Effects of Fetal Sex on Expression of the (Pro)renin Receptor and Genes Influenced by its Interaction With Prorenin in Human Amnion

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Abstract

Males are more likely to be born preterm than females. The causes are unknown, but it is suggested that intrauterine tissues regulate fetal growth and survival in a sex-specific manner. We postulated that prorenin binding to its prorenin/renin receptor (ATP6AP2) would act in a fetal sex-specific manner in human amnion to regulate the expression of promyelocytic zinc finger, a negative regulator of ATP6AP2 expression as well as 2 pathways that might influence the onset of labor, namely transforming growth factor β 1 (TGFB1) and prostaglandin endoperoxide synthase 2 (PTGS2). Our findings demonstrate that there are strong interactions between prorenin, ATP6AP2, and TGFB1 and that this system has a greater capacity in female amnion to stimulate profibrotic pathways, thus maintaining the integrity of the fetal membranes. In contrast, glucocorticoids or other factors independent of the prorenin/prorenin receptor pathway may be important regulators of PTGS2 in human pregnancy.

Keywords

amnion, prorenin, prorenin receptor, transforming growth factor, prostaglandin endoperoxide synthase 2

Introduction

There are a number of reports on the effects of fetal sex on fetal and neonatal morbidity and mortality. Male babies have an increased risk of adverse pregnancy outcomes including premature rupture of membranes (PROM), spontaneous preterm birth,¹⁻⁴ and delivery by cesarean section.^{3,5,6} In addition, there is a greater incidence of spontaneous abortions,⁷ miscarriages later in pregnancy,⁸ and stillbirths.^{9,10}

Very high levels of prorenin have been found in human amniotic fluid, decidua, chorion, and placenta,¹¹ and the decidua is the major site of prorenin production in the pregnant uterus.^{12,13} The expression and secretion of prorenin by decidua are sexually dimorphic. Decidua from women carrying female fetuses express renin messenger RNA (mRNA; *REN*) and secrete prorenin to a much greater extent than do decidua from women carrying male fetuses.¹⁴ Expression of *REN* in chorion and amnion is very low,¹³ so it is most likely that the high levels of renin-like enzyme activity found in human amnion and amniotic fluid¹¹ and the abundance of renin protein in human amnion¹³ are the result of decidual secretion of prorenin.

A prorenin/renin receptor ((pro)renin receptor, also called ATPase H⁺-transporting lysosomal accessory protein or ATP6AP2) that binds to and reversibly activates prorenin has

been described in term placenta¹⁵ and other late gestation intrauterine and fetal tissues.¹³ The identification of this receptor and its ability to interact with prorenin means that prorenin can have biological activity within the conceptus without being processed proteolytically to active renin. Binding of prorenin to the (pro)renin receptor can directly stimulate intracellular signaling or prorenin can be activated within the receptor complex to cleave angiotensin I (Ang I) from angiotensinogen (AGT).¹⁵ The (pro)renin receptor stimulates the production of transforming growth factor β 1 (TGF- β 1) as well as other profibrotic molecules, independent of the formation of Ang II.¹⁶⁻¹⁹ We

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Figure 1. Proposed mechanism by which the prorenin/(pro)renin receptor can regulate labor. Binding of prorenin to the (pro)renin receptor can stimulate transforming growth factor $\beta 1$ (TGF- $\beta 1$), which in turn stimulates fibronectin, plasminogen activator (PAI-1), and collagen to promote fibrosis and maintain the integrity of the fetal membranes, thus preventing labor onset. Alternatively, the prorenin–prorenin receptor interaction may also upregulate prostaglandin endoperoxide synthase 2 (PTGS2, or COX-2), which stimulates prostaglandin synthesis to induce labor onset.

postulate that prorenin–(pro)renin receptor interactions within amnion could also promote fibrosis, thus playing a role in maintaining the integrity of the amnion and reducing the risk of PROM and preterm labor (Figure 1).

