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ORIGINAL ARTICLE

Regulation of the human placental (pro)renin receptor-prorenin-angiotensin system by microRNAs

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STUDY QUESTION: Are any microRNAs (miRNAs) that target the placental renin-angiotensin system (RAS) in the human placenta suppressed in early gestation?

SUMMARY ANSWER: Overall, 21 miRNAs with predicted RAS mRNA targets were less abundant in early versus term placentae and nine were more highly expressed.

WHAT IS KNOWN ALREADY: Regulation of human placental RAS expression could alter placental development and therefore normal pregnancy outcome. The expression of genes encoding prorenin (REN), angiotensinogen, (pro)renin receptor, angiotensin converting enzyme 2, and the angiotensin II type 1 receptor are highest in early gestation, at a time when oxygen tension is at its lowest. Studies have shown that the human placental RAS is sensitive to oxygen, as are some miRNAs that regulate RAS mRNAs. We propose that in early pregnancy, the prevailing low O_2 tension, by suppression of levels of miRNAs that target RAS mRNAs, results in increased expression of RAS mRNAs and encoded proteins. As gestation proceeds and the prevailing oxygen tension rises, abundance of these miRNAs increases, and placental RAS mRNA expression is suppressed.

STUDY DESIGN, SIZE, DURATION: The expression of miRNAs was compared in human placentae collected in early (10–11 weeks; $n = 7$) and mid-gestation (14–18 weeks; $n = 8$) with placenta collected at term (38–40 weeks; $n = 8$). Expression of placental miRNAs in women with early (29–35.1 weeks; $n = 8$) or late-onset pre-eclampsia (PE) (>34-weeks gestation; $n = 8$) and gestational age matched preterm (31.6–35.1 weeks; $n = 8$) and term normotensive controls were also compared.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Agilent Human miRNA microarray v19 was used to detect up to 2006 miRNAs in four placentae from each group. Statistically different levels of expression were determined and refined using predictive modelling. Placental miRNAs predicted to target RAS mRNAs were identified in three databases. Differences detected on the array were confirmed for some miRNAs by semi-quantitative RT-PCR (qPCR, $n = 7$–8 for all groups). Two differentially expressed miRNAs that were known to target human renal *REN* mRNA (miR-181a-5p and miR-663) were transfected into human HTR-8/SVneo trophoblast cells to examine their effect on placental *REN* expression and prorenin levels.

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MAIN RESULTS AND THE ROLE OF CHANCE: In early gestation placentae, 186 miRNAs were differentially expressed compared with term placentae (109 increased, 77 decreased). Thirty of the differentially expressed miRNAs were predicted to target RAS components. In mid-gestation placentae, 117 miRNAs were differentially expressed compared with term placentae (69 increased, 48 decreased). Of these, 19 had RAS mRNAs as predicted targets. Eight miRNAs that were lower in early gestation and predicted to target RAS mRNAs were confirmed by qPCR. All showed an increase during gestation and could influence the transgestational profile of the human placental RAS. Additionally, on the array, three miRNAs predicted to target RAS mRNAs (miR-892c-3p, miR-378c and miR-514b-3p) were overexpressed in placentae from women with late-onset PE ($P = 3.6E{-}10$, $P = 1.8E{-}05$, $P = 5.3E{-}06$; respectively). miR-663, which suppresses renal *REN* mRNA expression, was overexpressed in early-onset PE placentae as determined by qRT-PCR analysis ($P = 0.014$). Transfection of miR-181a-5p and miR-663 into HTR-8/SVneo trophoblast cells suppressed *REN* mRNA expression ($P = 0.05$) and prorenin protein production ($P = 0.001$).

LARGE SCALE DATA: Data can be found via GEO accession number GSE109832.

LIMITATIONS, REASONS FOR CAUTION: Further validation that the differentially expressed miRNAs do indeed directly target RAS mRNAs and affect placental development and function is required. This study is limited by the small sample size. Therefore independent validation in a larger cohort is required.

WIDER IMPLICATIONS OF THE FINDINGS: We propose that suppression of miRNAs that target the placental RAS in early gestation is partly responsible for the increase in RAS expression at this time, in order to promote placental development. Later in pregnancy, we have detected overexpression of several miRNAs in placentae from women with PE. These may prove to be biomarkers for early detection of women at risk of developing PE. Since the placenta produces at least two miRNAs that were found in the kidney to target *REN* mRNA, and that also target placental *REN* mRNA, the escape of these miRNAs into the maternal circulation in excess amounts could affect maternal renal *REN* mRNA production and thereby disturb maternal fluid and electrolyte homoeostasis.

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Key words: miRNA / placenta / renin-angiotensin system / pre-eclampsia / pregnancy / HTR-8/SVneo cells / miRNA array

Introduction

There is strong evidence that angiotensin (Ang) II, acting via the Ang II type 1 receptor (AT_1R), can stimulate angiogenesis, cell proliferation and migration, which are the processes necessary for normal placental development (Cooper *et al.*, 1999; Takimoto-Ohnishi *et al.*, 2005; Hering *et al.*, 2010; Williams *et al.*, 2010). Therefore, appropriate regulation of human placental renin-angiotensin system (RAS) expression could be important in determining successful pregnancy outcome. In humans, placental expression of the renin gene (*REN*), angiotensinogen gene (*AGT*), prorenin receptor (ATPase H^+ transporting accessory protein) gene (*ATP6AP2*), angiotensin converting enzyme 2 gene (*ACE2*) and AT_1R gene (*AGTR1*) is greatest in early gestation (Pringle *et al.*, 2011).

In early gestation (<12 weeks) the placenta develops in a low oxygen environment (<20 mmHg), which is necessary for successful placentation (Jauniaux *et al.*, 2003a,b). At 12 weeks, the oxygen tension within the intervillous space rises to ~50 mmHg, then stays at that level until term (Jauniaux *et al.*, 2000). In animal models, the clinical signs of pre-eclampsia (PE) (i.e. hypertension, proteinuria, elevated soluble fms-like tyrosine kinase 1) can be induced by reducing uterine perfusion pressure (Intapad *et al.*, 2014).

Studies have shown that the human placental RAS is sensitive to oxygen (Delforce *et al.*, 2016; Kurlak *et al.*, 2016). Animal studies have also shown that microRNAs (miRNAs) that regulate some RAS genes are sensitive to oxygen (Goyal *et al.*, 2011a,b). We postulate that activity of the human placental RAS depends in part on the prevailing oxygen tension, and that the latter controls levels of placental miRNAs that regulate placental RAS mRNA expression. We propose that in early pregnancy the prevailing low O_2 tension, by suppression of levels of these miRNAs, results in increased expression of RAS mRNAs and encoded proteins. As gestation proceeds and the prevailing oxygen tension rises, abundance of these miRNAs increases, and placental RAS mRNA expression is suppressed.

To test this hypothesis, it was necessary to determine the changes that occur in the expression of human placental miRNAs across gestation and to identify those miRNAs that have RAS mRNAs as a predicted target. We therefore compared the expression of miRNAs in human placentae collected at 10–11 weeks (early) and 14–18 weeks (mid) gestation with miRNAs expressed at 38–40 weeks (term) gestation. We also determined the expression of placental miRNAs in women who had either early or late-onset PE and compared these with patterns of expression in women delivering preterm and at term.

