAS signaling is pro- or anti-proliferative.

Although there is strong evidence that Ang II via the AT_1R promotes placental cell growth, data on whether the Ang II/ AT_1R interaction promotes or inhibits trophoblast invasion are conflicting. Initial studies on the role of Ang II on trophoblast proliferation and invasion were carried out in trophoblast cell lines. An early report found that Ang II, acting via the AT_1R inhibited trophoblast invasion by HTR-8/SVneo cells by stimulating plasminogen activator inhibitor-1 (PAI-1) production [108], which inhibits matrix degrading enzymes. In contrast, studies in choriocarcinoma cell lines found that Ang II, acting via the AT_1R but not the AT_2R, increased trophoblast proliferation [22] and increased trophoblast migration and invasion, with no change in PAI-1 levels [18]. More recent studies using first trimester villous explants have reported similarly conflicting results. In 2008, Araki-Taguchi and colleagues reported that Ang II increased extravillous trophoblast outgrowth and the

number of cells in cell columns [81]. These effects were accompanied by increased markers of proliferation and an increase in PAI-1 suggesting that Ang II inhibited trophoblast differentiation towards an invasive phenotype. A subsequent study however, reported an increase in trophoblast invasion using an invasion assay, which was accompanied by increased in markers of proliferation and a decrease in caspase-3 expression [9]. Given the localization of AT_1R in extravillous trophoblasts and villous trophoblasts [9, 10, 13], it is likely that Ang II plays a role in both proliferation and trophoblast invasion, however better functional studies in the placenta are needed to fully elucidate its role. Similarly there have been no studies on the functional role of Ang 1-7 in the placenta. Given that this peptide is the predominant peptide during pregnancy this could be very important.

The placental renin-angiotensin system and its role(s) in preeclampsia and intrauterine growth restriction

In this section we discuss how the placenta contributes to the pathogenesis of preeclampsia (PreE) and intrauterine growth restriction (IUGR), which may coexist with PreE or occur independently.

PreE occurs only in the presence of trophoblasts and only resolves when the placenta is removed. As the gateway between the mother and fetus, the placenta controls the transfer of nutrients and oxygen; thus placental dysfunction is likely to be associated with IUGR. Since low birth weight is associated with an increased incidence of cardiovascular disease [109], the effects of abnormal placental development echo throughout life.

Preeclampsia (PreE) is classified as hypertension (blood pressure $\geq$140/90 mmHg) occurring after 20 weeks gestation accompanied by proteinuria [110]. It is associated with abnormal placental development in early pregnancy, which is characterized by shallow invasion of the spiral arterioles by extravillous trophoblasts. Consequently, these vessels do not lose their muscular coats nor do they lose their reactivity to vasoactive substances. As a result, maternal placental blood flow into the intervillous space becomes limited and no longer meets the demand of the growing fetus leading to hypoxia later in gestation. PreE is a potentially fatal disorder as it can progress to eclampsia, which usually results in fetal and maternal death. PreE accounts for 15% of maternal mortality and is the second most common cause of maternal death after embolism. It is also likely that women who develop PreE are at risk of developing hypertension in later life.

IUGR with or without PreE affects approximately 10% of all pregnancies and is associated with serious morbidity and mortality. IUGR is classified as a birthweight <5th centile using the GROW corrected birth weight centiles (http://www.gestation.net/birthweight_centiles/birthweight_centiles.htm).

As described above (Figure 5), there is a very high level of placental expression of *REN*, *ATP6AP2* and *AGTR1* in early gestation, and they are co-expressed with VEGF [13], suggesting a key role for the placental RAS in early placental development via the Ang II-AT_1R pathway. In one study in the mouse, a lack of the $AT_{1a}R$ receptor was associated with a 30% incidence of placental malformations incompatible with embryonic survival [111]. These malformations were so severe that only the endocrine trophoblast was present and active.

