

## Evidence for a Spatial and Temporal Regulation of Prostaglandin-Endoperoxide Synthase 2 Expression in Human Amnion in Term and Preterm Parturition

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**Context:** Prostaglandin-endoperoxide synthase 2 (PTGS2) is a key enzyme involved in parturition. PTGS2 mRNA was found to be differentially expressed between placental amnion (amnion overlying the placental disc) and reflected amnion (amnion of the extraplacental chorioamniotic membranes) in term placentas.

**Objective:** The aim was to evaluate the spatial and temporal regulation of PTGS2 expression in the amnion and the chorion-decidua.

**Design:** PTGS2 expression was analyzed in the amnion and chorion-decidua obtained from 32 women: term not in labor ( $n = 12$ ), term in labor ( $n = 12$ ), and preterm labor ( $n = 8$ ), by immunoblotting and densitometry. Prostaglandin  $E_2$  ( $PGE_2$ ) in the amnion and chorion-decidua was measured by a specific immunoassay.

**Results:** Compared to preterm labor cases, PTGS2 expression increased at term before the onset of labor far more prominently in placental amnion (4.5-fold;  $P = 0.002$ ) than in reflected amnion (1.4-fold;  $P = 0.007$ ). There was a significant increase in PTGS2 expression in reflected amnion (2.9-fold;  $P < 0.01$ ) but not in placental amnion with labor at term. PTGS2 expression was higher in reflected amnion than in chorion-decidua in labor at term (2.9-fold;  $P < 0.01$ ). PTGS2 was barely detected in amnion and chorion-decidua with preterm labor. Expression of  $PGE_2$  showed a good correlation with PTGS2 expression ( $r = 0.722$ ;  $P < 0.001$ ).

**Conclusion:** PTGS2 expression in the amnion shows a distinct spatial and temporal regulation. Spontaneous labor at term and pathological preterm labor clearly differ in amniotic PTGS2 and  $PGE_2$  abundance. Our observations underscore the biological significance of the amnion and amniotic fluid in human parturition. (*J Clin Endocrinol Metab* 95: E86–E91, 2010)

Prostaglandins play a critical role in the initiation of human labor. The expression pattern of prostaglandin-endoperoxide synthase 2 (PTGS2; cyclooxygenase 2), a key enzyme in the synthesis of prostaglandins, by chorioamniotic membranes, decidua, and myometrium has

been subject to investigation (1–3). The expression of PTGS2, but not PTGS1, in the amnion and chorion-decidua is thought to be important for parturition at term (4). Preterm labor associated with intraamniotic infection is also characterized by dramatic elevation of amniotic

fluid concentration of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and PGF<sub>2α</sub> (5). In conjunction with this, *in vitro* experimental studies have shown consistent and marked up-regulation of PTGS2 expression in amnion cells, trophoblasts, and decidual cells by proinflammatory cytokines such as IL-1β and TNFα (6–8).

We have recently shown that PTGS2 mRNA expression is significantly higher in the placental amnion (the amnion overlying the placental disc) than in the reflected amnion (the amnion of extraplacental chorioamniotic membranes) (9). This intriguing difference in the spatial PTGS2 mRNA expression pattern led us to postulate that it might be an important feature of human parturition. This study was conducted 1) to evaluate the spatiotemporal regulation patterns of amniotic PTGS2 expression in human pregnancy in the context of advancing gestational age and the presence of labor; and 2) to compare PTGS2 and PGE<sub>2</sub> expression between the amnion and the chorion-decidua.

## Materials and Methods

### Tissue samples

Placental amnion, reflected amnion, and chorion-decidua tissues were obtained as previously described (9) and flash-frozen using liquid nitrogen. The tissue samples were obtained from the following groups of patients: 1) women at term not in labor (TNL; n = 12); 2) women at term in labor (TIL; n = 12); and 3) women with spontaneous preterm labor (PTL) without clinical and histological chorioamnionitis (n = 8). Three cases in the PTL group were delivered by cesarean section due to a nonreassuring fetal heart rate pattern during labor, and five PTL cases were delivered vaginally. The samples were stored at –80 C until use. All patients provided written informed consent, and the Institutional Review Board of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Department of Health and Human Services approved the collection and use of materials for research purposes.

### Protein extraction

Each tissue was liquid nitrogen-pulverized using a mortar and pestle, and protein was isolated using T-PER Tissue Protein Extraction Reagents with a protease inhibitor cocktail (Thermo Scientific, Rockford, IL). Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Scientific).