Changes in concentration of several placental miRNAs that were differentially expressed across gestation and that were predicted to target RAS mRNAs were confirmed by semi-quantitative RT-PCR (qPCR). Finally, we chose two miRNAs known to target human renal *REN* mRNA (Marques *et al.*, 2011b) and transfected each into a first trimester human trophoblast cell line to determine their effects on *REN* mRNA expression and prorenin protein secretion by the cells.

Materials and Methods

The study was approved by the Hunter New England Health Human Research Ethics Committee (Approval No. 02/06/12/3.13) and the University of Newcastle (Australia) Human Research Ethics Committee

(Approvals: H-382-0602 and H-2015-0284). Written informed consent to participate was obtained from all women.

Samples

Term and preterm placentae were collected from the John Hunter Hospital, Newcastle, Australia. Term samples were collected from women with uncomplicated singleton pregnancies delivering at term (38.2–40.4 weeks gestation) by elective caesarean section in the absence of labour (n = 8). Women treated with non-steroidal anti-inflammatory drugs or who had a history of infection, chorioamnionitis, PE, or who were undergoing induction of labour, were excluded from this group. Placentae were also collected from women undergoing elective terminations of pregnancy at 10–11 weeks (n = 7) or 14.3–17.8 (n = 8) weeks gestation.

In addition, placentae were collected at 29–35.1 weeks from eight women who developed early-onset PE (PE that develops before 34 weeks' gestation) and, for comparison with equivalent gestational age placentae, from eight women who delivered preterm (at 31.6–35.1 weeks) after spontaneous labour/rupture of membranes and vaginal delivery with no evidence of hypertension. Furthermore, placentae were collected from eight women who developed late-onset PE (PE that develops at or after 34 weeks' gestation). Values obtained in these women were compared with those for term placentae, as described above.

PE was classified as sustained blood pressure ≥140/90 mmHg after the 20th week of pregnancy but before the onset of labour, and with significant proteinuria, i.e. dipstick ≥2+ or ≥300 mg/l or ≥ 500 mg/day or urinary protein:creatinine ratio ≥30 mg/mmol.

Term and preterm placental villous tissue was collected within 30 min of delivery from the maternal side of each placenta from four random sampling sites. Decidua and any contaminating membranes were removed and the tissues were washed in PBS to remove any maternal blood. Tissues from the four sites were pooled, snap frozen in liquid nitrogen and homogenized to provide a sample of the entire placenta. Placentae obtained prior to 18 weeks gestation were identified based on their villous structure. The entire placenta was frozen and subsequently homogenized for total RNA and protein extraction.

Total RNA preparation and microarray

Total RNA was extracted using the miRNeasy FFPE kit (Qiagen, Doncaster, Victoria, Australia). RNA quantity and purity was assessed by determination of A_{260}/A_{280} and A_{260}/A_{230} ratios using the Nanodrop™ spectrophotometer (ThermoFisher Scientific, North Ryde, New South Wales, Australia) (Avery-Kiejda *et al.*, 2014). Samples were excluded if A_{260}/A_{280} ratio was <1.8. The integrity of RNA in each sample was determined by gel electrophoresis. Total RNA quality was then assessed using an Agilent RNA 6000 kit with a Bioanalyzer (performed by the Ramaciotti Centre for Genomics, University of New South Wales, Sydney, New South Wales, Australia).

Labelling and hybridization to an Agilent Human miRNA microarray v19, was performed by the Ramaciotti Centre for Genomics. miRNA microarray analysis included four samples from each group (total sample n = 24). A robust multi-array analysis was performed using Genomic Suite 6.6 (Partek, St. Louis, MO, USA). This included $\log_2$ transformation, background correction, quantile normalization and summation of the probe features, resulting in a set of expression signal intensities (Mathe *et al.*, 2015).

Unsupervised hierarchical clustering was performed on miRNAs that were found to be significantly different ($P < 0.05$; fold change >1.5) when comparing early versus term samples, and mid-gestation versus term samples (Supplementary Fig. 1A and B) and when comparing preterm versus early-onset PE, and term versus late-onset PE (Supplementary Fig. S2A and B). Correction for multiple testing was performed using the Benjamini–Hochberg method.

Predictive modelling was used to refine the list of miRNAs that were differentially expressed on the array. Data can be found via GEO accession number GSE109832. The literature and the following databases: miRDB.org, targetscan.org and microRNA.org were employed to determine whether the miRNAs identified in the array were predicted to target any of the RAS mRNAs. For the purposes of this study miRNAs were classified as 'predicted to target a RAS miRNA' if listed in at least one of these databases, with >90% complementarity, or cited in the scientific literature.

Confirmation of miRNA expression levels by qPCR

Predesigned Taqman™ probes (ThermoFisher Scientific) were used to determine miRNA levels in placental samples. Total RNA (5 ng) was reverse transcribed to generate cDNA using the Taqman™ miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. cDNA was amplified using the TaqMan™ ProAmp Master Mix (Applied Biosystems). qPCR analysis was performed using TaqMan™ Universal PCR mix No AmpErase UNG and TaqMan™ miRNA Assays (Applied Biosystems). Results were quantified on a 7500 Real-Time PCR System (Applied Biosystems). The expression levels of miR-181a-5p, miR-181a-3p, miR-181c, let-7, miR-625, miR-454, miR-34c, miR-483-3p and miR-663b were determined by calculating $2^{-\Delta\Delta CT}$ using RNU44 (a highly conserved small nucleolar RNA in the growth arrest specific 5 transcript) as the house-keeping gene.

Messenger RNA expression

REN, *ATP6AP2*, *AGT*, *ACE* and *ACE2* mRNA expression levels were determined using methods and primers described previously (Pringle *et al.*, 2011). Ten micrograms of total RNA was DNase I-treated (Qiagen GmbH, Hilden, Germany) and the RNA was reverse transcribed using a Superscript III RT kit with random hexamers (Invitrogen, Carlsbad, CA, USA). qPCR was performed using an Applied Biosystems 7500 real-time PCR system with SYBR green for detection. Each reaction contained 5 µl of SYBR green PCR master mix (Applied Biosystems), primers, cDNA reverse transcribed from 10 ng total RNA, and water to 10 µl. β-actin was used as the housekeeping gene. RAS primer sequences were as described previously (Pringle *et al.*, 2011).

Transfection of cells with miRNA mimics

MiExpress precursor miRNA clones, constructed in a non-viral vector-based system with enhanced green fluorescent protein reporter gene, for hsa-miR-181a and has-miR-663b, were obtained from GeneCopoeia™ (Rockville, MD, USA) (Marques *et al.*, 2011a). In addition, a 'scrambled' and an 'empty vector' control was used.

HTR-8/SVneo-immortalized first-trimester human trophoblast cells were cultured in 6-well plates in RPMI-1640 medium, and transfected with vectors containing the miExpress precursor, a scrambled control or an empty vector control (all at 1000 ng per well) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 48 h, cells were collected and stored for total RNA extraction, and the incubation medium was stored at –20°C for later measurement of secreted proteins. Each experiment was performed on three separate occasions, each in triplicate.