Placental oxygen levels could regulate the placental RAS. The early placenta develops in a hypoxic milieu, when there is high expression of *REN*, *ATP6AP2* and *AGTR1* [13]. At the end of the first trimester maternal blood ruptures through the cytotrophoblast plugs occluding the spiral arterioles and pours into the intervillous space so that the placental pO_2 increases [96]. Normally, expression of placental *REN*, *ATP6AP2, ACE2* and *AGTR1* is reduced in late gestation [13]. As explained above, the ambient pO_2 may exert these effects on the placental RAS through miRNAs that target key placental RAS mRNAs.

If the placenta fails to develop normally, then placental function is restricted in late pregnancy. We postulate that not only does this reduce oxygen and nutrient supply to the fetus but the associated placental hypoxia activates placental RAS genes just as it did in early pregnancy when the spiral arterioles were plugged by trophoblast. In the pregnant mouse, maternal hypoxia has been shown to be associated with increased expression of placental *REN* [95]. Activation of the placental RAS in late gestation has adverse effects on the fetus, because it amplifies effects of limited nutrient supply by mechanisms described below. It also contributes to the pathogenesis of PreE. Therefore, in both PreE and IUGR the primary placental defect may occur because of inadequate activation of the early placental RAS. This defect then leads to inadequate placental function in late pregnancy, which in turn results in inappropriate reactivation of the placental RAS. Indeed, *AGT*, *REN*, *ACE* and *AGTR1* expression is upregulated in preeclamptic placentae compared to normal term placentae [48, 49, 56], as is placental ACE activity [56] and Ang II peptide levels in the chorionic villi [48].

The kidney RAS provides an excellent example of this biphasic role of a tissue RAS. In the developing kidney, high levels of RAS expression are essential for early development. Knockout or blockade of key enzymes and receptors in the RAS cascade, e.g. ACE and AT_1R, are associated with severe renal defects [112]. In particular, pharmacological blockade of the AT_1R causes abnormal vascular development of the kidney, with stunted arterioles and few glomeruli [113]. There is also failure of development of the urinary concentrating mechanisms, i.e. the medulla and collecting duct [114]. In adult life however, sustained over activity of the renal and therefore the circulating RAS cause hypertension and exacerbate the renal damage caused by diabetes. Drugs that block the activity or actions of the adult RAS are mainstays in the treatment of hypertension, heart failure and diabetic nephropathy [115].

Because the maternal renal and circulating RASs have a multitude of factors and feedback mechanisms that influence their activities it is very difficult to find out anything about the contributions of the various components of the placental RAS to the maternal RAS or to the pathogenesis of PreE.

The maternal circulating RAS is activated very early in normal pregnancy. Initially renin levels are increased, subsequently angiotensinogen levels rise. These changes cause sustained high levels of Ang II and aldosterone. In response to the high circulating levels of Ang II, vascular reactivity to Ang II both in terms of vascular sensitivity [116] and blood pressure responsiveness [117] are reduced in normal pregnant women through down regulation of AT_1R.

This is not the case in women with PreE. In these women there is upregulation of pressor sensitivity to Ang II in late gestation [117]. This could be due to 2 changes, either upregulation of AT_1R as shown by Broughton Pipkin *et. al.* [118] or secretion of autoantibodies to AT_1R which bind to the second extracellular domain of the AT_1R (AT_1R-AA, [119]). Binding of autoantibodies to this domain restores vascular sensitivity of the pregnant animal to Ang II [120]. These actions of AT_1R-AA can be blocked by the AT_1R

blocking drug, losartan [121]. The source of AT_1R-AAs is currently unknown. Animal studies have however, provided convincing evidence that they cause the symptoms of PreE (namely hypertension and proteinuria, [122]) and that they result from pathological changes in placental production of renin (as a result of transgenically induced placental expression of human *REN* or in response to a reduction in uteroplacental perfusion). Takimoto *et. al.* [123] developed a transgenic mouse model, whereby a female mouse transgenic for human angiotensinogen, when mated with a male transgenic for human renin, developed hypertension in the latter stages of pregnancy coincident with the appearance of human renin in the maternal circulation. Dechend *et. al.* [124] characterised this model further, showing that these animals had many of the features of PreE including proteinuria and that there were AT_1R-AAs in plasma. In the rat, LaMarca *et. al.* [125] were able to show that reduced uteroplacental perfusion plus infusion of tumour necrotic factor (TNF)-α caused PreE like symptoms in the mother and that AT_1R-AA was present in the maternal circulation. While these animal models clearly implicate the late gestational placental RAS in PreE and its association with the presence of AT_1R-AAs in maternal plasma, which restore vascular reactivity and may cause proteinuria, there are other pathways via which the placental RAS could alter maternal cardiovascular and renal function in PreE.