### Immunoblotting

Ten micrograms of proteins were electrophoresed in 10% SDS-PAGE gels under nonreducing conditions and transferred onto a polyvinylidene difluoride membrane. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20, incubated with 1:250 diluted mouse monoclonal anti-Cox-2 antibody (clone no. 29; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at room temperature, and then probed with peroxidase-conjugated antimouse IgG (1:5000; Santa Cruz Biotechnology, Inc.). Membranes were probed

with a murine monoclonal anti-β-actin antibody (Sigma-Aldrich Co., St. Louis, MO) as a loading control. Chemiluminescent signals were detected using ChemiGlow West reagents (Alpha Innotech Corporation, San Leandro, CA). The PTGS2/β-actin ratio was analyzed by FluorChem SP densitometry using Alpha-Ease FC Software (version 4.1.0; Alpha Innotech Corporation).

### ELISA

For the measurement of PGE<sub>2</sub> content, 6.25 μg of protein lysates (5 μl) from the amnion and chorion-decidua were added to 95 μl of assay buffer for immunoassay, respectively. Meclofenamic acid (10 μg/ml; Sigma-Aldrich Co.) was added to all samples. The assays were done using the Prostaglandin E<sub>2</sub> Enzyme Immunoassay Kits (Assay Designs, Inc., Ann Arbor, MI). The interassay coefficient of variation was 8.1%, and the intraassay coefficient of variation was 6.7%.

### Immunohistochemistry

To determine PTGS2 expression patterns, immunohistochemistry was performed in TNL (n = 3), TIL (n = 3), and PTL (n = 3) cases. Formalin-fixed, paraffin-embedded, 5-μm-thick sections of the chorioamniotic membranes were placed on silanized slides and stained using a Ventana Discovery automatic staining system (Ventana Medical Systems, Tucson, AZ). Immunostaining was performed using 1:50 diluted, mouse monoclonal anti-Cox-2 antibody (clone no. 29; Santa Cruz Biotechnology, Inc.).