ELISAs

Commercially available ELISAs were used to measure levels of prorenin (Molecular Innovations, MI, USA), angiotensinogen (IBL International, Hamburg, Germany) and ACE (Duoset, R&D systems, MN, USA) using methods described previously (Pringle *et al.*, 2015).

Western blotting

Proteins (10 μg for the (pro)renin receptor ((P)RR) and ACE2, and 20 μg for AT_1R, loaded in duplicate), were separated on a 4–12% Bis-Tris NuPAGE gel (Invitrogen) and transferred to a polyvinylidene fluoride membrane (Amersham Hybond P 0.2 μM pore-size, GE Healthcare, Parramatta, New South Wales, Australia). After blocking overnight at 4°C in 5% skim milk, 5% bovine serum albumin (BSA), membranes were incubated with a primary antibody against (P)RR (Abcam, Cambridge, UK; ab40790, 1:1000), ACE2 (ab15348; 1:1000), or AT_1R (ab9391; 1:400) for 2 h. Blots were exposed to a goat anti-rabbit IgG (Millipore, Burlington, MA, USA; #12-348, 1:5000) for (P)RR and ACE2, and rabbit anti-mouse IgG (Cell Signaling Technology, Danvers, MA, USA; #7076, 1:3000) for AT_1R, for 1 h. Proteins were detected using an Amersham ECL detection kit (GE Healthcare Life Science) and Amersham Imager 600. Blots were washed and re-probed for β-actin using a rabbit polyclonal anti-β-actin antibody (Abcam; ab8227, 1:5000). The ratio of the protein of interest to β-actin was averaged for duplicate lanes and differences between blots were corrected for using an internal control (a pooled placental sample).

Statistics

To determine differences between groups, samples were compared using the Kruskal–Wallis test with correction by Dunnett's multiple comparisons test. Mann–Whitney tests were employed to determine if there were differences between placentae from women with PE and their gestational age-matched controls. Spearman non-parametric correlations were used to assess associations between gene expression and protein levels. Graph Pad Prism V7 (La Jolla, CA, USA) was used for all analyses. The null hypothesis was rejected at $P < 0.05$.

Results

Gestational changes in placental miRNA expression

Comparison of 2006 miRNAs on a human miRNA microarray in samples of early gestation placentae (<12 weeks, range 10–11 weeks, $n = 4$) with samples of normal term placentae showed significant differences in expression of 186 miRNAs (after correction for multiple comparisons). We found 109 miRNAs were highly expressed in early gestation placenta relative to term placenta and 77 miRNAs were suppressed. Thirty (16.1%) of the differentially expressed miRNAs had RAS mRNAs as predicted targets. Overall, 21 miRNAs with predicted RAS targets were less abundant in early versus term placentae and nine were more highly expressed (Table I).

One hundred and seventeen miRNAs were differentially expressed in mid-gestation placentae compared to term placentae. Of these, 69 were more highly expressed and 48 were less abundant. Nineteen (16.1%) had RAS mRNAs as predicted targets. Of these miRNAs, 10 were less abundant and nine were more abundant relative to term (Table I).

No miRNAs were differentially expressed between early and mid-gestation placentae. The expression of miRNAs in term and preterm placentae from normotensive pregnancies was similar.

miRNA expression in placenta from women with PE

There were no differences in placental miRNA expression in women with early-onset PE compared with matched preterm controls.

Placental expression of miR-892c-3p, miR-378c and miR-514b-3p was significantly higher in women with late-onset PE than in term controls (Table II). Placentae from women with late-onset PE also had significantly higher levels of miR-892c-3p, miR-670-5p, miR-378c and miR-514b-3p than placentae from women with early-onset PE (Table III).

Confirmation of miRNA expression

Eight of the miRNAs identified as being differentially expressed by microarray, and that were predicted to target specific RAS mRNAs, were confirmed by qPCR (n = 7–8/group). These miRNAs are highlighted in bold in Table I. Seven confirmed miRNAs were less abundant in early-gestation placentae than term placentae (Fig. 1), confirming the microarray results. One miRNA, miR-936, was undetectable by qPCR.

Although the microarray results showed no difference in miRNA expression between placentae collected in early gestation and those in mid-gestation, qPCR in a larger sample set detected significant differences in expression of some of these miRNAs, i.e. miR-181a-3p, miR-181c and miR-34c (Fig. 1).

qPCR also confirmed the changes in miR-663, a renal miRNA differentially expressed in human hypertension and shown to downregulate *REN* mRNA by reducing its stability in transfected kidney cells (Marques *et al.*, 2011a). The miRNA microarray results showed miR-663 to be another miRNA that was significantly upregulated in early gestation placentae compared to term placentae (Table I). The qPCR analysis demonstrated that miR-663 expression was higher in placentae from women with early-onset PE compared with preterm control placentae (Fig. 2). There were no other differences in the expression of the eight qPCR-confirmed miRNAs (shown in Fig. 1) between preterm and term control placentae, and between early and late-onset PE (data not shown).

Gestational changes in placental expression of RAS mRNAs and protein

The relative abundance of *REN* mRNA and prorenin protein levels decreased through gestation ($P = 0.0002$ and $P = 0.001$, respectively; Fig. 3A and B). Placental *REN* mRNA was highest in early gestation compared with both mid-gestation and term ($P = 0.009$ and $P = 0.0003$, respectively). Moreover, *REN* mRNA abundance in mid-gestation placentae was greater than at term ($P = 0.0006$; Fig. 3A). Prorenin protein levels showed a similar gestational profile. Prorenin was highest in early gestation compared with mid-gestation ($P = 0.002$ and $P = 0.0003$, respectively) and mid-gestation prorenin levels were higher than levels at term ($P = 0.0008$; Fig. 3B).

Neither *ATP6AP2* mRNA levels nor (P)RR protein levels changed with gestational age (Fig. 3C, D).

AGT mRNA levels did not change with gestational age (Fig. 3E). On the other hand, AGT protein levels were lowest in early gestation placentae and highest in term placentae ($P = 0.0005$). Early and mid-gestation placental AGT protein levels were similar and in each case were lower than at term ($P = 0.0003$ and $P = 0.0002$, respectively; Fig. 3F).

Neither *AGTR1* mRNA nor AT_1R protein levels changed with gestational age (Fig. 3G, H).