Ang II acting via the AT_1R increases sFLT-1 expression [104] as do AT_1R-AAs [126]. sFLT-1 binds to VEGF preventing its access to the VEGF receptors and so acts as an anti-angiogenic factor. It is implicated in the symptomology of PreE as infusions of adenoviruses transfected with sFLT-1 RNA induce a PreE like condition [127].

The reaction of renin with its substrate, angiotensinogen, is limited by the amount of angiotensinogen available, as normal levels of angiotensinogen are not saturating. During pregnancy circulating levels of angiotensinogen increase because estrogens stimulate expression of the hepatic angiotensinogen gene [128]. Thus, in normal pregnancy active renin levels actually fall as the rising levels of angiotensinogen maintain Ang II production, which via negative feedback suppresses renin release. High levels of estrogens are a known risk factor for PreE.

In 2010, Zhou *et. al.* [129] showed that oxidised angiotensinogen in the presence of prorenin receptor (which can exist in a soluble form in the circulation) has a much lower K_m than reduced angiotensinogen (0.22±0.09 μM compared with 2.5±0.5 μM for reduced angiotensinogen). Women with PreE had higher levels of oxidised angiotensinogen [129]. Since maternal blood perfuses the placenta, either locally produced reactive oxygen species (ROS) or loss of ROS scavengers could result in placental oxidation of circulating angiotensinogen. Oxidative stress is strongly implicated in adverse pregnancy outcomes [130], and in particular in preeclampsia.

Placental levels of Ang II are high in PreE (see below). Ang II stimulates the formation of placental ROS [131] as do the AT_1R-AAs [132]. Thus placental Ang II or AT_1R-AAs in the mother probably create a milieu in which the oxidised form of angiotensinogen predominates, so accelerating the production of Ang II.

Hepatic angiotensinogen expression is stimulated by Ang II and by the cytokine interleukin (IL)-6 [133]. Infusions of IL-6 cause a rise in angiotensinogen levels. In a pregnant rat model of reduced uteroplacental perfusion with hypertension [134] high levels of IL-6 were found. IL-6 infusions into non-pregnant rats increased plasma renin activity, caused hypertension and reduced renal function (the latter are defining symptoms for diagnosing PreE).

Another component of the placental RAS that could also be involved in the pathogenesis of PreE is ACE2 (Figure 1). ACE2 is located in fetal vascular endothelium, cytotrophoblasts and syncytiotrophoblasts (Figure 4). The catalytic activity of ACE2 in generating Ang 1-7 from Ang II is about 500 times that of other Ang 1-7 forming pathways [135]. Ang 1-7 acting through the Mas receptor is a vasodilator and is associated with increased production of NO [61]. Syncytiotrophoblastic ACE2 is ideally situated to convert maternally produced Ang II to Ang 1-7. This has two consequences. First, delivery of Ang II back into the maternal circulation is reduced with the potential for an overall reduction in its inflammatory, hypertensinogenic and renal effects. Second, maternal Ang 1-7 levels will be increased, contributing to vasodilation and increased NO production. In normal pregnancy Ang 1-7 levels, like the other components of the RAS, are increased, but in PreE they are reduced [136].

One of the defining features of PreE is proteinuria. The reason why PreE causes proteinuria has not been studied but in view of the potential role of the intratubular RAS in essential hypertension and in the proteinuria of diabetic and membranous nephropathy as described by Navar and others [137, 138], it is tempting to suggest that the proteinuria of PreE could be caused by activation of the intrarenal RAS.