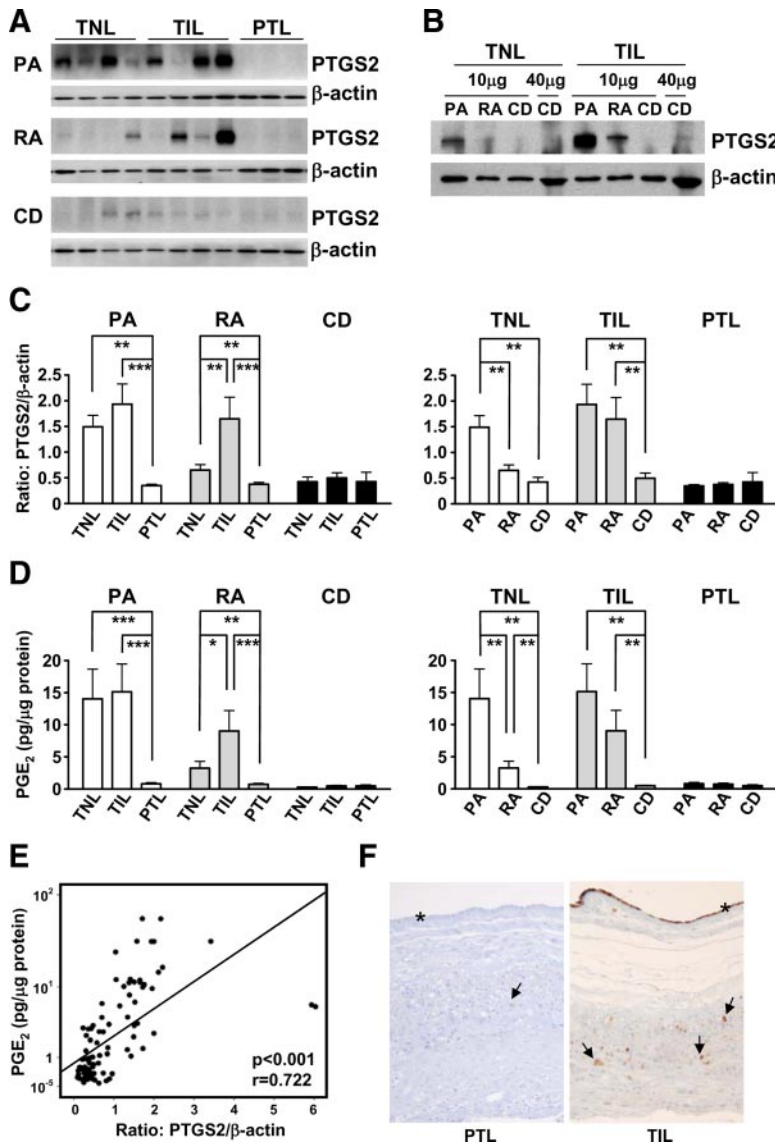
### Statistical analysis

Kruskal-Wallis tests were used to test for differences among the groups. When differences existed, Mann-Whitney *U*-tests were used to investigate which pairs were significantly different. The median protein expressions among placental amnion, reflected amnion, and chorion-decidua in each group were compared using Friedman tests and Wilcoxon signed ranks tests. The correlation between the expression of PTGS2 and PGE<sub>2</sub> was tested using the Spearman rho test. SPSS version 15.0 (SPSS Inc., Chicago, IL) was employed. All *P* values are two-sided, and *P* values of less than 0.05 were considered to be significant.

## Results

The median gestational ages of TNL, TIL, and PTL cases were 39 wk (range, 38–40 wk), 39 wk (range, 37–40 wk), and 32 wk (range, 28–34 wk), respectively. A distinct feature of PTGS2 expression observed in immunoblotting was its relative abundance in the amnion compared with the chorion-decidua (Fig. 1, A and B).

Densitometric analysis of PTGS2 expression for TNL cases showed higher expression of PTGS2 in the placental amnion than in the reflected amnion [median PTGS2/β-actin ratio, 1.44 (range, 0.23–3.42) *vs.* 0.48 (range, 0.31–1.51); *P* < 0.01] (Fig. 1, A and C). PTGS2 expression in placental and reflected amnion at TNL was 4.5-fold (*P* = 0.002) and 1.4-fold (*P* = 0.007) higher, respectively, than in those with PTL. Interestingly, increases in PTGS2 expression were more prominent in reflected amnion than in



**FIG. 1.** Expression patterns of PTGS2 and PGE<sub>2</sub> in the chorioamniotic membranes (amnion and chorion-decidua) obtained from term and preterm pregnancies. A, Immunoblotting data showing characteristic differences in PTGS2 expression among TNL, TIL, and PTL cases. PTGS2 abundance in the placental amnion (PA) at term is in stark contrast with placental amnion obtained from PTL cases. Increase in PTGS2 expression is more prominent in reflected amnion (RA) than in placental amnion after the onset of labor at term. PTGS2 expression is barely detected in the chorion-decidua (CD) of all groups studied. B, Additional immunoblotting data confirming relative robustness of PTGS2 expression in the amnion compared with the chorion-decidua in TNL and TIL cases. PTGS2 is more abundant in 10  $\mu$ g amnion proteins compared with 40  $\mu$ g chorion-decidua proteins. C, Densitometric analysis data of PTGS2/ $\beta$ -actin ratio obtained from immunoblotting experiments show significant increases of PTGS2 expression in the placental amnion at term compared with PTL cases. Increase in PTGS2 expression is also more prominent in reflected amnion than in placental amnion with labor at term. The graphs show means and SE. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . D, Comparisons of PGE<sub>2</sub> concentrations in each tissue type and according to the context of labor. The graphs show means and SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . E, A scatter plot showing good correlations between PTGS2/ $\beta$ -actin ratio and PGE<sub>2</sub> abundance in the tissues ( $r = 0.722$ ;  $P < 0.001$ ). F, Immunostaining for PTGS2 in the chorioamniotic membranes. Very faint PTGS2 immunoreactivity is found in chorionic trophoblasts (arrow) of a PTL case. PTGS2 immunoreactivity is strong and diffuse in amnion epithelial cells \* of a TIL case. Amnion mesenchymal cells and scattered chorionic trophoblasts (arrows) also show distinct immunoreactivity, whereas decidual expression of PTGS2 is minimal.

