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Molecular characterization of renin-angiotensin system components in human intrauterine tissues and fetal membranes from vaginal delivery and cesarean section

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Abstract

A prorenin-angiotensin system (RAS) could, via the (pro)renin receptor (ATP6AP2), have various effects in human intrauterine tissues, either directly by prorenin/ATP6AP2 cell signaling, or indirectly via angiotensin II and/or angiotensin 1-7. Here we describe RAS components in fetal membranes, decidua and placenta collected at elective cesarean section (non-laboring), after spontaneous delivery (after labor, n=38), and in myometria (n=16) from elective (non-laboring) or emergency cesarean (laboring) deliveries. Angiotensinogen *(AGT),* angiotensin-converting enzyme 1 and 2 *(ACE; ACE2),* angiotensin receptor 1 and 2 *(AGTR1; AGTR2)* and angiotensin 1-7 receptor *(MAS1)* mRNAs were measured by qRT-PCR and proteins were localized by immunohistochemistry. In myometrium, prorenin (*REN)*, *ATP6AP2*, and downstream signaling proteins zinc finger and BTB domain-containing protein 16 (*ZBTB16*), transforming growth factor-β1 (*TGFβ1*) and prostaglandin-endoperoxide synthase 2 *(PTGS2)* mRNAs were also measured. RAS mRNAs, except *AGTR1* and *AGTR2*, were abundant in decidua and lowest in amnion compared to the other tissues. *ACE, AGT* and *PTGS2* mRNAs were higher in laboring than non-laboring myometrium, suggesting that the myometrial RAS is involved in labor. Angiotensinogen and prorenin staining in amnion, chorion and decidua was pervasive despite their mRNAs being low in amnion and chorion. In placenta, prorenin, angiotensinogen and AGTR2 were present in syncytiotrophoblasts, ACE was in fetal endothelium, while ACE2 distribution was diffuse. *AGTR1* and *AGTR2* mRNAs and proteins were abundant. No differences were evident in the staining patterns with labor. These results are consistent with the hypothesis that fetal vascular ACE might contribute angiotensin II to the fetus, whilst syncytial ACE2 might hypothetically have a role in converting angiotensin II to angiotensin 1-7 in maternal blood.

Keywords: renin-angiotensin system; pregnancy; labor; cesarean; gene expression.

Introduction

Although only the kidney secretes active renin, its precursor prorenin is produced by a variety of tissues. Very high levels of prorenin-like activity are found in human amniotic fluid, decidua, chorion and placenta [1]. Although the decidua is the major site of prorenin production in intrauterine tissues [2], lower prorenin (*REN*) mRNA has been identified in late gestation placenta, amnion and chorion [3]. The (pro)renin receptor (ATPase H^+-transporting lysosomal accessory protein 2: ATP6AP2) has been described in placenta [4] and recently in other late gestation intrauterine and fetal tissues [3]. Since prostaglandin-endoperoxide synthase 2 (PTGS2, also known as COX-2 or PGHS-2) is upregulated in human decidua by renin [5] and in the kidneys of rats transgenic for ATP6AP2 [6], it is reasonable to propose that the renin-angiotensin system (RAS) might be involved in labor, possibly through effects on PTGS2.

By interacting with ATP6AP2, prorenin can directly stimulate cell signaling, activating ERK1/ERK2 [4] and heat shock protein (HSP)27/p38 pathways [7], and cause increased expression of transforming growth factor (TGFβ1) [8]. Prorenin bound to ATP6AP2 also causes translocation of the zinc finger and BTB domain-containing protein 16 (ZBTB16, also known as PLZF) to the nucleus, where it represses transcription of *ATP6AP2* [9].

Binding of prorenin to ATP6AP2 activates it and catalyses the formation of angiotensin I (Ang I) from angiotensinogen (AGT). Ang I is converted to angiotensin II (Ang II), by angiotensin converting enzyme (ACE). Ang II can bind to one of two receptors (AGTR1 and AGTR2) or it can be converted by ACE2 to angiotensin 1-7 (Ang 1-7) [10]. Ang II, acting via AGTR1 and AGTR2, has a wide range of potential actions that are often opposite [11,

12]. Ang 1-7 acting via a MAS protooncogene receptor (MAS1) has opposite actions to Ang II on vascular growth and causes a reduction in Ang II-induced ERK1/2 activities [13].

