

Inflammatory and steroid receptor gene methylation in the human amnion and decidua

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Abstract

Correct timing of parturition requires inflammatory gene activation in the gestational tissues at term and repression during pregnancy. Promoter methylation at CpG dinucleotides represses gene activity; therefore, we examined the possibility that DNA methylation is involved in the regulation of labour-associated genes in human pregnancy. Amnion and decidua were collected at 11–17 weeks of gestation and at term following elective Caesarean delivery or spontaneous labour. Methylation of the inflammatory genes *PTGS2*, *BMP2*, *NAMPT* and *CXCL2* was analysed using the Methyl-Profiler PCR System and bisulphite sequencing. Methylation of the glucocorticoid, progesterone and oestrogen receptor genes, involved in the hormonal regulation of gestational tissue function, and the expression of the DNA methyltransferases *DNMT1*, *-3A* and *-3B* were also determined. Variable proportions of inflammatory and steroid receptor gene copies, to a maximum of 50.9%, were densely methylated in both tissues consistent with repression. Densely methylated copy proportions were significantly different between genes showing no relationship with varying expression during pregnancy, between tissues and in individuals. Methylated copy proportions of all genes in amnion and most genes in decidua were highly correlated in individuals. *DNMT1* and *-3A* were expressed in both tissues with significantly higher levels in the amnion at 11–17 weeks than at term. We conclude that the unmethylated portion of gene copies is responsible for the full range of regulated expression in the amnion and decidua during normal pregnancy. Dense methylation of individually variable gene copy proportions happens in the first trimester amnion influenced by sequence context and affected strongly by individual circumstances.

Key Words

- ▶ methylation
- ▶ pregnancy
- ▶ amnion
- ▶ decidua

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Introduction

Parturition is associated with inflammation in the fetal membranes and the decidua. Array-based studies assessing the expression of a large number of genes simultaneously as well as studies determining the expression level of

proinflammatory candidate genes have shown that the activity of many inflammatory genes increases at term even in the absence of histological inflammation or detectable infectious insult (Haddad *et al.* 2006, Johnson

et al. 2006, Mazaki-Tovi *et al.* 2008, Li *et al.* 2011, Lim *et al.* 2012). This changing pattern of gene expression has been described as an 'acute inflammation gene expression signature' (Haddad *et al.* 2006), which transforms the membranes into an activated state producing uterotonic stimulants such as prostaglandins and undergoing structural remodelling in preparation for rupture during labour. The correct timing of inflammatory fetal membrane activation is critical for normal parturition, as pathological inflammation during pregnancy may cause serious complications such as preterm birth (Marvin *et al.* 2002, Shankar *et al.* 2010). The mechanisms that determine the timing of proinflammatory fetal membrane activation and the responsiveness of inflammatory genes to endogenous and exogenous stimuli are essentially unknown.

Stable, but reversible, covalent modifications of DNA and histones control gene transcription potential in eukaryotic cells by shaping the chromatin without altering DNA sequence (Bird 2007, Berger *et al.* 2009). The best characterised of these modifications is the C5-methylation of cytosine in CpG dinucleotide motifs. Methylation of CpGs (meCpG) at gene promoters results in transcriptional repression either by interfering with transcription factor binding or by recruiting meCpG-binding proteins that establish a closed chromatin structure (Klose & Bird 2006). Cell-specific DNA methylation is a key contributor to gene expression patterns that define cell identity (Ehrlich 2003, Straussman *et al.* 2009). DNA methylation marks are established early during development and maintained by the DNA methyltransferases DNMT3a, -3b and -1 respectively. CpG methylation levels can change, however, in response to developmental and environmental cues, underlying the importance of this chromatin modification in cell differentiation and susceptibility to disease (Bogdanovic & Veenstra 2009).