ACE1 mRNA expression increased with gestational age ($P = 0.0016$; Fig. 4A). Early-gestation placentae had lower *ACE1* mRNA levels than seen in both mid-gestation and term placentae ($P = 0.0003$ and $P = 0.0022$, respectively). There was no difference in ACE1 protein levels

Table I Human placental miRNAs that are predicted to target renin-angiotensin system mRNAs and that have different levels of expression in early gestation (<12 weeks) and mid-gestation (14–18 weeks) compared with levels measured in term placentae*

miRNA expression relative to term placentae			
HSA-miR-	**Predicted RAS target**	***P*-value**	**Fold change**
10–11 weeks of gestation			
34c-5p	***AGTR1* mRNA**	**2.4E–03**	**–91.2**
181c-5p	***AGT*, *AGTR1* mRNA**	**1.8E–07**	**–90.7**
181a-3p	***REN* mRNA**	**4.1E–04**	**–73.2**
625-5p	***ATP6AP2* mRNA**	**7.7E–06**	**–19.4**
144-3p	*ACE2* mRNA	8.0E–04	–8.8
519d-3p	*AGTR2* mRNA	4.6E–03	–7.0
135b-5p	*ATP6AP2* mRNA	4.0E–04	–6.9
539-5p	*AGT*, *AGTR2* mRNAs	3.7E–03	–4.8
424-5p	*AGTR2* mRNA	8.6E–04	–4.6
24-3p	*ACE* mRNA	1.3E–05	–3.2
335-5p	*AGTR2* mRNA	4.0E–03	–3.8
23a-3p	*AGTR2* mRNA	3.2E–05	–3.8
149-5p	*ACE2* mRNA	4.4E–03	–2.8
181a-5p	***REN* mRNA**	**3.5E–06**	**–3.2**
143-3p	*ACE* mRNA	2.5E–03	–3.0
27a-3p	*ACE* mRNA	3.6E–05	–3.0
483-3p	*ACE*, *ACE2*, *AGT*, *AGTR1*	3.2E–03	–3.0
16-5p	*AGTR1* mRNA	2.5E–03	–2.9
141-3p	*AGT*, *ACE2* mRNA	2.4E–03	–2.6
376b-3p	*ATP6AP2*, *AGTR2*, *ACE2* mRNA	1.1E–03	–2.5
25-3p	*AGTR2* mRNA	8.6E–04	–2.4
6510-5p	*REN* mRNA	4.8E–03	2.7
663	*REN* mRNA	4.3E–04	4.7
5787	*ACE* mRNA	1.2E–03	5.3
1207-5p	*ACE* mRNA	3.3E–05	5.9
4763-3p	*ACE* mRNA	8.5E–07	5.2
125a-3p	*AGT* mRNA	5.9E–09	6.1
4505	*ACE* mRNA	9.3E–06	10
3202	*REN* mRNA	1.6E–04	38
936	***ACE2* mRNA**	**2.1E–03**	**75**
10–11 weeks of gestation			
454-3p	***ATP6AP2* mRNA**	**5.3E–04**	**–93**
34c-5p	***AGTR1* mRNA**	**2.4E–03**	**–91**
7-5p	***AGTR1* mRNA**	**4.8E–04**	**–63**
181c-5p	***AGT*, *AGTR1* mRNA**	**1.3E–03**	**–8.2**
16-5p	*AGTR2* mRNA	7.1E–05	–4.7
143-3p	*ACE* mRNA	7.2E–04	–3.6
374a-5p	*AGTR2* mRNA	3.0E–03	–3.0
376b-3p	*ATP6AP2*, *AGTR2*, *ACE2*	1.2E–03	–2.5
106b-5p	*AGTR2* mRNA	4.7E–06	–2.4
20a-5p	*AGTR2* mRNA	2.9E–03	–2.1
134-5p	*AGTR1* mRNA	1.2E–03	2.7
125a-3p	*AGT* mRNA	2.5E–05	2.7
5001-5p	*ACE* mRNA	2.9E–04	3.3
4463	*AGT* mRNA	2.5E–03	3.3
4763-3p	*ACE* mRNA	3.9E–05	3.4
4270	*ACE*/*ACE2* mRNA	6.3E–04	4.3
1207-5p	*ACE* mRNA	1.1E–04	4.9
4505	*ACE* mRNA	4.2E–05	7.9
936	***ACE2* mRNA**	**2.7E–03**	**66**

*These were detected using an Agilent miRNA microarray. Data were analysed by robust multi-array analysis. Correction for multiple testing was performed using the Benjamini–Hochberg method. Shown are adjusted P values and fold-change relative to term placentae. Those miRNAs that were confirmed by semi-quantitative RT-PCR are shown in bold.
ACE, angiotensin converting enzyme 1; *ACE*, angiotensin converting enzyme 2; *AGT*, angiotensinogen; *AGTR1*, angiotensin II type 1 receptor; *AGTR2*, angiotensin II type 2 receptor; *ATP6AP2*, (pro)renin receptor; RAS, renin-angiotensin system; *REN*, prorenin.

Table II Human placental miRNAs that have different levels of expression in women with late-onset pre-eclampsia compared with levels measured in term placentae of women without pre-eclampsia*

miRNA expression in late-onset PE placentae relative to term placentae			
HSA-miR-	**Predicted RAS target**	***P*-value**	**Fold change**
892c-3p	*AGT* mRNA	3.6E–10	–76
378c	*REN*, *ACE* mRNA	1.8E–05	–99
514b-3p	*AGT*, *AGTR1* mRNA	5.3E–06	–158

*Detected using an Agilent miRNA microarray. These miRNAs are predicted to target RAS mRNAs. Data were analysed by robust multi-array analysis. Correction for multiple testing was performed using the Benjamini–Hochberg method.
HSA, *homo* sapiens; PE, pre-eclampsia.

between mid-gestation and term placentae or between early and mid-gestation (Fig. 4B).

ACE2 mRNA levels decreased with gestational age ($P = 0.006$). The levels of *ACE2* mRNA in early and mid-gestation placentae were similar, and mid-gestation *ACE2* mRNA expression was greater than *ACE2* mRNA expression at term ($P = 0.005$; Fig. 4C). On the other hand, placental ACE2 protein levels at term were higher than levels in early and mid-gestation ($P = 0.06$ and $P < 0.002$, respectively; Fig. 4D).

Effects of transfection with miR-181a-5p and miR-663 on expression of REN mRNA and prorenin protein in HTR-8/SVneo cells

To determine if there was a causal link between the gestational patterns of expression of placental miR-181a-5p or miR-663, both of

Table III Human placental miRNAs that have different levels of expression in women with late-onset PE compared with early-onset PE*

miRNA expression in late-onset PE relative to early-onset PE placentae			
HSA-miR-	**Predicted RAS target**	***P*-value**	**Fold change**
892c-3p	*AGT* mRNA	3.6E–10	76
670-5p	*AGT* mRNA	2.2E–05	92
378c	*REN*, *ACE* mRNA	1.8E–05	99
514b-3p	*AGT*, *AGTR1* mRNA	5.3E–06	158

*Detected using an Agilent miRNA array. These miRNAs are predicted to target RAS mRNAs. Data were analysed by robust multi-array analysis. Correction for multiple testing was performed using the Benjamini–Hochberg method.

which target renal *REN* mRNA (Marques *et al.*, 2011a), HTR-8/SVneo cells were transfected with vectors containing either miR-181a-5p or miR-663 precursors. Non-transfected cells and cells transfected with scrambled precursors, or with empty vectors, served as controls. Cells that were transfected with either miR-181a-5p or miR-663 had increased expression of miR-181a-5p and miR-663, respectively (Supplementary Fig. S3). The cells also exhibited significantly reduced expression of both *REN* mRNA ($P = 0.05$) and prorenin protein ($P = 0.001$ by Kruskal Wallis test; Fig. 5).