Thus, the human intrauterine RAS could have a large number of biological actions depending on which pathways in the cascade predominate within a particular tissue. We have described the distribution of prorenin previously and showed that ATP6AP2 is expressed ubiquitously throughout late gestational tissues [3]. One aim of the present study was to characterize which RAS pathways are predominant in fetal membranes, decidua and placenta.

The factors controlling human labor are complex and although the role of the intrauterine RAS in the onset of labor has not been studied extensively, it is known that active renin can stimulate prostaglandin E (PGE) synthesis by amnion and decidual cells in culture, independent of Ang II [5, 14]. Prostaglandins (PGs) are key hormones involved in human labor [15]. There is a positive correlation between decidual *REN* and amnion *PTGS2* mRNA expression [3] and between *PTGS2* and *ATP6AP2* mRNA levels in human amnion after labor [3], supporting the involvement of the intrauterine RAS in control of amniotic *PTGS2* expression.

Nothing is known about the expression of RAS genes in term human myometrium and the effects of labor. In the heart it is well established that stretch induces AGT expression, and that the RAS is intimately involved in myocardial hypertrophy and contractility [16]. The interaction of prorenin with ATP6AP2 in neonatal cardiomyocytes activates the p38/HSP27 pathway [7], which affects the assembly of β-actin filaments. Phosphorylation of HSP27 occurs during contraction of human myometrial strips [17]. Therefore, since a myometrial RAS could potentially influence myometrial growth and contractility, a further aim of the

present study was to identify the molecular components of the RAS cascade in myometrium collected before and during labor.

Materials and Methods

Tissues

Fetal membranes (amnion and chorion), decidua and placenta were collected from normal pregnancies at term (37–41 weeks gestation), with delivery either by elective cesarean section in the absence of labor (non-laboring; n=18), or after spontaneous vaginal delivery (after labor; n=20). Samples of myometrium were obtained at elective cesarean section (non-laboring; n=8) or at cesarean section carried out as an emergency procedure during labor (laboring; n=8). Exclusion criteria included women treated with non-steroidal anti-inflammatory drugs, or having a history of infection, chorioamnionitis or asthma, or undergoing labor induction. Informed consent was obtained from all participants, as approved by the Hunter Area Research Ethics Committee and the University of Newcastle Human Research Ethics Committee.

Fetal membranes and decidua were dissected at least 1 cm away from the adjacent placenta. Amnion was peeled from the chorio-decidua and the chorion laeve was separated from the decidua by sharp dissection. Biopsies of the placental bed were taken from 3–4 different sites of each placenta. Myometrial samples were taken adjacent to the lower segment incision for cesarean sections. Samples were collected within 30 minutes of delivery. For RNA analyses, tissues were snap frozen and crushed in liquid nitrogen and 0.2 g of tissue was used for subsequent RNA extraction. For immunohistochemical analysis, strips of full thickness membranes (amnion, chorion and decidua), dissected away from the adjacent

placenta and cervix, were fixed in 4% paraformaldehyde overnight, before embedding in paraffin.

Quantitative real-time RT-PCR (qPCR)

Total RNA was extracted from intrauterine tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Samples were purified and treated with DNase on spin columns (Qiagen). RNA was reverse transcribed using a SuperScript III RT-kit with random hexamers (Invitrogen). qPCR was performed in a Corbett Research Rotor-Gene 3000 Real Time PCR System (Corbett Life Science) using SYBR Green for detection. Each reaction contained 12 µl of Express SYBR-Greener master mix (Invitrogen), primers (listed in Table 1), cDNA (10 ng reverse-transcribed total RNA) and water to 25 µl. The primers for *AGTR2* have been described previously [18]. Samples were assayed in triplicate. Cycling conditions were as follows: incubation at 50ºC for 2 minutes, followed by 95ºC for 5 minutes, and 45 cycles of 15 seconds at 95ºC, 30 seconds at the annealing temperature (Table 1) and 30 seconds at 72ºC. mRNA abundance was calculated relative to β-actin (*ACTB*) mRNA using the ΔCT method. Comparisons of mRNA abundance were made by incorporating a calibrator sample in each run and determining relative abundance as 2-ΔΔCT [19]. HeLa cell cDNA was used as the calibrator for all RAS mRNAs in amnion, chorion, decidua and placenta, except for AGTR2 mRNA, where HL60 cell cDNA was used as the calibrator. For myometrial samples, mRNA abundance was calculated relative to *ACTB* mRNA and relative abundance determined as 2-ΔCT. Dissociation curves, to detect non-specific amplification, were generated for all reactions, and no-template control samples were included in all assays. The predicted sizes of the PCR products were verified by agarose gel electrophoresis (data not shown).