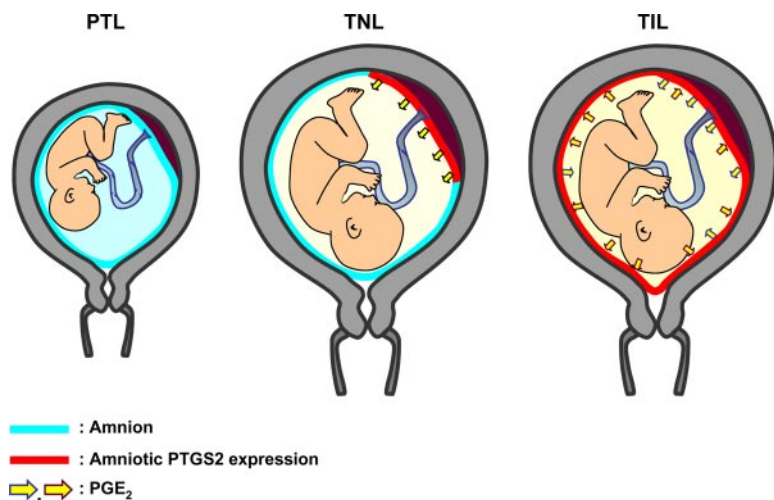
placental amnion in TIL cases compared with those of TNL cases. Therefore, there was no difference in the PTGS2/ $\beta$ -actin ratio between the placental amnion and the reflected amnion in tissues derived from women who had undergone labor [median, 1.73 (range, 0.44–5.94) vs. median, 1.38 (range, 0.36–6.03)] (Fig. 1, A and C).

PTGS2 expression in the placental amnion (median PTGS2/ $\beta$ -actin ratio, 0.32; range, 0.26–0.50) and the reflected amnion (median PTGS2/ $\beta$ -actin ratio, 0.34; range, 0.27–0.58) of PTL cases was significantly lower compared with those of the placental amnion and the reflected amnion from women at term in spontaneous labor ( $P < 0.001$  for each).

PTGS2 expression was barely detected by immunoblotting in the chorion-decidua of all groups of patients with small PTGS2/ $\beta$ -actin ratios [TNL median, 0.33 (range, 0.09–0.96); TIL median, 0.50 (range, 0.12–1.43); PTL median, 0.25 (range, 0.15–1.73)]. The PTGS2/ $\beta$ -actin ratios of the chorion-decidua were significantly lower than the corresponding placental amnion in women TNL ( $P < 0.01$ ) as well as both corresponding placental amnion and reflected amnion in women TIL ( $P < 0.01$  for each). In contrast to reflected amnion, PTGS2 expression in the chorion-decidua did not increase with labor at term.

The median concentration of PGE<sub>2</sub> in placental amnion was not different between women in labor and those not in labor at term. However, women who had preterm parturition had a significantly lower median concentration of PGE<sub>2</sub> in placental amnion than those at term regardless of labor status [TNL median, 9.0 pg/ $\mu$ g total protein (range, 1.2–55.8); TIL median, 11.3 pg/ $\mu$ g total protein (range, 2.6–56.0); PTL median, 0.6 pg/ $\mu$ g total protein (range, 0.3–2.1)].

The median concentration of PGE<sub>2</sub> in the reflected amnion of TIL cases was significantly higher than that of



**FIG. 2.** A schematic representation of proposed model for labor at term. Amniotic expression of PTGS2 is minimal before term in the absence of clinical and histological chorioamnionitis (PTL), whereas there is a robust increase in the placental amnion at term before the onset of labor (TNL). Labor at term is largely associated with increased PTGS2 expression in the reflected amnion (TIL). Arrows indicate PGE<sub>2</sub> expression in the placental amnion and release into the amniotic fluid (TNL), which induces PTGS2 expression in the reflected amnion by a positive feedback mechanism (TIL). PTL, Preterm labor without clinical and histological chorioamnionitis.

TNL cases ( $P < 0.05$ ). Interestingly, the median concentration of PGE<sub>2</sub> was lower in the reflected amnion of women who underwent preterm parturition [TNL median, 1.7 pg/μg total protein (range, 0.5–11.4); TIL median, 3.9 pg/μg total protein (range, 1.4–31.9); PTL median, 0.5 pg/μg total protein (range, 0.4–1.9)] (Fig. 1D). There was a good correlation between PGE<sub>2</sub> and the PTGS2/β-actin ratio ( $r = 0.722$ ;  $P < 0.001$ ) (Fig. 1E).

An additional immunohistochemical assessment of PTGS2 expression in the chorioamniotic membranes demonstrated immunoreactivity consistent with immunoblotting results. Trace or weak PTGS2 immunoreactivity was observed in PTL and TNL cases, mostly in chorionic trophoblasts. On the other hand, diffuse and strong PTGS2 immunoreactivity was evident in the amnion, particularly in the epithelial cells, of TIL cases. Amnion mesenchymal cells were also frequently positive, but the immunoreactivity was not as strong as amnion epithelial cells. In the chorion-decidua, scattered chorionic trophoblasts were immunoreactive, but decidual immunoreactivity was barely detected, even in TIL cases (Fig. 1F).