Discussion

The present study was undertaken to determine whether the human placenta expresses miRNAs predicted to target RAS mRNAs and if their expression profile could explain why the placental RAS is highly expressed early in gestation. We showed that a cluster of miRNAs,

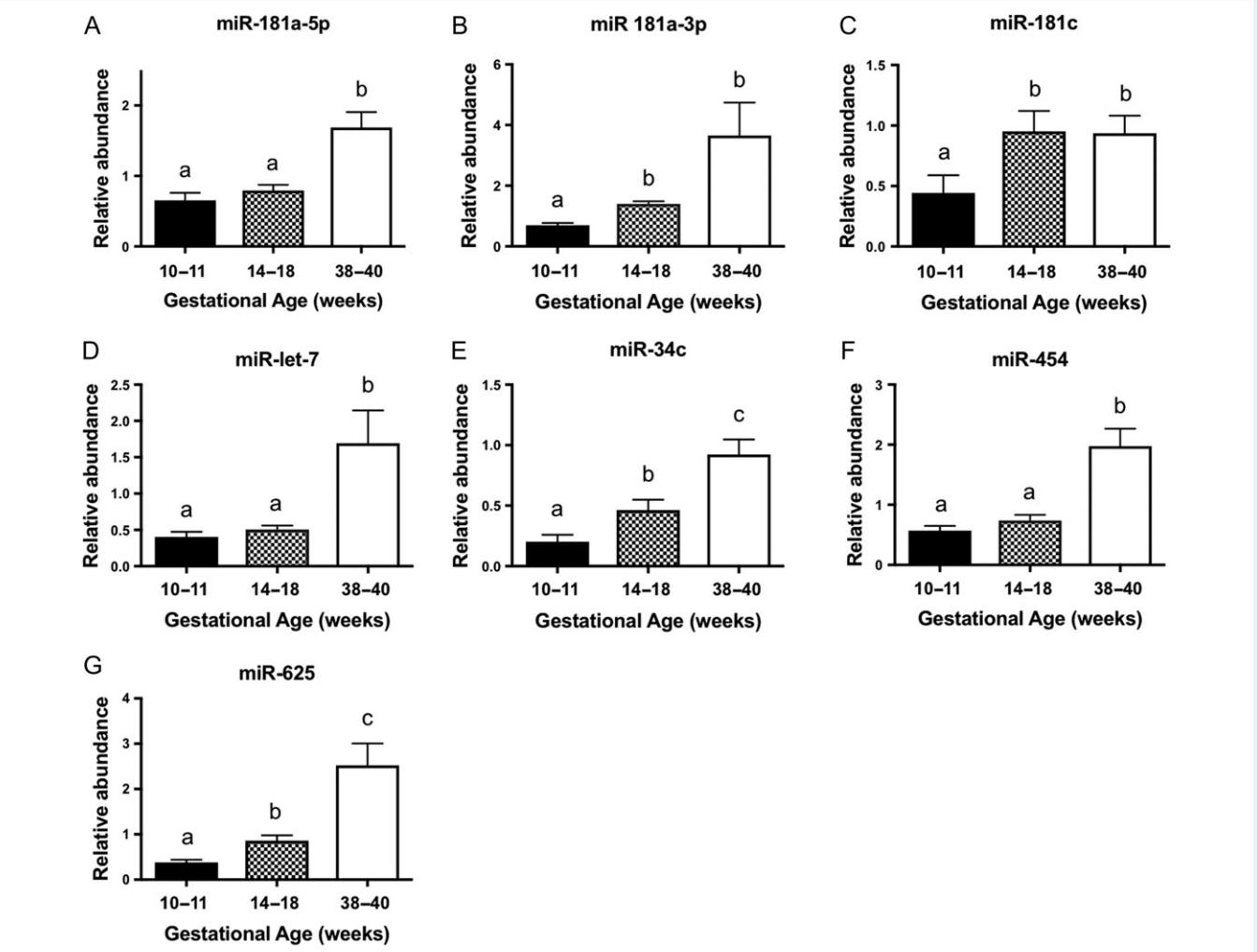

Figure 1 Relative levels across gestation of (**A**) miR-181a-5p, (**B**) miR-181a-3p, (**C**) miR-181c, (**D**) miR-Let-7, (**E**) miR-34c, (**F**) miR-454 and (**G**) miR-625 in human placenta that are predicted to target renin-angiotensin system mRNAs. The placental microRNAs (miRNAs), detected using a miRNA microarray, were found to be significantly lower in early gestation. Different superscripts indicate significant differences between groups (all $P < 0.02$). Data were analysed by the Kruskall Wallis with Dunnett's Multiple Comparison test and are presented as mean ± SEM; $n = 7$–8 placentae per group.

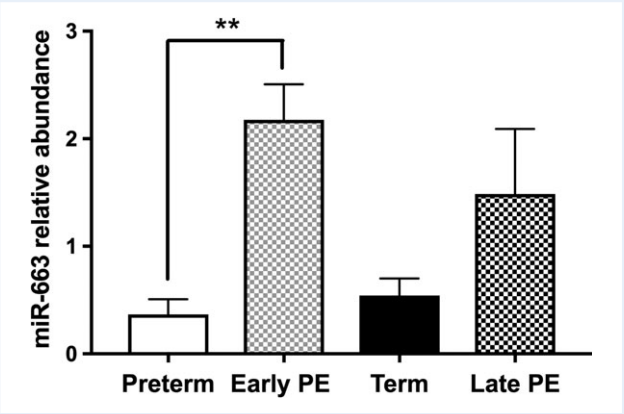

Figure 2 Expression of placental miR-663. Expression of miR-663 was highest in placentae from women with early-onset pre-eclampsia (PE) (Early PE) compared with preterm control (Preterm) placenta (**$P = 0.01$). There was no significant difference in the expression of miR-663 between term control placentae (Term) and placentae from women with late-onset PE (Late PE). Data were analysed by Mann Whitney test and are presented as mean ± SEM; $n = 8$ per group.

not only those that target prorenin mRNA but additional miRNAs targeting other mRNAs in the RAS pathway, have low levels of expression in early-gestation compared with expression at term (Fig. 1). Transfection of HTR-8/SVneo cells with two of these miRNAs, miR-181a-5p or miR-663, which are known to target renal *REN* mRNA, reduced the level of secreted prorenin protein (Fig. 5). We also demonstrated differences in the expression of miRNAs in placentae from women with early or late-onset PE compared with gestational age-matched controls. All of these miRNAs have RAS mRNAs as predicted targets.

In the present study, only *REN* mRNA and prorenin protein levels showed the gestational profile of high early expression and secretion, which might result from early suppression of miRNAs that target the 3′-untranslated region (UTR) of *REN* mRNA. miR-181a-5p and miR-663 are found in the human kidney and are known to destabilize renin mRNA leading to reduced expression levels in transfected kidney cells (Marques *et al.*, 2011a). miR-181a-5p and miR-663 expression in the renal cortex is reduced in non-pregnant hypertensive subjects compared with normotensive subjects (Marques *et al.*, 2011b). Circulating levels of miR-181a-5p correlated with blood pressure (Marques *et al.*, 2015) and suppression of miR-181a-5p by the sympathetic nervous system plays a key role in upregulating renal *REN* mRNA in neurogenic hypertension (Head *et al.*, 2015).