Immunohistochemistry (IHC)

Four µm thick formalin-fixed paraffin embedded sections were deparaffinized, and antigen retrieval for prorenin, ATP6AP2, ACE, AGT, AGTR1 and AGTR2 was performed using a microwave oven with Epitope Retrieval Solution™ pH 6.0 (Novacastra) for 10 min. No antigen retrieval was required for ACE2. Antibodies used were: ACE (Novacastra, NCL-CD143), ACE2 (Abcam, ab15348), AGT (R&D Systems, AF3156), AGTR1 (Abcam, ab9391), renin propeptide (R&D Systems, MAB4447), ATP6AP2 (Abcam, ab64957) and AGTR2 (Abcam, ab78747). Positive control tissue included normal human small intestine, basal forebrain, kidney and heart as well as fetal guinea pig heart. Matched samples without the use of the primary antibody were used as negative controls. Sections were blocked with 2% skim milk powder in Tris-buffered saline (TBS) and then incubated for 1 h with primary antibody. Immunostaining was performed on a Bond-XTM automated immunostainer (Vision BioSystems) with the Bond Polymer Refine Detection System (Leica MicroSystems) consisting of polymer conjugated anti-mouse/rabbit secondary antibody and diaminobenzidine (DAB, brown) as the chromogen. Images were captured and analyzed using the Aperio Scanscope XT slide scanner (Aperio Technologies).

Data analyses

Data were tested for normal distribution using the skewness and kurtosis test. Non-normally distributed data were logarithmically transformed to achieve normal distribution. Independent sample *t*-tests (with unequal variances, 2-tailed) were used to compare data for the laboring, non-laboring and after labor groups. Between-tissue comparisons were carried out by one-way analysis of variance on ranks, followed by correction for multiple testing using the Bonferroni post-hoc test, to determine significance of differences between the groups.

Pearson correlations were calculated for bivariate normally distributed data and Spearman correlations were determined for non-normally distributed data. The SPSS statistical package (SPSS for Windows, Release 17.0.0. Chicago) was used for the analyses. Significance was set at $P<0.05$.

Results

RAS gene expression in fetal membranes, decidua and placenta and differences associated with labor

Messenger RNAs for *ACE, ACE2 AGT, AGTR1* and *AGTR2* were found in amnion, chorion, decidua and placenta (Figure 1). *MAS1* mRNA was very low to undetectable in these tissues (data not shown).

In amnion, the abundance of all RAS mRNAs, except *AGTR2*, were lower than in the other tissues. Specifically, *ACE* and *ACE2* mRNA levels were lower than placental or decidual levels ($P<0.001$; Figures 1A and B), *AGT* and *AGTR1* mRNAs levels were lower than in all other tissues ($P<0.001$, Figures 1C and D). Only in amnion were there any differences in mRNA abundance with labor, in that *ACE* mRNA abundance was lower ($P=0.014$; Figure 1A) and *AGTR2* abundance higher ($P=0.010$; Figure 1E) relative to the non-laboring samples.

In chorion, *ACE* and *ACE2* mRNA abundance was low relative to decidua and placenta ($P<0.001$; Figures 1A and B). *AGTR2* mRNA abundance, however, was over 3 times higher in chorion than in decidua (Figure 1E; $P=0.030$).