Alternatively, prorenin–(pro)renin receptor interactions could stimulate expression of amniotic prostaglandin endoperoxide synthase 2 (PTGS2, commonly known as COX-2), the key enzyme of prostaglandin biosynthesis and therefore may be involved in the induction of labor (Figure 1). PTGS2 is upregulated in human decidua by renin,²⁰ in amnion explants by prorenin,¹³ and in the kidneys of rats transgenic for the (pro)renin receptor.²¹

Prorenin signaling through the (pro)renin receptor can also activate a negative feedback pathway whereby the transcription factor promyelocytic zinc finger protein (PLZF, also known as zinc finger and BTB domain-containing protein 16; *ZBTB16*) is translocated to the nucleus where it represses transcription of the (pro)renin receptor (*ATP6AP2*).²² Interestingly, expression of *ZBTB16* is upregulated by progesterone and glucocorticoids in endometrial and myometrial cells.²³ Glucocorticoids are important regulators of prostaglandin production and dexamethasone, a synthetic glucocorticoid, inhibits PTGS2 in amnion explants.²⁴ It is unknown however what role PLZF plays in this process. It may be that PLZF is also implicated in PTGS2 production in the amnion and that this is regulated by the interactions between prorenin and the (pro)renin receptor.

Since the expression and secretion of prorenin by the maternal decidua are sexually dimorphic and interactions between

prorenin and the (pro)renin receptor can stimulate factors known to be important in influencing the onset of labor, we aimed to observe whether there were any sex-specific differences in the expression of the (pro)renin receptor gene and potential downstream targets of the prorenin–(pro)renin receptor interaction, namely *PTGS2*, *TGF β 1*, and *ZBTB16* in late-gestation amnion explants. In addition, we aimed to determine whether dexamethasone or other factors present in amniotic fluid influenced (pro)renin receptor (*ATP6AP2*), PLZF (*ZBTB16*), or PTGS2 (*PTGS2*) mRNA abundance in amnion explants.

Methods

Amnion Explant Culture

Amnion was collected from uncomplicated singleton pregnancies at term (37–41 weeks gestation), delivered by elective cesarean section in the absence of labor ($N = 11$, for reasons including breach presentation or previous cesarean section), and fetal sex recorded. Women treated with nonsteroidal anti-inflammatory drugs, or having a history of infection, chorioamnionitis, or asthma, or undergoing labor induction were excluded. 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 and amnion was peeled from the choriodecidua.²⁵ Amnion membrane was then cut into approximately 2-cm² pieces and distributed as 0.5-g portions. Each portion was then washed briefly in sterile phosphate-buffered saline and incubated in 25 mL Dulbecco modified Eagle medium (DMEM)/F12 medium (DMEM/F12 supplemented with 15 mmol/L HEPES, 1.2 g/L NaHCO₃, 1 mg/mL L-glutathione reduced, 0.1 g/L albumin fraction V, 0.65 μ g/mL aprotinin, and 40 μ g/mL gentamicin) with 0, 5, or 50 ng/mL recombinant human prorenin (Cayman Chemical Company, Ann Arbor, Michigan). These concentrations were chosen because in maternal plasma the prorenin concentration ranges between 1 and 8 ng/mL in healthy pregnancies,²⁶ and in amniotic fluid, prorenin concentrations are reported to be up to 10 times higher than in maternal plasma at term.^{27,28} Amnion explants were incubated for 0, 0.5, 4, 16, or 24 hours at 37°C. In separate experiments, amnion explants ($n = 3$; 2 females and 1 male) were also treated with amniotic fluid (pooled sample was collected from 5 to 7 women delivering by elective cesarean section in the absence of labor, approximately half of these women were carrying a male fetus and half were carrying a female fetus). Alternatively, amnion explants ($n = 4$; 3 females and 1 male) were treated with 100 μ mol/L dexamethasone for 24 hours and compared with vehicle-treated controls (25 μ L dimethyl sulfoxide in 25 mL media). After incubation, the tissues were removed from the medium, blotted, and frozen in liquid nitrogen until extraction of RNA. Zero-hour samples were frozen immediately without incubation.