## Discussion

The novel and primary findings of this study are: 1) there is a stark contrast in amniotic PTGS2 expression in spontaneous labor at term and in preterm parturition; 2) the placental amnion, but not the reflected amnion, is the primary location of PTGS2 expression before the onset of labor at term; 3) however, the labor-associated increase in

PTGS2 expression is more prominent in the reflected amnion than in the placental amnion; 4) PTGS2 is far more abundant in the amnion than in the chorion-decidua; and 5) there was a good correlation between PTGS2 and PGE<sub>2</sub> expression in the tissues.

A stark contrast in amniotic PTGS2 and PGE<sub>2</sub> expression is additional evidence that spontaneous PTL in the absence of clinical and histological chorioamnionitis and term labor are biologically different. Spontaneous labor at term is a PTGS2/PGE<sub>2</sub>-rich process, whereas PTL is characterized by a relative deficiency of amniotic PTGS2/PGE<sub>2</sub>. This is consistent with the results of previous observations of amniotic fluid PGE<sub>2</sub> and PGF<sub>2α</sub> (5, 10). Although several studies have reported PTGS2 expression by decidua (11–13), significantly higher expression of PTGS2

and PGE<sub>2</sub> in the amnion than in the chorion-decidua confirmed in this study clearly underscores the importance of amnion in spontaneous labor at term. Our findings are consistent with those of Fuentes *et al.* (14) who elegantly showed an amniotic dominance of PTGS2 expression at the time of labor at term. Their densitometric analysis revealed that the amniotic PTGS2 expression, but not that of the decidua, increases in labor at term. Osman *et al.* (15) also demonstrated that PTGS2 mRNA expression was higher in the amnion than in the chorion-decidua. Furthermore, the amniotic PTGS2/PGE<sub>2</sub> expression pattern in the current study is consistent with the results of our analysis of PGE<sub>2</sub> and PGF<sub>2α</sub> in the amniotic fluid (16). Collectively, the findings indicate that there is minimal expression of PTGS2 and PGE<sub>2</sub> during the preterm gestation in the absence of clinical and histological chorioamnionitis and an abrupt increase as term approaches even in the absence of labor. Because the amnion is in direct contact with the amniotic fluid, we propose that placental amnion is primarily responsible for the surge in the amniotic fluid PGE<sub>2</sub> at term.

Several investigators have pointed out the importance of fetal signals, particularly from the fetal hypothalamo-pituitary-adrenal axis, in the onset of labor (17, 18). Given that CRH and cortisol up-regulate PTGS2 production by amnion, the surge of PTGS2 expression in the placental amnion before the onset of labor at term is biologically relevant. The basic difference between the placental amnion and the reflected amnion is the absence of fetal vas-

culature in the latter, whereas the placental amnion resides on top of numerous ramifications of fetal chorionic vessels. Once a marked increase in the expression of PTGS2 and prostaglandins by the placental amnion is induced by fetal signals in the chorionic/placental circulation (e.g. CRH), it is very likely that prostaglandins released into the amniotic fluid function as a paracrine signal for a subsequent surge of PTGS2 and prostaglandin expression in the avascular reflected amnion, which lines a large uterine surface. Indeed, increased PTGS2 expression by PGE<sub>2</sub>-mediated positive feedback has been reported in different types of cells, notably by macrophages that are present in amniotic connective tissue (19, 20). We propose that the placental amnion transforms fetal signals into paracrine signals (e.g. PGE<sub>2</sub>) in the amniotic fluid at term, and this in turn induces increased expression of PTGS2 and prostaglandins in the reflected amnion. This model would provide an effective pathway to transmit signals to the rest of the amnion and uterine contents during labor (Fig. 2).

The findings in this study indicate that the amnion is functionally compartmentalized for parturition at term. One interpretation is that the placental amnion seems to be responsible for the baseline production of prostaglandins before the onset of labor at term. Of major interest is that such a functional subdivision of the amnion is absent in PTL that presents no infection/inflammation. Our novel observations are further evidence that seemingly homogeneous human chorioamniotic membranes have region-specific biological characteristics as shown in the morphological and biological changes occurring at the chorioamniotic membranes of the lower uterine pole (zone of extreme altered morphology) in preparation for rupture and labor (21, 22).

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