Changing CpG methylation is one possible mechanism regulating gene activity in the gestational tissues during pregnancy. It is plausible to hypothesise that methylation may suppress labour-associated inflammatory genes in early pregnancy while loss of methylation increases inflammatory gene expression potential at term and during labour. The aim of the present investigation was to explore this possibility by determining the CpG methylation density in the promoters of four inflammatory genes that show increased expression in the fetal membranes and decidua at term labour. These genes encode prostaglandin endoperoxide synthase-2 (*PTGS2*, the key prostaglandin biosynthetic enzyme), bone

morphogenetic protein-2 (*BMP2*), nicotinamide phosphoribosyltransferase (*NAMPT* also called PBEF, or visfatin) and chemokine (C-X-C motif) ligand-2 (*CXCL2*) (Haddad *et al.* 2006, Li *et al.* 2011). We have assessed the methylation status of these genes in amnion collected from normal pregnancies at 11–17 weeks of gestation, at term (37–41 weeks) in the absence of labour and following term labour. Decidua was also analysed from term pregnancies not in labour and following labour. Steroid hormones are fundamentally important regulators of gestational tissue function including inflammatory reactions. The responsiveness to these hormones varies between tissues and changes during pregnancy, which is partially due to changing steroid hormone receptor levels (Sun *et al.* 1996, Zakar & Hertelendy 2007, Merlino *et al.* 2009, Jiang *et al.* 2012). We have assessed the methylation status of the glucocorticoid receptor (*GR*, *NR3C1*), progesterone receptor (*PGR*, *NR3C3*) and oestrogen receptor (*ESR1*, *NR3A1*) genes in the amnion and decidua to delineate the contribution of receptor gene methylation to the differences in steroid receptor expression levels. In addition, we have measured *DNMT1*, -3A and -3B expression in the same tissues to determine whether varying DNA methyltransferase (DNMT) levels may contribute to the methylation differences observed between gestational tissues, genes and individuals.

Materials and methods

Patients and tissue collection

Placentas with attached fetal membranes were collected at the John Hunter Hospital obstetrics ward after deliveries at term either by elective Caesarean section (in the absence of labour, CS, $n=8$) or following spontaneous labour (SL, $n=8$). All pregnancies were uncomplicated and singleton. Amnion and decidua were isolated from the reflected membranes within 30 min of birth as described previously (Mitchell & Powell 1984, Johnson *et al.* 2002). Amnion tissues were also collected from women undergoing early elective termination between 11 and 17 weeks of pregnancy (ea, $n=8$) at a day-surgery. Tissues were snap-frozen in liquid nitrogen and stored at -80°C . Exclusion criteria included the presence of clinical or histological inflammation, induced labour or steroid treatment within 24 h of delivery. Informed written consent was obtained from all participants. Approval of the collection and use of these tissues was granted by the Hunter New England Health Human Ethics Committee and the University of Newcastle Human Ethics Committee.

DNA extraction

DNA was extracted from 20 to 25 mg crushed frozen tissue using the QIAmp DNA Mini Kit (Qiagen). DNA concentration and purity was determined by spectrophotometry (NanoDrop ND-1000, NanoDrop Technologies, Rockland, DE, USA). DNA integrity and fragment size were examined by agarose gel electrophoresis to ascertain whether DNA fragments were in the optimal range of up to 50 kb in length, with 20–30 kb fragments predominating.

DNA methylation profiling

Methylation density at target genes was determined by the Methyl-Profiler DNA methylation PCR System (SABiosciences, distributed as EpiTect Methyl qPCR Array by Qiagen Pty Ltd) according to the manufacturer's instructions. This assay uses a combination of methylation-sensitive and methylation-dependent restriction enzymes (provided in the Methyl-Profiler Methylation Enzyme Kit) followed by qPCR analysis of the digestion-resistant DNA to quantify the proportion of densely, sparsely and intermediately methylated target sequences in the molecular population present in the DNA sample. The procedure is suitable to perform serial tissue analyses for multiplex gene-specific DNA methylation with high sensitivity and in a convenient bisulphite free manner. Its principle, data analysis method and validation have been described (Ordway *et al.* 2006, Holemon *et al.* 2007). For this study, a customised 96-well multiplex assay was developed by the manufacturer based on the genomic sequences of NCBI Homo sapiens Build Number 36 Version 1. The following gene-specific assays were used to construct the customised array: *PTGS2*, MePH28527-1A; *BMP2*, MePH25532-1A; *NAMPT*, MePH10682-1A; *CXCL2*, MePH06436-1A; *GR*, MePH28267-1A; *PGR*, MePH06436-1A and *ESR1*, MePH28494-1A. The PCR conditions including the primer sequences and amplicon positions have been optimised and are held proprietary by the manufacturer. All screened regions included the transcriptional start site, several hundred base pair sequences upstream and downstream of it and at least one CpG island including or near the transcriptional start site. Further information about each gene-specific assay is available on the SAB-Qiagen website ([http://sabiosciences.com/dna_methylation_info.php?pcatn=\[assay i.d.\]](http://sabiosciences.com/dna_methylation_info.php?pcatn=[assay i.d.])). The arrays were run on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) using one cycle of 10 min at 95 °C to activate the DNA polymerase, followed by 40 cycles of 97 °C for 15 s and 72 °C

for 1 min. Dissociation curve analysis followed to confirm homogeneity of the amplification products.