Table 1. Primers Used for Quantitative Real-Time Polymerase Chain Reaction.

Official Gene Symbol	GenBank Accession No.	Primer Sequence (5'→3')	Concentration, $\mu\text{mol/L}$
<i>ACTB</i>	NM_001101	Fw: CGCGAGAAGATGACCCAGAT Rv: GAGTCCATCACGATGCCAGT	200
<i>ATP6AP2</i>	NM_005765	Fw: CCTCATTAGGAAGACAAGGACTATCC Rv: GGGTTCCTTCGCTTGTTTTGC	200
<i>PTGS2</i>	NM_000963	Fw: GAATCATTACCCAGGCAAATTG Rev: TCTGTACTGCGGGTGGAACA	400
<i>TGFB1</i>	NM_000660	Fw: CAAGGGCTACCATGCCAACTT Rv: CCGGGTTATGCTGGTTGTACA	600
<i>ZBTB16</i>	NM_001018011	Fw: TAGGGTGCACACAGGTGAGA Rv: GTGCAGATGGTGCACCTGGTA	200

Abbreviations: Fw: forward primer; *PTGS2*, prostaglandin endoperoxide synthase 2; Rv: reverse primer; *TGFB1*, transforming growth factor B1; *ZBTB16*, zinc finger and BTB domain-containing protein 16.

Quantitative Real-Time Reverse Transcription–Coupled Polymerase Chain Reaction

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. Samples were purified and DNase treated on spin columns (Qiagen, Hilden, Germany) to eliminate contaminating genomic DNA. Total RNA was spiked with Alien RNA (Stratagene, La Jolla, CA; 10^7 copies/ μg of total RNA), which served as reference RNA for internal standardization.²⁹ The RNA was reverse transcribed using the Superscript III RT-kit with random hexamers (Invitrogen). Quantitative real-time polymerase chain reaction (PCR) was performed in an Applied Biosystems 7500 real-time PCR system using SYBR Green for detection. Each reaction contained 5 μL of SYBR Green PCR master mix (Life Technologies, Carlsbad, California) primers (listed in Table 1), complimentary DNA reversed transcribed from 10 ng total RNA, and water to 10 μL . The primer pairs were designed using Primer Express software (Life Technologies, Carlsbad, California) with 1 primer spanning an exon–exon junction. Messenger RNA abundance was calculated relative to β -actin (*ACTB*) mRNA (for dexamethasone and amniotic fluid experiments) or alien mRNA (for prorenin experiments) using the ΔC_T method. Comparisons of mRNA abundance between PCR runs were made by incorporating a calibrator sample³⁰ in each set of PCR amplifications and determining relative abundance as $2^{-\Delta\Delta C_T}$. Dissociation curves, to check for homogeneity of amplification products, were generated for all reactions, and no-template control samples were included in all assays to confirm the absence of nonspecific amplification products due to primer interactions. The predicted sizes of the PCR products were verified by agarose gel electrophoresis.

Data Analyses

All data were logarithmically transformed to achieve normal distribution. Independent sample *t* tests (with unequal variances, 2-tailed) were used to compare expression levels of target genes in amnion collected from pregnancies carrying a male or female fetus prior to incubation. For incubated amnion,

data were expressed as fold change relative to the corresponding 0-hour sample prior to log transformation and univariate analysis of variance and Bonferroni post hoc analysis was performed to determine the effect of incubation time, fetal sex, and prorenin treatment. Pearson correlations were determined using log-transformed data. For dexamethasone- and amniotic fluid-treated amnion explants, *t* tests (with unequal variances, 2 tailed) were used to compare expression levels of target genes in amnion between treated and nontreated explants incubated for 24 hours. The statistical package, SPSS for Windows, release 22.0.0 (Chicago), was used for the analyses. Significance was set at $P < .05$.