Decidua had high levels of *ACE, ACE2* and *AGT* mRNAs, but *AGTR1* and *AGTR2* mRNA levels were low (Figures 1D and E). The placenta had the highest levels of AGTR1 and AGTR2 mRNA ($P<0.001$) compared to all other tissues.

We noted a significant positive correlation between *ACE* and *ACE2* mRNA levels in amnion (r=0.64, $P<0.001$; Figure 2A). This correlation was stronger when the analysis was confined to just non-laboring samples (r=0.84, $P<0.001$; Figure 2B). A positive correlation between *ACE* and *AGTR1* mRNA in placenta was noted as well (r=0.40, $P=0.013$; Figure 2C). The correlation was seen in both non-laboring and after labor placenta samples.

RAS expression and changes with labor in myometrium

Prorenin mRNA (*REN*) and that for its receptor (*ATP6AP2*), as well as *AGT, ACE, ACE2, AGTR1, AGTR2* and *MAS1* mRNAs were all present in the myometrium (Figure 3A and B). Messenger RNAs for genes involved in signaling pathways affected by prorenin binding to its receptor, i.e. *ZBTB16, TGFB1* and *PTGS2* were also present (Figure 3B). Laboring myometrium had higher levels of *ACE* ($P=0.033$) and *AGT* ($P=0.021$) mRNAs compared to non-laboring myometrium. *PTGS2* mRNA abundance was also increased in laboring myometrium ($P=0.022$).

Localization of RAS proteins in the fetal membranes, decidua and placenta

Immunohistochemical localization of prorenin, ATP6AP2 [3], ACE, ACE2, AGT, AGTR1 and AGTR2 proteins in placenta and full-thickness membrane sections are shown in Figure 4. The density of immunostaining of prorenin, AGT, AGTR1 and AGTR2 was similar in amnion, chorion and decidua (Figure 4; left hand panel). This is in contrast to the respective levels of mRNAs in these three tissues (Figures 1B, C and D) [3]. In placenta, prorenin AGT and AGTR2 showed strong immunostaining in the syncytiotrophoblast layer of the placental

villi. ACE was located only in fetal capillary endothelial cells, while ACE2 and AGTR1 were present in both sites (Figure 4). Immunostaining was not seen in the negative controls, which contained no primary antibody. No differences in localization were seen between non-laboring and after labor samples (data not shown).

Discussion

We have described and compared, for the first time, the levels of *ACE*, *ACE2*, *AGT*, *AGTR1, AGTR2* and *MAS1* mRNAs in human fetal membranes, decidua and placenta from non-laboring and after labor deliveries, as well as in non-laboring and laboring myometrial samples. We have also localized prorenin, ACE, ACE2, AGT, AGTR1 and AGTR2 proteins in fetal membranes, decidua and placenta. RAS components in the placenta and endometrium/decidua have been reported previously [18, 20-23], but little attention has been paid to their distribution across membranes and in the myometrium. Furthermore, the distribution of the more recently discovered components of the RAS (*ATP6AP2*, *ACE2* and *MAS1*) and labor-associated changes in their expression have not been examined.

We detected higher *AGT*, *ACE* and *PTGS2* mRNA levels in laboring compared to non-laboring myometrium (Figure 3). This is consistent with a possible influence of locally produced Ang II in myometrium during spontaneous labor.

AGTR2 mRNA and protein were detected in all intrauterine tissues (Figures 1E and 4). The high levels of *AGTR2* mRNA in amnion and chorion is not surprising since AGTR2 is abundant in fetal tissues of other species [24]. Since expression of *AGTR2* occurs in adult tissues when there is cellular damage [11], perhaps the high levels of *AGTR2* mRNA in after

labor fetal membranes reflects a tissue response to the inflammatory processes associated with labor.