Bisulphite modification and sequence analysis

Genomic DNA was extracted from 1 µg amnion tissue and bisulphite-converted using the Cells-to-CpG Bisulfite Conversion Kit (Life Technologies). PCR amplifications of bisulphite-modified *PTGS2* gene sequences were performed with primer sets A, B and C described by Song *et al.* (2001) using the TopTaq Master Mix Kits (Qiagen) in 50 µl reaction volume. PCR conditions included an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min and a final extension step at 72 °C for 10 min. The first PCR was performed with primer set A, covering the 869 bp region from –668 to +201 of the *PTGS2* gene (transcription start site is +1). The forward and reverse primer sequences of set A were 5'-TGTATATTGAAGGTAGTTATTTTAT-3' and 5'-ACCAAATACTCACCTATATAACT-3' respectively. Two microlitres of a 20-fold diluted aliquot of this reaction mixture were used as template for the second PCRs using primer sets B and C nested within the sequence amplified by primer set A. The B set forward and reverse sequences were 5'-TTGGAGAGGAAGTTAAGTGTTT-3' and 5'-AT-CCCCACTCTCCTATCTAAT-3' respectively. The C set forward sequence was 5'-ATTAGATAGGAGAGTGGG-GAT-3' and the reverse sequence was 5'-TCTAAAAACGTC-TAACTATAAAACT-3'. The nested PCR products were gel purified with the Wizard SV Gel and PCR Clean-up Kit and cloned using the pGEM-T easy vector system (both from Promega). Plasmid DNA was purified from the white colonies using the GenElute Plasmid Mini Prep Kit (Sigma–Aldrich) and the presence of inserts was verified after digestion with EcoRI. Inserts from six to nine clones from each PCR amplification were sequenced by the Australian Genomic Research Facility (Brisbane, QLD, Australia).

RNA isolation and qRT-PCR analysis

Total RNA was extracted from 0.2 g tissue with TRIzol reagent (Life Technologies) according to the manufacturer's instructions. The RNA was purified using RNase-free DNase and RNeasy Mini kits (Qiagen) and the quality was assessed by agarose gel electrophoresis. Total RNA (1 µg) was converted to cDNA using the Superscript III First Strand Synthesis system and random hexamer primers (Life Technologies). *PTGS2*, *DNMT1*, *DNMT3A* and *DNMT3B* mRNA abundance was determined by

Table 1 qRT-PCR primer sequences and positions

Primer	Primer sequence (5'–3')	Position ^a	GenBank no.
DNMT1	F: CCTCATTGCCGAATACATTCTG	1670/1741	NM_001130823
	R: TCTCCTGCATCAGCCCAAATA		
DNMT3A	F: TCCCGGCTCCAGATGTTT	2145/2220	NM_175629.1
	R: CTGGGACAGGTGGGTAAACCT		
DNMT3B	F: CCTCAGTTCCTCTTGCTCAGT	3001/3098	NM_006892.3
	R: AGCAGCCAGGTCTGATTTGTG		

^aNucleotide base position of the amplified mRNA sequence relative to the transcription start site (+1).

quantitative real-time RT-PCR (qRT-PCR) using an Applied Biosystems 7500 Real-Time PCR System. β -Actin mRNA was used as the internal reference RNA. The primer sequences, positions and concentrations for *PTGS2* mRNA and β -actin mRNA have been published (Johnson *et al.* 2002, Mitchell *et al.* 2011). Primer sequences for *DNMT1*, *DNMT3A* and *DNMT3B* mRNA were designed using Primer Express Software v3.0 (Applied Biosystems) and are listed in Table 1. *DNMT* primer optimisation was performed in preliminary experiments using RNA from BeWo choriocarcinoma cells, which express the three *DNMT* mRNAs. The optimised primer concentrations were 1000 nM for *DNMT1* and *DNMT3A* and 800 nM for *DNMT3B* mRNAs. Amplifications were performed in duplicate with each reaction mix containing SYBR green PCR master mix supplied by Applied Biosystems, primer and template to a total volume of 25 μ l. No-template and no-reverse transcriptase controls were included to test for non-specific amplification and residual DNA template respectively and dissociation curves were examined to ascertain the homogeneity of amplification products.