Results

Effect of Fetal Sex and Prorenin Treatment on (Pro)renin Receptor (*ATP6AP2*) mRNA Expression

In freshly isolated amnion, there were no fetal sex-specific differences in the expression of *ATP6AP2* or *TGFB1*. In incubated amnion, however, there was a significant overall effect of fetal sex on *ATP6AP2* mRNA abundance ($P < .001$), where incubated female amnion had greater *ATP6AP2* mRNA levels than male amnion. When separated by fetal sex, we found that there was a significant overall effect of prorenin treatment on *ATP6AP2* mRNA abundance in male amnion ($P = .03$; Figure 2A). Specifically, treatment with 50 ng/mL prorenin resulted in a significant decrease in *ATP6AP2* mRNA abundance compared with vehicle-treated controls ($P < .05$). Treatment with either 5 or 50 ng/mL prorenin had no effect on *ATP6AP2* mRNA expression in female amnion (Figure 2B).

Effect of Fetal Sex and Prorenin Treatment on *TGFB1* mRNA Expression

There was no fetal sex–associated difference in *TGFB1* mRNA levels ($P = .08$). However, when separated by fetal sex, a significant effect of incubation time on *TGFB1* mRNA levels in male amnion was observed ($P = .001$; Figure 2C). In addition, there was a significant overall effect of prorenin treatment ($P = .03$) on *TGFB1* mRNA expression in male amnion

Figure 2. (Pro)renin receptor *ATP6AP2*, transforming growth factor B1 (*TGFB1*), prostaglandin endoperoxide synthase 2 (*PTGS2*), and zinc finger and BTB domain-containing protein 16 (*ZBTB16*) messenger RNA (mRNA) abundance in incubated amnion treated with vehicle (control), 5 or 50 ng/mL recombinant human prorenin. Data are presented as mean $\pm$ standard error mean (SEM) and are expressed relative to baseline (0 hour) amnion mRNA levels (ie a fold change from 1). $N = 5$ male and 6 female amnions. A and B, *ATP6AP2* mRNA abundance in (A) male and (B) female amnions. There was a significant effect of fetal sex on *ATP6AP2* mRNA expression. *ATP6AP2* mRNA abundance was greater in female amnion but in male amnion prorenin treatment (50 ng/mL) was associated with reduced expression of *ATP6AP2* mRNA levels. C and D, *TGFB1* mRNA abundance in (C) male and (D) female amnions. Treatment with 50 ng/mL prorenin was associated with a significant reduction in *TGFB1* mRNA abundance in male amnion explants. No effect of prorenin was seen in female amnion incubations. E and F, Both (A) male and (B) female amnions showed a significant effect of incubation time on *PTGS2* mRNA abundance. There was, however, a significant effect of fetal sex on *PTGS2* mRNA expression and a significant interaction between fetal sex and incubation time. Female amnion incubations increased their expression of *PTGS2* mRNA abundance to a greater extent than male amnion incubations. *PTGS2* mRNA levels in amnion treated with exogenous prorenin were not different from controls in either male or female amnion incubations. G and H, *ZBTB16* mRNA abundance in (C) male and (D) female amnions. Neither fetal sex nor treatment with exogenous prorenin had any effect on *ZBTB16* mRNA levels but there was a significant decrease in *ZBTB16* mRNA expression with incubation time in both male and female amnions.

Figure 3. Correlations between (pro)renin receptor (*ATP6AP2*) and both transforming growth factor B1 (*TGFβ1*) and prostaglandin endoperoxide synthase 2 (*PTGS2*) messenger RNA (mRNA) in incubated amnion. A, There was a significant positive correlation between $\log ATP6AP2$ and $\log TGFβ1$ in incubated amnion ($r = .641$, $P < .001$). When separated by fetal sex, this correlation remained in both male ($r = .395$, $P = .012$) and female ($r = .717$, $P < .001$) amnions. B, There were significant associations between *ATP6AP2* and *PTGS2* ($r = .360$, $P = .001$), which were not evident when correlations were carried out on female and male amnions separately. Pearson correlations were determined using log-transformed data from all time points (0.5, 4, 16, and 24 hours) and all treatments.

incubations. Specifically, treatment with 50 ng/mL prorenin significantly decreased *TGFβ1* mRNA levels compared to controls ($P < .024$; Figure 2C); this was not found with the lower dose of prorenin, and neither prorenin treatment nor incubation time had any effect on *TGFβ1* mRNA abundance in female amnion (Figure 2D).