Previously, we showed that among the gestational tissues, decidua had the greatest abundance of *REN* mRNA [3]. Here we demonstrate that the decidua also seems to be the major site of *ACE*, *ACE2* and *AGT* production. Fetal membranes contained lower levels of the RAS mRNAs studied, apart from *ATP6AP2 and AGTR2*. Since the staining of proteins in the fetal membranes and decidua could be compared directly in full thickness membrane sections (Figure 4) it appeared that levels of AGT and AGTR1 proteins in amnion and chorion were higher than expected considering the low levels of their mRNAs in these tissues relative to decidua (Figure 1). It is possible that the decidua could supply prorenin and AGT proteins to amnion and chorion. Alternatively it is possible that prorenin and *AGT* mRNA levels are independent of their final protein concentrations due to translational or post-translational regulation. ACE and ACE2 protein abundances were in accordance with their mRNA levels (Figure 4). The early finding of high levels of renin-like activity in amnion and chorion [1] agrees with the intensity of prorenin staining we saw using IHC (Figure 4).

Amnion is an avascular tissue and the role of ACE in this tissue is unknown. Expression of *ACE* mRNA and protein was low in both amnion and chorion, despite its presence in the nearby decidua (Figure 4). There was, however, a strong positive correlation between *ACE* and *ACE2* mRNA levels in amnion, especially in non-laboring samples (Figure 2, 2B). This might indicate counter-balancing effects between the peptide products of these two enzymes (Ang II and Ang 1-7, respectively) [25]. Any action of Ang 1-7 might involve, however, receptor(s) other than MAS1, since *MAS1* mRNA was not detected in fetal membranes.

The higher *ACE* mRNA in laboring myometrium (Figure 3A) is in accord with data from genome-wide gene expression arrays [26]. The increase in *AGT* and *ACE* mRNA in this

tissue at labor could generate increasing amounts of Ang II and promote myometrial cell hypertrophy in late gestation [27]. A similar mechanism has been described in the myocardium, where stretch upregulates both *AGT* and *ACE* [28] and Ang II induces markers of cardiac hypertrophy, including *TGFβ1* [29], which is abundant in the myometrium (Figure 3B). The increase in *AGT* and *ACE* expression in laboring myometrium (Figure 3A) could also affect labor by upregulating PTGS2, which is associated with the onset of term and preterm labor (Figure 3B) [30, 31], in agreement with previous studies [32, 33]. It is also possible that prorenin might be acting through Ang II-independent pathways. Prorenin, via the prorenin receptor and HSP27/p38 pathways, influences assembly of actin filaments within cardiac myocytes independently of Ang II [7]. Interestingly, the phosphorylation status of HSP27 changes substantially in the human myometrium at labor [17], but the involvement of the prorenin/ATP6AP2 system in this regulatory phenomenon remains to be explored.

REN and *AGT* mRNAs are expressed in the term placenta [3] (Figure 1) and prorenin and AGT protein are abundant in the syncytial layer (Figure 4). Perhaps these proteins are taken up by the placenta from the maternal circulation. Despite a claim that prorenin is secreted from the uteroplacental unit into the maternal circulation [34], a study of ACE activity in venous maternal and cord blood did not find any statistical difference between laboring and non-laboring groups at term [35], consistent with our mRNA results showing no labor-related differences in *ACE* in decidua and placenta. Exposure to syncytial ACE2 could also generate RAS peptides, including Ang 1-7, which could then enter the maternal circulation or have effects locally. Alternatively, the presence of ACE2 in the syncytiotrophoblast may indicate a role for this enzyme independent of the RAS. In the intestine, the neutral amino acid transporter SLC6A19 (B^0AT1) requires ACE2 coexpression for full activity [36, 37]. At this

time there is no literature relating the co-expression of B^0AT1 and ACE2 in the human placenta, but a role for ACE2 in placental amino acid transport is an intriguing possibility.

The differences in location of RAS proteins lead us to propose that different local RASs are operating within the placenta. In contrast to ACE2, ACE protein was exclusively localized to fetal endothelium (Figure 4). It has been reported that plasma ACE levels are higher in infants compared to their mothers [35]. Because ACE activity in the lung is low until birth or stimulation by cortisol [38], we suggest that fetal placental endothelial ACE may contribute to fetal Ang II levels through conversion of fetal Ang I to Ang II. Therefore, during intrauterine life, maintenance of fetal Ang II levels may depend upon such placental conversion. The correlation between *ACE* and *AGTR1* mRNA levels in placenta and the high abundance of AGTR1 immunoreactive protein in fetal endothelium are also consistent with the possible vasoconstrictor action of Ang II in fetal vessels. Indeed, human umbilical venous Ang II levels are 5–8 times the normal adult levels at vaginal delivery [39].