The intratubular/intrarenal RAS is activated by circulating Ang II. All components of the RAS are located in the nephron. Renin is secreted into the interstitium from juxtaglomerular (JG) cells, angiotensinogen is synthesised in the proximal convoluted tubule and both Ang II and IL-6 can stimulate its synthesis. ACE and AT_1Rs are located along the tubule and prorenin and (P)RR are found in the distal nephron [137]. It is thought that this system plays a role in stimulating sodium reabsorption. In view of the high glomerular filtration rate occurring in normal human pregnancy, which delivers more salt to the renal tubules and the increased activity of the circulating RAS, the intratubular RAS is probably activated in normal pregnancy compared with the non-pregnant state. We postulate that in PreE, Ang II, AT_1R-AAs and IL-6 could all stimulate proximal tubular angiotensinogen production so driving the activity of the intratubular RAS and causing glomerular damage as well as salt and water retention and hypertension. There is one report suggesting that renal sensitivity to Ang II is upregulated in an animal model of PreE [139].

Not surprisingly PreE is associated with IUGR, but are there any associations between abnormal expression of the placental RAS and IUGR independent of concurrent PreE?

Placental AT_1R levels in normotensive pregnant women are inversely correlated with infant birth weight [140] and other studies have shown that although IUGR fetuses have high levels of Ang II, placental AT_1Rs are not (as might be expected) down regulated [141].

In the ACE2 deficient mouse, fetal weight was reduced [142] and placental Ang II levels were higher, a finding similar to the higher levels of Ang II found in chorionic villi from women with PreE and in transgenic models of PreE which also have fetal growth retardation [48, 143].

Ang II in this location could cause placental vasoconstriction and therefore reduce materno-fetal exchange of O_2 and nutrients, as we have found AT_1R on fetal placental endothelium (Figure 4) but there are, also, other pathways via which abnormal expression of the late gestational placental RAS can affect fetal growth.

Ang II inhibits system A amino acid transport across placental cells because it inhibits Na-K-ATPase activity in primary villous cells [144]. Since fetal growth is dependent upon accretion of amino acids, and 20-40% of fetal energy expenditure is dependent upon maternal

supply of amino acids [144] this action of placental Ang II could be profoundly growth limiting.

Furthermore, in the late gestation placenta, ACE (unlike ACE2) is only found in fetal vascular endothelial cells (Figure 4, [10, 13]). In this location it is ideally situated to convert Ang I entering the placenta via the umbilical arteries to Ang II and in fact, Yagami *et. al.* [145] and Broughton Pipkin *et. al*. [118], found that Ang I levels were higher than Ang II levels in umbilical arteries and Ang II levels were higher than Ang I levels in umbilical veins, providing evidence of conversion of Ang I to Ang II across the placenta. Ito *et. al.* [56] showed that *ACE* mRNA, protein and activity were upregulated in stem villous venous endothelial cells. This conversion of Ang I to Ang II is therefore occurring distal to syncytiotropoblast amino acid transporter systems but clearly proximal to the fetal pulmonary and systemic vasculature where Ang II would cause vasoconstriction resulting in decreased supply of nutrients to peripheral tissues and the relative decrease in body to brain weight ratio ('brain-sparing') characteristic of IUGR.

While this review has examined the role of the placental RAS in the pathogenesis of PreE and IUGR, it is possible that its functions are influenced by another intrauterine tissue, namely the decidua. The decidua vera (which is not in direct contact with the placenta expresses very high levels of renin, in a sex specific manner [146]. Shah *et. al.* [147] have shown decidual expression is increased in PreE and Herse *et. al.* [148] have shown upregulation of the decidual AT_1R in PreE. Thus there are novel levels of interactions between these tissue intrauterine renin-angiotensin systems and we need to explore the paracrine and autocrine as well as endocrine interrelationships between them and the circulating fetal and maternal RASs before we fully understand the role(s) of the placental RAS in PreE and IUGR.