Statistical analysis

Primary data generated by the Methyl-Profiler DNA methylation PCR System (C_T values with mock, methylation-sensitive, methylation-dependent and double-digested templates) were imported into the Excel macro spreadsheet provided by the manufacturer. The macro performed quality control of data and quantified the percentage/proportion of densely, intermediately and sparsely methylated template copies in the molecular population of the DNA sample. Assays where delta- C_T values between mock- and double-digests were ≤ 2 ($\geq 25\%$ digestion-resistant template), were reported as failures and removed from subsequent analysis. As no intermediately methylated target sequence was detected in any sample (see later), statistics were calculated using the proportion of densely methylated template copies. The data were

non-normally distributed; therefore, we employed non-parametric statistic procedures. Comparisons involving more than two groups were performed by Kruskal–Wallis ANOVA followed by pair wise comparisons using the Mann–Whitney *U* test with Bonferroni-adjusted significance thresholds for multiple comparisons. Two-group comparisons were done with the Mann–Whitney *U* test. Variances were compared using the Brown & Forsythe's robust variance test. Relationships were assessed by Spearman rank correlation with Bonferroni-adjusted significance values for multiple comparisons. The relative mRNA abundance for *DNMT1*, *DNMT3A* and *DNMT3B* was determined by the delta- C_T method (Livak & Schmittgen 2001) with β -actin mRNA as the reference, and group comparisons were performed as described earlier. The levels of the reference mRNA (β -actin) were compared between the gestational groups employing the delta- C_T method using the calibrator sample, which contained β -actin and was included in all multi-well PCR plates. There was no significant difference between average β -actin mRNA abundance in amnion from the different gestational groups (ANOVA); however, β -actin mRNA abundance was 10% higher in the decidua following SL than in the absence of labour. Relative mRNA abundance values measured in the after-labour decidua samples were therefore multiplied by 1.1 to compensate for this difference in the average reference mRNA level. In all statistical analyses, $P < 0.05$ was considered significant. Analyses were carried out using STATA version 11.0 software package (College Station, TX, USA).

Results

Methylation of inflammatory genes

We have used the Methyl-Profiler DNA Methylation PCR Array System to assess the methylation status of the genes selected for the study. Methyl-Profiler returns result as the

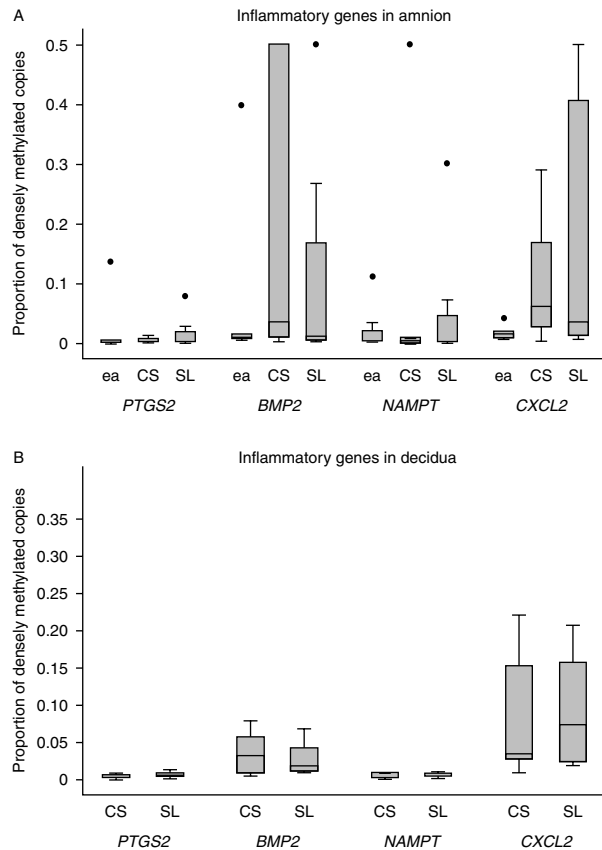
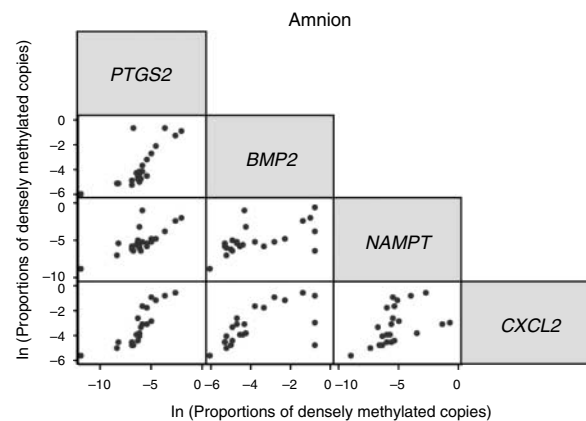