In conclusion, we have shown for the first time that the majority of RAS components are expressed in fetal (amnion and chorion), maternal (decidua and myometrium) and placental tissues in term pregnancies. Particularly intriguing was the localization of ACE, ACE2 and AGTR2 within the placenta, suggesting the existence of an endothelial and syncytial RAS and/or an alternative role for ACE2 in amino acid transport [36, 37]. The labor-associated changes in *ACE*, *AGT* and *PGTS2* in myometrium, and of *ACE* and *AGTR2* in amnion indicates a role of a local RAS in labor and provide impetus for further research.

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Figure 1

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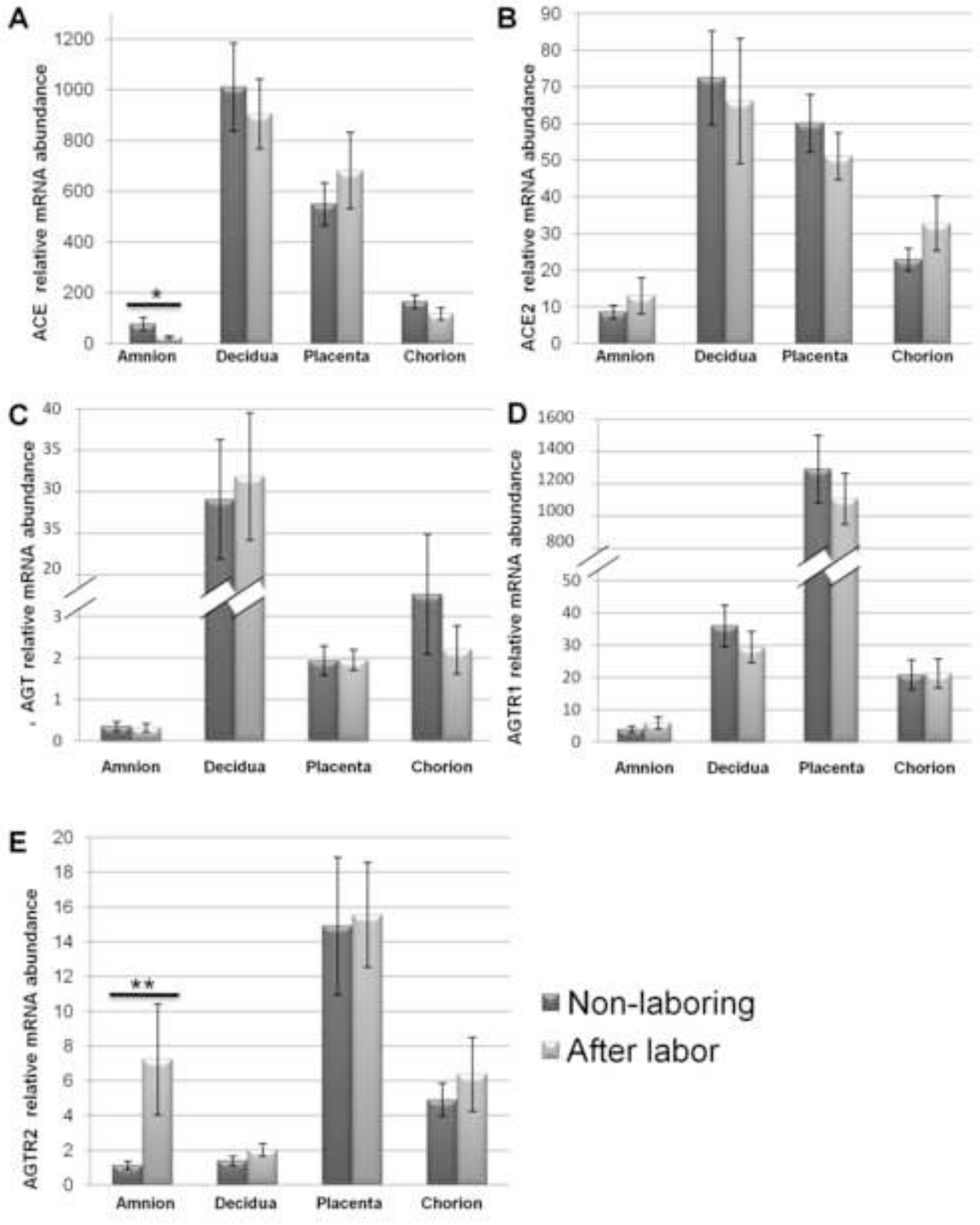