Conclusions

The placenta has its own renin angiotensin system, which has been implicated in placental angiogenesis as well as trophoblast proliferation and migration. As well, placental prorenin activated by binding to the prorenin receptor or through proteolytic activation by endogenous proteases may react with maternal and fetal circulating angiotensinogen. Conversely, placental angiotensin converting enzymes (ACE and ACE2) may convert angiotensin I in fetal blood perfusing the placenta to Ang II and Ang II or, alternatively, may convert Ang I in maternal blood perfusing the placenta to Ang 1-7. Little is known about how the placental RAS is regulated in normal and pathological pregnancies but hypoxia and miRNAs are likely to play key roles. In preeclampsia, the expression of the RAS is dysregulated. Whether this is a cause or a consequence of the disease is not clearly known. Since the placental RAS is positioned between maternal and fetal circulating RASs, it may not only directly affect placental growth and development but also, through interactions between the placental RAS and the two circulating RASs, have effects in the developing fetus and its mother. Since these postulated roles of the placental RAS may affect both maternal and fetal health, further research into its regulation and function(s) throughout gestation are important in order to fully understand its role(s) in both normal and abnormal placental development and function.

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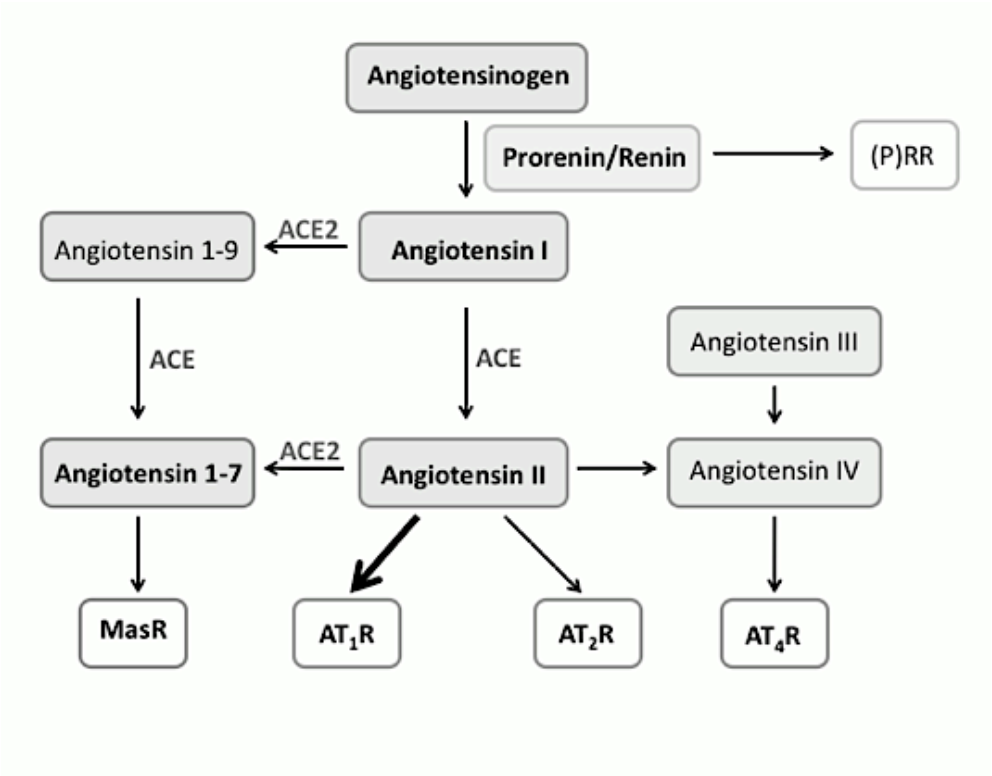