Since the prorenin–(pro)renin receptor interaction has been reported to stimulate $TGF-β1$ ^{16–19} and given the sex-specific differences in *ATP6AP2* and *TGFβ1* mRNA expressions seen in incubated amnion (Figure 2), we wanted to observe whether there were any sex-specific correlations between *ATP6AP2* and *TGFβ1*. Overall, there was a significant positive association between *TGFβ1* and *ATP6AP2* ($r = 0.641$, $P < .001$; Figure 3A). When separated by fetal sex, it was found that this association held true in both male ($r = 0.395$, $P = .012$) and female ($r = 0.717$, $P < .001$) amnion.

Effect of Fetal Sex, Prorenin Treatment, and Incubation Time on *PTGS2* mRNA Expression

In amnion prior to incubation, there were no sex-specific differences in the expression of *PTGS2*. In incubated amnion, however, there was a significant overall effect of fetal sex on *PTGS2* expression ($P < .001$), where incubated female amnion had greater *PTGS2* mRNA levels than male amnion. In addition, there was a significant effect of incubation time ($P < .001$) on *PTGS2* mRNA expression as well as a significant interaction between fetal sex and incubation time ($P = .004$) such that the increase in *PTGS2* expression with incubation time was greater in female compared with male amnion explants (Figure 2E and F). Prorenin treatment had no effect on *PTGS2* mRNA expression in either male or female amnion. When all data were pooled, there were significant associations between *ATP6AP2* and *PTGS2* ($r = 0.360$, $P = .001$; Figure 3B), which was not evident when correlations were carried out on female and male amnions separately.

Effect of Fetal Sex, Prorenin Treatment, and Incubation Time on *PLZF* (*ZBTB16*) mRNA Expression

Since *PLZF* interacts with the (pro)renin receptor and is a negative regulator of (pro)renin receptor gene expression,²² we wanted to determine whether prorenin treatment altered *ZBTB16* mRNA abundance in amnion explants and whether *ZBTB16* mRNA was negatively correlated with *ATP6AP2*, *TGFβ1*, or *PTGS2* mRNA expression. In incubated amnion, there was a significant negative effect of incubation time on *ZBTB16* mRNA expression ($P < .001$); however, we found no evidence that *ZBTB16* mRNA abundance was influenced by fetal sex or prorenin treatment (Figure 2G and H). We found no significant association between *ZBTB16* and *ATP6AP2* in either male or female amnion incubations. However, we observed significant negative correlations between *ZBTB16* and *TGFβ1* in male amnion ($r = -.441$, $P = .005$) and between *ZBTB16* and *PTGS2* in female amnion ($r = -.313$, $P < .02$).

Effect of Amniotic Fluid and Dexamethasone on (Pro)renin Receptor (*ATP6AP2*), *PLZF* (*ZBTB16*), and *PTGS2* mRNA Expression

The strong decrease in *ZBTB16* and increase in *PTGS2* mRNA levels in incubated amnion suggest that there may be a *PTGS2* inhibitory factor normally present in vivo, possibly in amniotic fluid that is washed out in the in vitro environment allowing *PTGS2* to be expressed, perhaps through a reduction in *PLZF*. Indeed, amnion incubated in the presence of amniotic fluid showed a significant decrease in *PTGS2* levels at 24 hours compared to those cultured in media alone ($P = .027$; Figure 4A); this was accompanied by a significant increase in *ZBTB16* mRNA levels ($P = .007$; Figure 4B).