Figure 2

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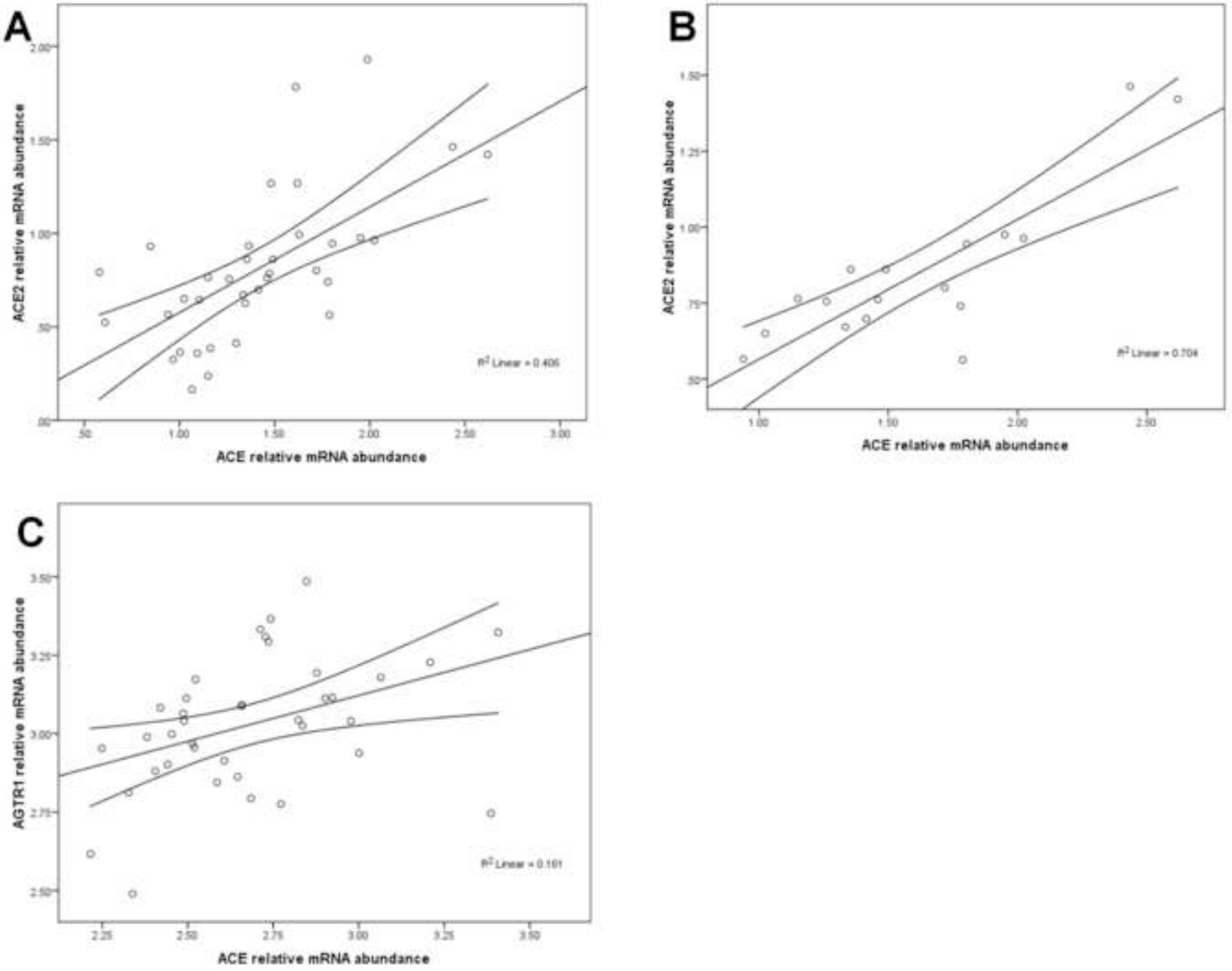