Figure 1. Tissue-Based Renin Angiotensin Systems

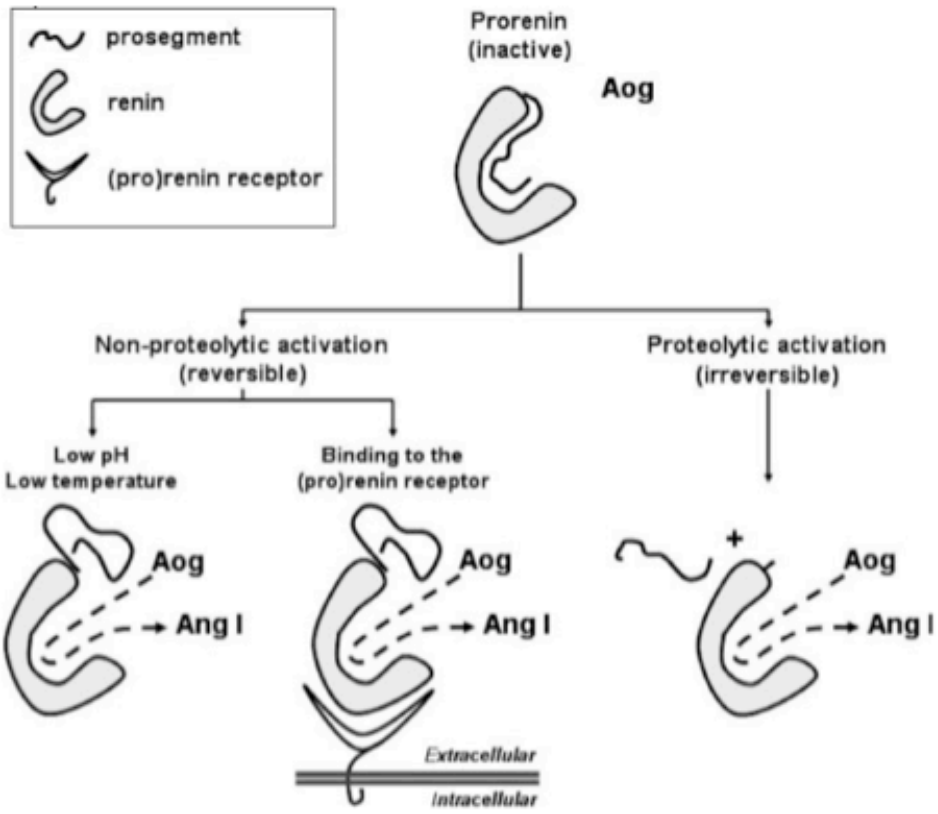

Figure 2. Proteolytic and non-proteolytic activation of prorenin. Aog, angiotensin; Ang, angiotensin. Danser, A.H.J., W.W. Batenburg, and J.H.M. van Esch, Prorenin and the (pro)renin receptor - an update. Nephrol. Dial. Transplant, 2007. 22(5): p. 1288-1292**,** by permission of Oxford University Press.