Since glucocorticoids are known to increase *PLZF* in endometrial and myometrial cells²³ and are important regulators of prostaglandin production in amnion, we hypothesised that it was glucocorticoids in amniotic fluid that regulate *ZBTB16* and

Figure 4. Effect of amniotic fluid and dexamethasone on prostaglandin endoperoxide synthase 2 (*PTGS2*) and zinc finger and BTB domain-containing protein 16 (*ZBTB16*) messenger RNA (mRNA) abundance in amnion after 24 hours. A, Amniotic fluid significantly reduced *PTGS2* mRNA expression in amnion explants after 24 hours ($P = .027$). B, This was accompanied by a significant increase in *ZBTB16* mRNA expression ($P = .007$). C, Treatment with dexamethasone (100 $\mu\text{mol/L}$) significantly reduced *PTGS2* mRNA expression in amnion explants after 24 hours ($P = .001$). D, This was accompanied by a significant increase in *ZBTB16* mRNA expression ($P < .001$). Data are presented as mean $\pm$ standard error mean (SEM) and are expressed relative to baseline (0 hour) amnion mRNA levels (ie, a fold change from 1). $N = 3$ amnion for Figure 2A and B (2 females and 1 male), $N = 4$ amnion for Figure 2C and D (3 females and 1 male). *indicates $P < .05$.

PTGS2 expressions in amnion. Amnion cultured in the presence of the synthetic glucocorticoid dexamethasone showed a significant reduction in *PTGS2* mRNA expression compared to controls after 24 hours of incubation ($P = .001$; Figure 4C), and this was accompanied by a significant increase in *ZBTB16* mRNA abundance ($P < .001$; Figure 4D). Importantly, neither amniotic fluid nor dexamethasone influenced *ATP6AP2* mRNA expression (data not shown).

Discussion

We aimed to determine whether there were any sex-specific differences in the expression of pathways that are known to be influenced by the interactions of prorenin with its (pro)renin receptor in human amnion and to observe whether any differences in their response to treatment with exogenous prorenin could help explain the high prevalence of PROM and preterm birth of male infants. We have recently demonstrated that decidual prorenin expression and secretion are much greater in decidua from women carrying a female fetus compared to decidua from women carrying a male fetus.¹⁴ In addition, we have found that there is a positive correlation between decidual *REN* and amnion *ATP6AP2* mRNA levels.¹³ Based on these findings, we proposed that the expression of *ATP6AP2* in the amnion may be regulated in a fetal sex-specific manner and that this sexual dimorphism in both decidual prorenin secretion

and amniotic (pro)renin receptor levels might underpin a functional role for pathways controlled by the prorenin–(pro)renin receptor in maintaining the integrity of the amnion or in the induction of labor. The 2 pathways we were particularly interested in were those that lead to increased production of *TGF β 1* because this molecule, through stimulation of collagen, fibronectin, and PAI-1, promotes fibrosis and may play a role in maintaining the integrity of the amnion, and those that stimulate prostaglandin synthesis (ie *PTGS2*), which plays an active role in induction of labor.

Here we demonstrate that in incubated amnion, there were sex-specific effects on *ATP6AP2* mRNA expression and the mRNA of its potential downstream targets, namely, *TGF β 1* and *PTGS2*. In addition, we have provided evidence that male and female amnions respond differently to treatment with exogenous prorenin in vitro. Specifically, prorenin treatment of male amnion decreased *ATP6AP2* and *TGF β 1* mRNA abundance (Figure 2). This effect was not seen in female amnion.

The reason why *ATP6AP2* expression was lower in male amnion than in female amnion might be related to the high levels of expression of *ZBTB16* at 0.5 hour of incubation in male amnion (Figure 3A). PLZF is known to translocate to the nucleus and causes repression of *ATP6AP2* transcription.²² However, we observe no sex-specific effect of fetal sex in *ZBTB16* mRNA expression and no significant correlation between *ATP6AP2* and *ZBTB16* mRNA expressions in either male or female amnion

explants suggesting that in amnion at least, PLZF may not be regulating (pro)renin receptor expression.