Figure 3

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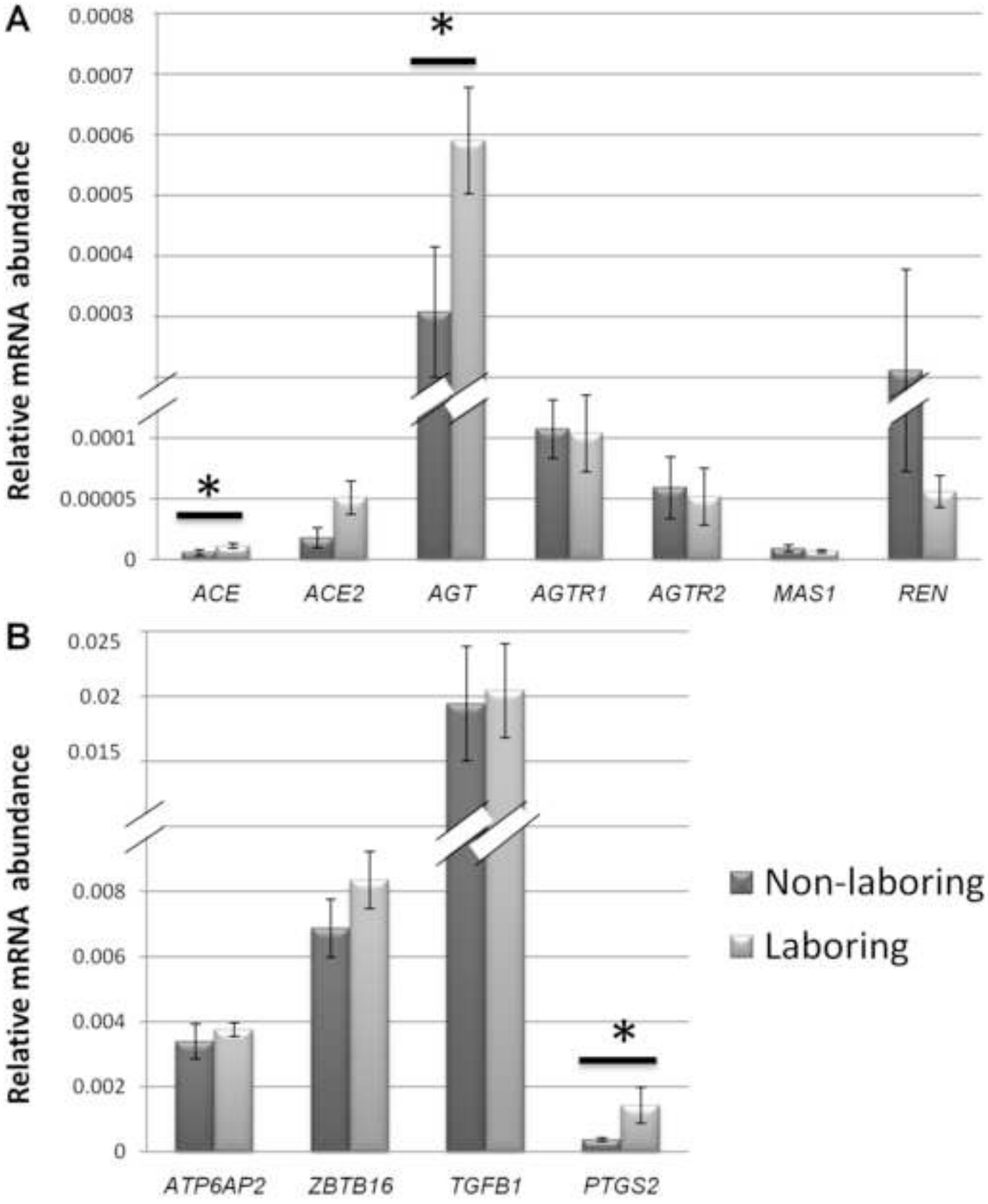

Figure 4

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