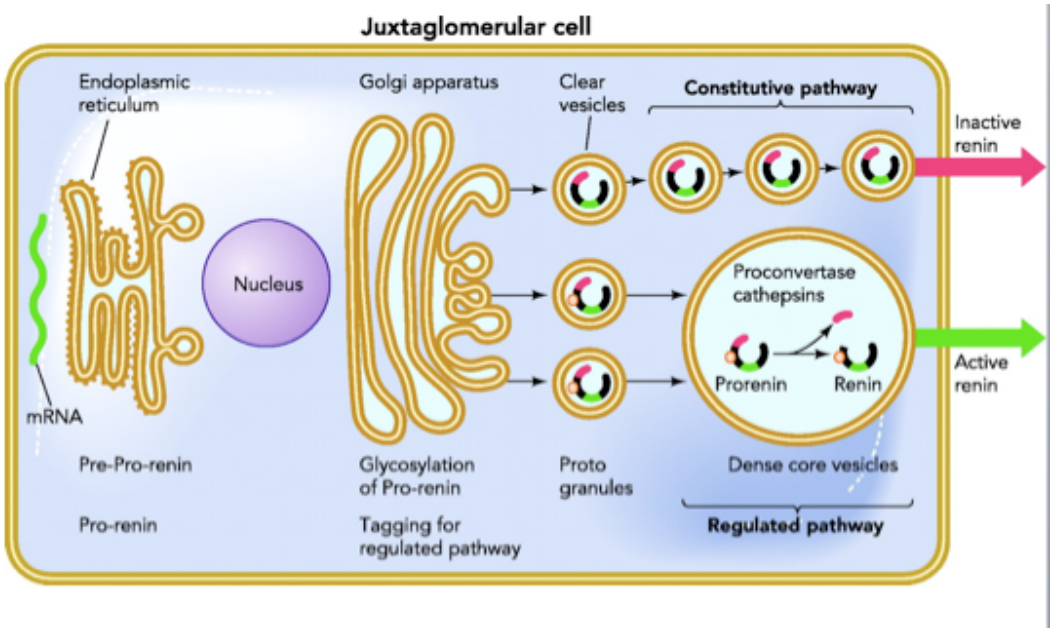

Figure 3. Synthesis and secretion of prorenin and renin. After translation, pre-pro-renin enters the endoplasmic reticulum. The pre-part is cleaved and prorenin moves to the golgi-apparatus, where glycosylation and tagging for the regulated pathway takes place. Untagged prorenin is constitutively released in clear vesicles from the trans-Golgi. Prorenin tagged for the regulated pathway is released in proto granules which form mature renin granules. Here the prosegment is cleaved off and renin is activated. Active renin is stored in these mature granules and can be released rapidly by regulated exocytosis. Schweda F., Friis U., Wagner C., Skott O. and Kurtz A. (2007). Renin Release. Physiology, 22(5), 310-9, with permission from the American Physiological Society.