A number of studies have examined the expression of miRNAs in PE (see review: Sheikh *et al.*, 2016). These have identified miR-181a as immune-modulating, but they did not study effects on the RAS as we have (Fig. 5). The unique feature of our study is that we attempted to define the profiles of expression of miRNAs that target the RAS across gestation.

We found that the human placenta contains both miR-181a-5p and miR-663. Each miRNA has been shown to bind to the 3′-UTR of prorenin mRNA and destabilize reporter gene constructs transfected into HEK293 kidney cells in culture (Marques *et al.*, 2011a). Furthermore, we demonstrated that transfection of HTR-8/SVneo cells with miR-181a-5p or miR-663 resulted in overexpression of these miRNAs and reduced the levels of secreted prorenin protein (Fig. 5). These data support our hypothesis that the reduced expression of miR-181a-5p in early gestation placentae (Fig. 2) was driving the increased expression of prorenin mRNA and protein (Fig. 3) at this time.

In contrast, miR-663 was more highly expressed in early and mid-gestation placentae compared to term (Table I and Fig. 2). We also found a higher level of miR-663 expression in placentae from women who had early-onset PE compared with gestational age matched control placentae (Fig. 2). Although transfection of miR-663 into trophoblast cells *in vitro* inhibited prorenin production, its expression in human placenta was not inversely related to the level of expression of *REN* mRNA and prorenin protein. Therefore, its role in regulating placental prorenin expression and production *in vivo* requires further investigation. High levels of miR-663 expression in late gestation placenta could nevertheless be a feature of early-onset PE.

While we did not detect any differences in expression of any miRNAs between early-onset PE and gestational age-matched controls on the array, high levels of miR-663 were detected by qPCR. This may be due to the small sample size used in the array and large variation between samples. No miRNAs were differentially expressed in preterm compared with term placentae. Placentae from women with late-onset PE expressed three miRNAs more abundantly (miR-378a, that targets *REN* mRNA and *ACE* mRNA; miR-514-5p, that targets *AGT* mRNA and *AGTR1* mRNA; and miR-892a, that targets *AGT* mRNA; Table II). These three miRNAs were more highly expressed in late-onset PE placentae compared with early-onset PE. This was also the case for miR-670-5p and miR-1258. In contrast, miR-4323 was lower in late-onset PE placentae compared with early-onset PE. It should be noted that miR-514-5p belongs to the cluster of miRNAs that have been described as 'placenta-specific' (Lagana *et al.*, 2017).

Although we predicted that suppression of miRNAs that target the placental RAS in early-gestation would allow increased expression of prorenin and other mRNAs of the RAS, some of the most marked differences in expression of RAS mRNAs at 6–16 weeks shown previously in human kidney (Pringle *et al.*, 2011), were not evident in the present study. This may have been because the sample size here was smaller or because placentae were collected later in early gestation (10–11 weeks), whereas previously we collected samples at 6–9 weeks of gestation, along with samples at 10–11 weeks and 14–16 weeks (Pringle *et al.*, 2011). Fig. 3 shows that in early gestation, *REN* mRNA expression was, as predicted, higher than at term, as was *ACE2* mRNA. Similarly, *ACE* mRNA was most highly expressed at term and *AGT* and *AGTR1* mRNAs had profiles similar to those described previously (Pringle *et al.*, 2011). The level of *ATP6AP2* mRNA expression in early gestation was not, however, significantly different from term, contrary to our previous study in which it was higher in early gestation (Pringle *et al.*, 2011).

Apart from the very strong correlation between *REN* mRNA and prorenin protein levels ($P < 0.0001$, $rho = 0.9$), no significant correlations were found between the levels of expression of other placental RAS mRNAs and their proteins (data not shown).

There were striking differences in the gestational patterns of *AGT* mRNA and *ACE2* mRNA abundance and placental levels of AGT and ACE2 proteins (Fig. 2). At the end of pregnancy, placental levels of AGT and ACE2 proteins were increased (Figs 2 and 4). However, of the various mRNAs, only *ACE* mRNA levels were higher than levels