Expressions of *ATP6AP2* and *TGFB1* mRNA were highly correlated in both male and female amnion explants. Treatment with 50 ng/mL prorenin in male amnion, however, resulted in significant decreases in both *ATP6AP2* and *TGFB1* mRNA expressions (Figure 2). The association between *TGFB1* and *ATP6AP2* mRNA and the effect of prorenin on both *ATP6AP2* and *TGFB1* mRNA expressions suggest that interactions between the decidual prorenin/amnion (pro)renin receptor might influence the integrity of the amnion through regulation of expression of *TGFB1*. In the kidney, there is strong evidence that this pathway is “profibrotic”¹⁷; the same may be the case in the amnion. There is some evidence that this pathway may be more active in female amnion because of the increased production of prorenin by the “female” decidua¹⁴ and the increased expression of the (pro)renin receptor in incubated female amnion (Figure 2B). This pathway could therefore play a role in protecting the female fetus from PROM and premature delivery.

We also postulated that *PTGS2* and PLZF (*ZBTB16*) mRNA expressions would be influenced by interactions between prorenin and the (pro)renin receptor in a sex-specific manner; however, in this study, we observed no effect of prorenin on *PTGS2* or *ZBTB16* mRNA expression and no significant correlations between *ATP6AP2* and either *PTGS2* or *ZBTB16* mRNA expressions. Importantly, although we found a significant spontaneous increase in *PTGS2* mRNA expression and a decrease in *ZBTB16* mRNA levels with incubation time in both male and female amnions. Both *ZBTB16* and *PTGS2* expressions are however influenced by factors other than prorenin. PLZF is upregulated in endometrial and myometrial cells by progesterone and glucocorticoids²³ and *PTGS2* is inhibited by glucocorticoids.²⁴ Therefore, in our amnion explants, the decline in expression of *ZBTB16* and increase in the expression of *PTGS2* with time could be related to the withdrawal of these steroid hormones.

To test this hypothesis, we incubated amnion in the presence of amniotic fluid (which contains a variety of factors including glucocorticoids) or dexamethasone alone. We found that neither amniotic fluid nor glucocorticoids influenced (pro)renin receptor mRNA expression, but they significantly inhibited the spontaneous increase in *PTGS2* mRNA abundance and prevented the decrease in *ZBTB16* mRNA expression that was observed with incubation time (Figure 3). This suggests that glucocorticoids or other factors in amniotic fluid besides prorenin and its interaction with the prorenin receptor are important regulators of *PTGS2* in human pregnancy. The functional role of PLZF in amnion is yet to be determined.

In conclusion, in late gestation, when a woman is carrying a female fetus, her decidua expresses and secretes more prorenin and there are strong interactions between prorenin, the (pro)renin receptor, and TGF- β 1. Therefore, this system has a greater capacity in female amnion to stimulate profibrotic pathways and maintain the integrity of the fetal membranes, thus protecting against PROM and preterm labor. More research is needed

however to investigate whether this pathway and other profibrotic molecules (collagen, PAI-1, and fibronectin) play a functional role in the fetal sex-specific regulation of membrane integrity and whether this pathway is dysregulated in women with preterm PROM. In this study, we also demonstrated that there is no interaction between prorenin, the (pro)renin receptor, and *PTGS2*. We have however provided evidence to support the novel hypothesis that glucocorticoids stimulate PLZF in amnion in order to suppress *PTGS2* during pregnancy possibly preventing the onset of labor. We suggest that the relatively low prevalence of preterm birth of female babies may be due to the sustained exposure of their amnion to high levels of decidual prorenin. If this is the case, then perhaps production of prorenin by decidua in women carrying female babies has to be “switched off” for labor to occur. Interestingly, after labor, prorenin expression is suppressed in these decidua to levels seen in decidua from male pregnancies.¹⁴ Thus, our data further support the hypothesis that sexually dimorphic differences in pregnancy outcome are related to sexually determined differences in the biology of the intrauterine membranes.

Declaration of Conflicting Interests

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