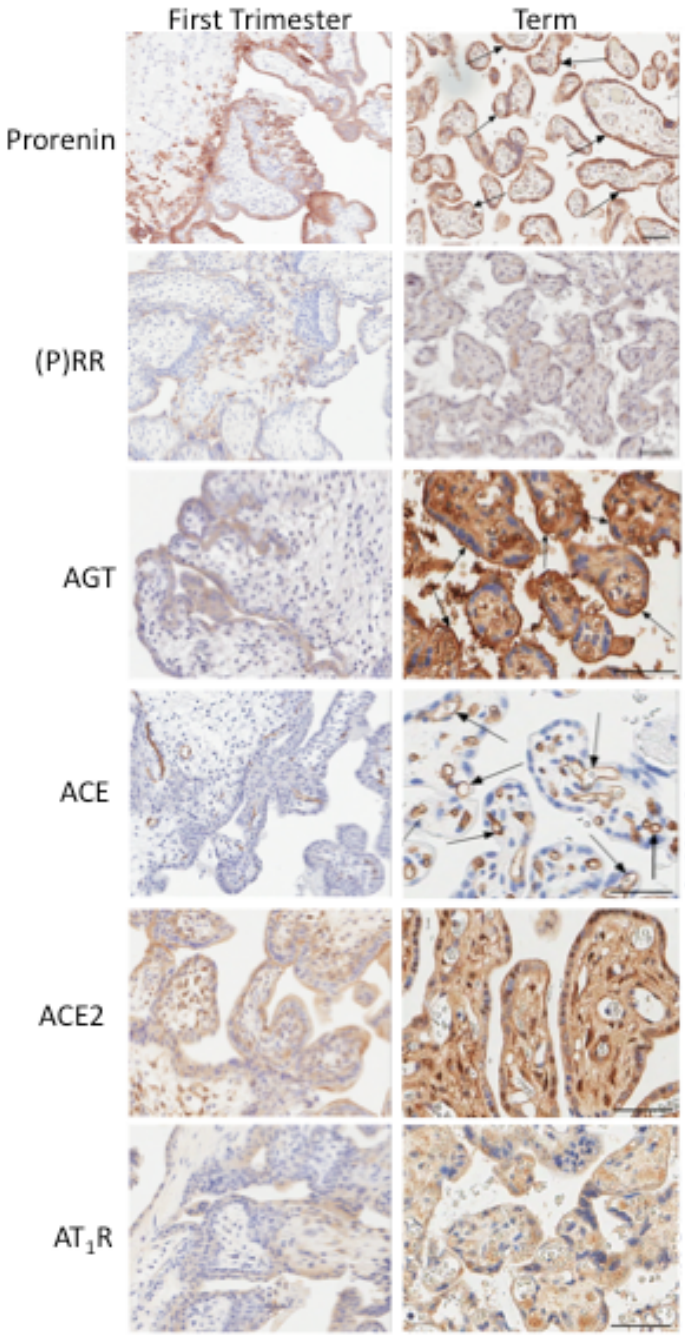

Figure 4. Localisation of Renin Angiotensin System components in first trimester (left) and term (right) human placentae. Reprinted from Placenta 32(3), Marques, F.Z., et al., *Molecular characterization of renin-angiotensin system components in human intrauterine tissues and fetal membranes from vaginal delivery and cesarean section*, p. 214-221. Copyright (2011) and Placenta 32 (12), Pringle K. G., et al., *The expression and localization of the human placental prorenin/renin-angiotensin system throughout pregnancy: roles in trophoblast invasion and angiogenesis?* P. 956-62. Copyright (2011) with permission from Elsevier.

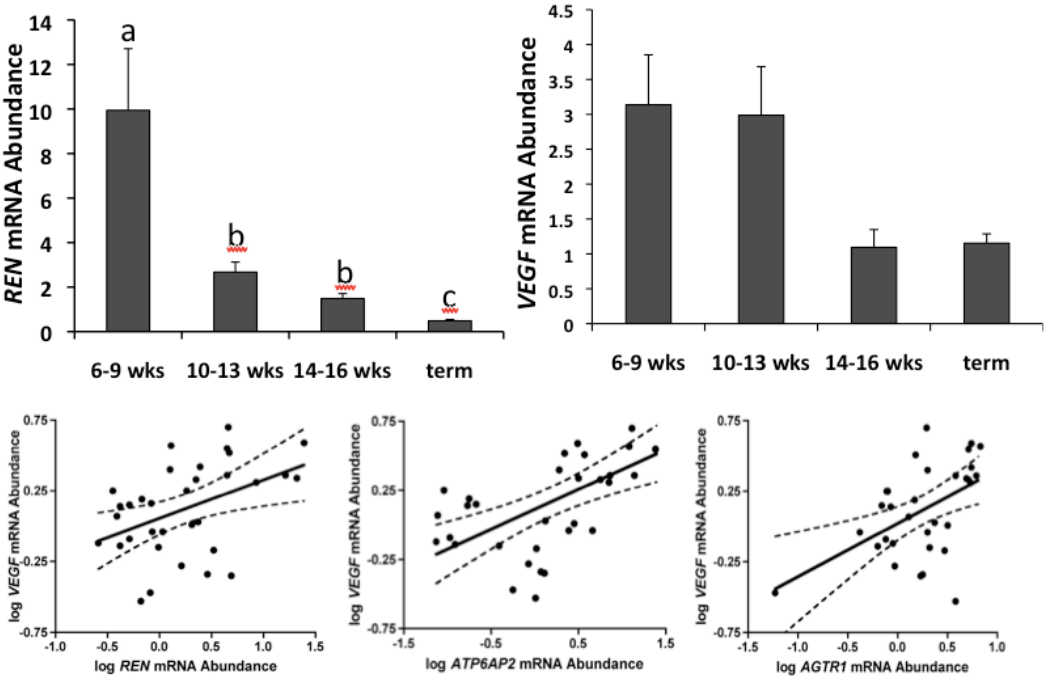

Figure 5. (A) Prorenin (*REN*) and (B) *VEGF* mRNA abundance in early and late gestation human placenta. *VEGF* mRNA expression in early and late gestation placentae is highly correlated with (C) *REN*, (D) *ATP6AP2* and (E) *AGTR1* mRNA abundance. Adapted from Placenta 32 (12), Pringle K. G., et al., *The expression and localization of the human placental prorenin/renin-angiotensin system throughout pregnancy: roles in trophoblast invasion and angiogenesis?* P. 956-62. Copyright (2011) with permission from Elsevier.