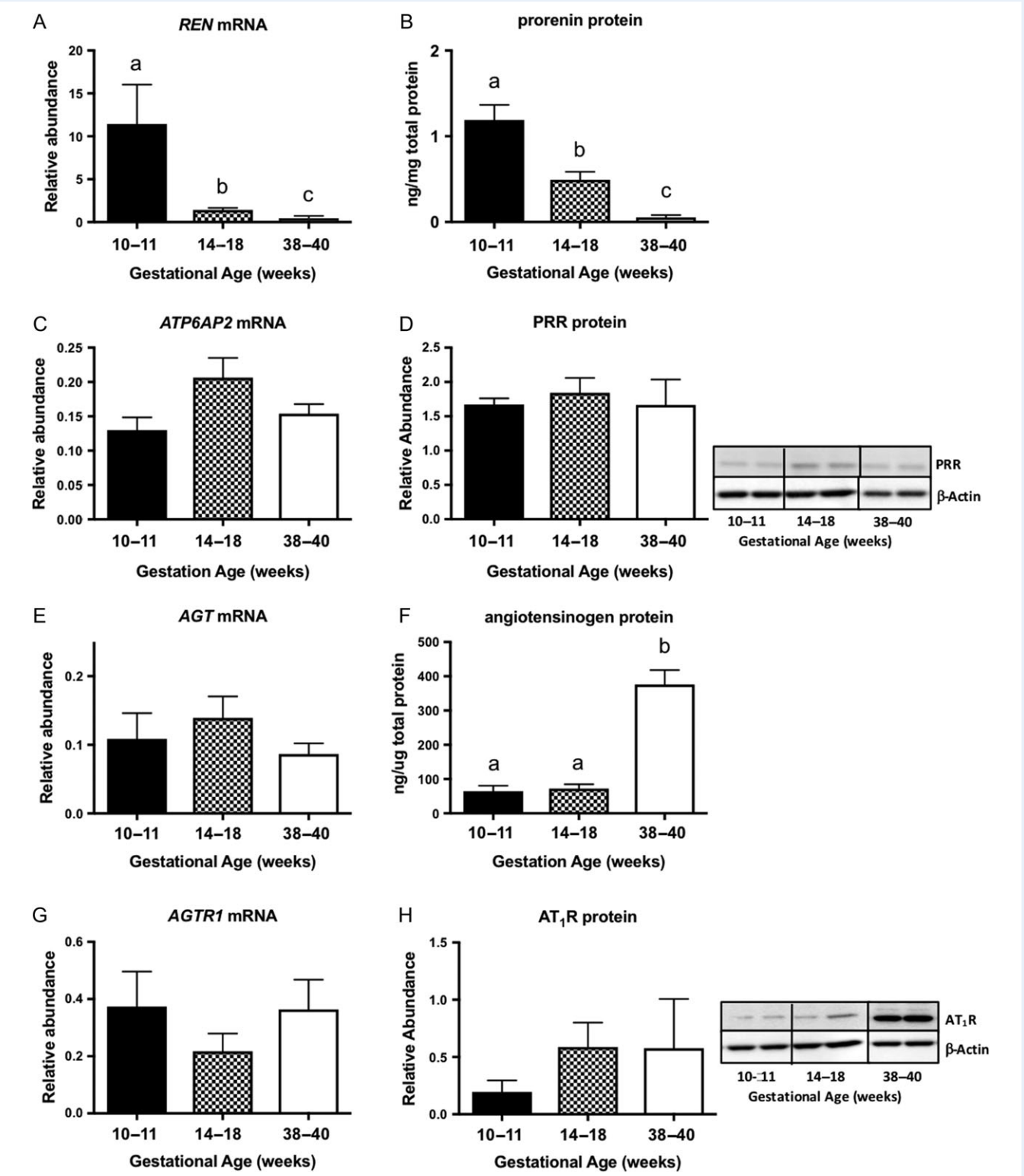

Figure 3 Abundance of different mRNAs and proteins across human gestation. (**A**) Prorenin (REN) mRNA, (**C**) prorenin receptor (ATP6AP2) mRNA, (**E**) angiotensinogen (AGT) mRNA and (**G**) angiotensin II type I receptor (AGTR1) mRNA were measured using RT-qPCR. (**B**) Prorenin and (**F**) angiotensinogen (AGT) protein levels were measured by ELISA, (**D**) prorenin receptor (PRR) and (**H**) angiotensin II type I receptor (AT_1R) proteins were measured by western blot. Representative western blots of proteins quantified by this method are shown. The AT_1R antibody produced two bands, one at ~42 kDa and one at ~55 kDa. The predicted band size was ~40 kDa. Therefore, the band detected at 42 kDa was ascribed as being AT_1R. Different superscripts indicate significant differences between groups (all $P < 0.02$). Data were analysed by Kruskall Wallis with Dunnett's Multiple Comparison test and are presented as mean ± SEM; $n = 7$–8 placentae per group.

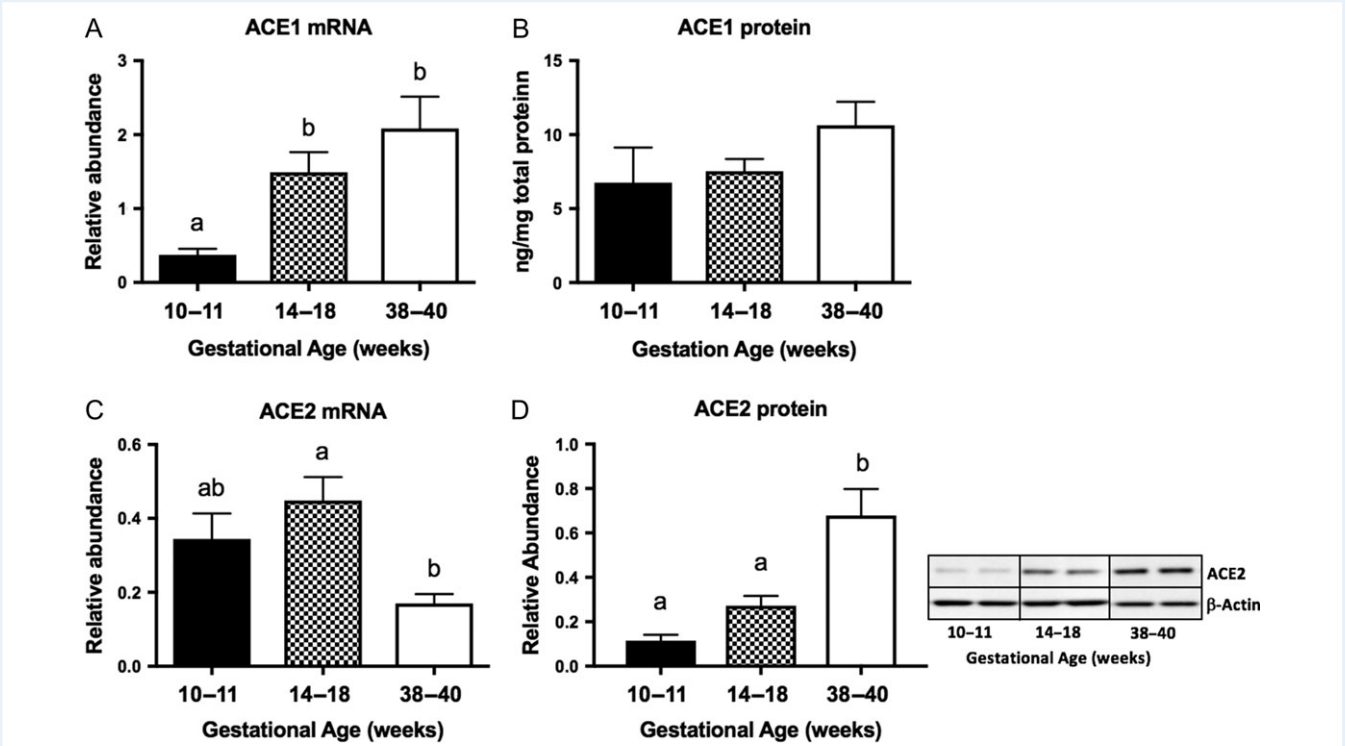

Figure 4 Levels of the mRNAs encoding (**A**,**B**) angiotensin converting enzyme 1 (ACE1) and (**C**,**D**) ACE2 and of the proteins across human gestation. Angiotensin converting enzyme 1 (ACE1) protein levels were measured by ELISA, ACE2 protein was measured by western blot. Representative western blots of ACE2 proteins quantified by this method are shown. Different superscripts indicate significant differences between groups (all $P < 0.02$). Data were analysed by Kruskall Wallis with Dunnett's Multiple Comparison test and are presented as mean ± SEM; $n = 7$–8 placentae per group.

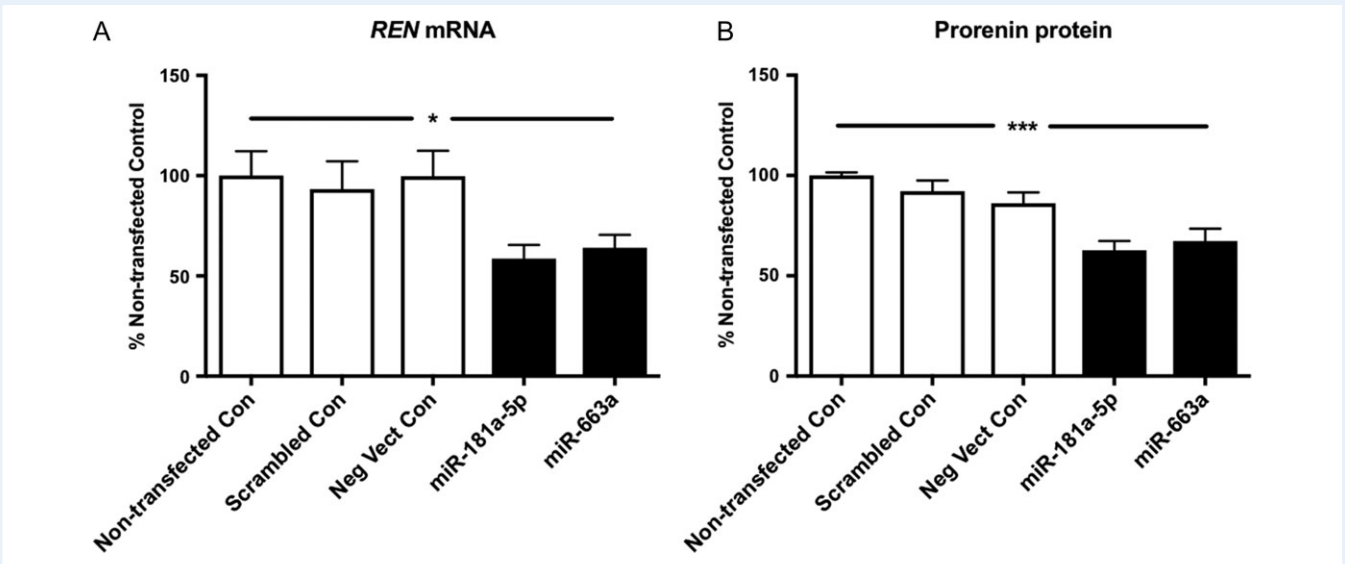

Figure 5 HTR-8/SVneo cells that expressed miR-181a-5p or miR-663 had a reduced level of both (**A**) *REN* mRNA and (**B**) prorenin protein. Data were analysed by Kruskall Wallis with Dunnett's Multiple Comparison test and are presented as mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$; $n = 3$ in triplicate. CON: control, Neg Vect: negative vector.

seen in early gestation. Circulating AGT levels increase throughout pregnancy (Skinner *et al.*, 1972) and it is possible that AGT protein is taken up from the maternal circulation by the syncytiotrophoblast. Very dense staining of AGT in the syncytiotrophoblast of late gestation placentae has been described (Marques *et al.*, 2011b). A soluble form of ACE2 (sACE2) has been shown to be a biomarker for systolic dysfunction and hypertension (Epelman *et al.*, 2008; Uri *et al.*, 2014). It is not known if levels of sACE2 are increased in pregnancy or if sACE2 is taken up by the placenta. We cannot therefore explain the disconnection between the levels of *ACE2* mRNA expression and its protein.

The most definitive evidence for control of the placental RAS by miRNAs concerns data for placental *REN* mRNA. Levels of renin in first trimester human gestational sacs (measured by an enzyme kinetic assay) are extremely high (Itskovitz *et al.*, 1992), and prorenin levels measured in the present study by an ELISA were ~24-fold lower at term compared with levels of prorenin in early gestation placentae (Fig. 3). Enzyme kinetic assays have, moreover, shown that placental total renin levels at term are ~88-fold lower than levels in amniotic fluid (Skinner *et al.*, 1972). No other RAS mRNAs or proteins show such marked changes across human gestation. Levels of prorenin, particularly in early gestation, are sufficiently high to enable hydrolysis of Ang I from angiotensinogen after binding to the (P)RR (Batenburg *et al.*, 2011).

The changes in placental renin with gestation are probably not solely a result of altered expression of miRNAs that target *REN* mRNA. In a previous study, we showed that expression of *REN* mRNA and secretion of prorenin protein was stimulated in a primary trophoblast cell line by cAMP, by global hypomethylation using 5′-AZA-2′-deoxycytidine, and by sex steroids (medroxyprogesterone acetate and 17-β-oestradiol) (Wang *et al.*, 2013). Global hypomethylation combined with cAMP was more effective at stimulating *REN* mRNA expression than either treatment alone (Wang *et al.*, 2013). It is not known if any of these factors cause the changes in human placental *REN* mRNA levels across gestation we observed here or if they are mediated through effects on expression of placental miRNAs. Both placental *REN* mRNA and prorenin protein levels show a strong negative correlation with placental angiotensinogen, suggesting that, in addition, a negative feedback mechanism operates within the RAS of this tissue, and that this may presumably be mediated by Ang II (Johns *et al.*, 1990).

A full understanding of the roles of miRNAs in regulating the expression of placental prorenin across gestation requires further validation of the placental RAS as a target for placental miRNAs. A limitation of the present study is the small sample size. Therefore, these findings will require validation in a larger, independent, cohort. To determine a role for these miRNAs in the aetiology of the shallow placentation that has been suggested to predispose to PE (McMaster *et al.*, 2004), we need to validate the RAS mRNA targets of other miRNAs detected in our study, determine their sensitivity to oxygen and determine whether any of them are released into maternal plasma and whether this may affect the maternal RAS. If the miRNAs that we have found to target placental RAS mRNAs in the present study are released into the maternal circulation, and are 'placental specific' – that is, they are only expressed by the placenta, such as is the case for miR-514-5p (Lagana *et al.*, 2017) – then these miRNAs could potentially be used as circulating biomarkers of placental pathologies. Placenta-specific and placenta-derived miRNAs have been noted in patient plasma and serum as biomarkers of placental dysfunction, having been released mostly by syncytiotrophoblasts as exosomes (Luo *et al.*, 2009; Hromadnikova *et al.*, 2013, 2017). Levels of these circulating miRNAs are also known to be altered in women with PE, and thus trophoblast-derived circulating miRNAs are the most likely valuable biomarkers (Donker *et al.*, 2012; Hromadnikova *et al.*, 2013, 2017; Sarker *et al.*, 2014; Tsochandaridis *et al.*, 2015).

In conclusion, we have shown that miRNAs that target renal *REN* mRNA also target placental *REN* mRNA, and that there are differences in the levels of expression of miRNAs in early and late-onset PE. Whether or not these findings can be translated into the development of useful biomarkers for early detection of PE remains to be determined and will also depend on measurement of levels of various RAS components in maternal plasma throughout pregnancy.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* Online.

Authors' roles

Y.W.: Substantial contributions to design of study, acquisition of data, analysis and interpretation of data, in revising draft article for content and in final approval of the version submitted for publication.

E.R.L.: Substantial contributions to conception and design, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content and in final approval of the version to be published.

A.L.A.: Involved in acquisition of data and analysis and interpretation of data, in revision of drafted article for intellectual content and in final approval of the version to be published.

C.C.d.M.: Involved in acquisition of data, data analysis and interpretation of data.

A.M.: Involved in acquisition of data, analysis and interpretation of data, critical revision of draft article for important intellectual content and final approval of the version to be published.

K.A.A.-K.: Involved in acquisition of data, analysis and interpretation of data, in critical revision of draft article for important intellectual content and in final approval of the version to be published.

C.T.R.: Contribution to conception; involved in revision of draft article and in final approval of the version to be published.

F. B. P.: Contribution to conception; Involved in revision of draft article and in final approval of the version to be published.

F.Z.M.: Contributions to conception and design and in acquisition of data. Involved in revision of draft article and in final approval of the version to be published.

B.J.M.: Contributions to conception and design, analysis and interpretation of data, in revising drafts of article critically for important intellectual content and in final approval of the version submitted for publication.

K.G.P.: Substantial contributions to conception and design, analysis and interpretation of data in revising drafts of article critically for important intellectual content and in final approval of the version to be published.

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Conflict of interest

None declared.

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