Figure 1 Proportions of densely methylated copies of the *PTGS2*, *BMP2*, *NAMPT* and *CXCL2* genes in amnion (A) and decidua (B). Tissues were obtained at 11–13 weeks of pregnancy (ea), after term elective Caesarean delivery (CS) and term spontaneous birth (SL). Boxes span the 25th–75th percentiles with horizontal lines indicating the median, whiskers showing the range of adjacent values and dots showing the outside values. $n = 7–8$ per group; there are no significant differences between gestational groups for any gene in both tissues. Variance of *CXCL2* methylation increases significantly in amnion with advancing gestation. Methylated copy proportions are significantly different among the genes in both the amnion and the decidua ($PTGS2 = NAMPT < BMP2 = CXCL2$).

proportion/percentage of densely, intermediately and sparsely methylated target sequences in the DNA samples. A surprisingly consistent feature of the methylation results was that no intermediately methylated sequence was detected in any of the analysed genes in any tissue sample at any gestational state studied. Therefore, the methylation results are presented as proportions of densely methylated sequences, and the rest of the target DNA (1 minus the proportion of densely methylated sequences) is determined as a sparsely methylated or unmethylated molecular population. The box-plots in Fig. 1A and B show the proportions of the densely methylated copies of the inflammatory genes in the

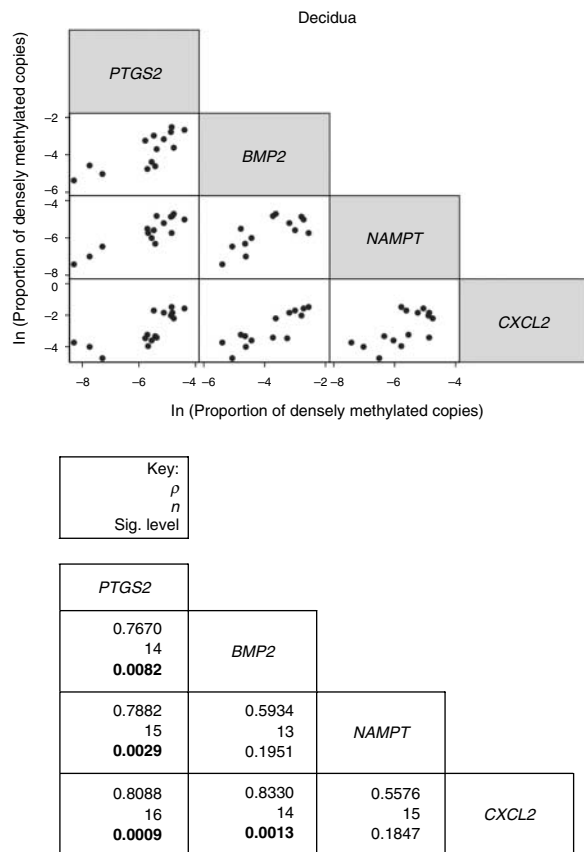
amnion and decidua respectively. The proportions varied widely between individuals in the amnion (range: 0–13.56% for *PTGS2* and 0.01–50% for the other genes) and somewhat less in the decidua (range: 0.02–1.25% for *PTGS2*, 0.46–7.78% for *BMP2*, 0.06–0.78% for *NAMPT* and 0.89–21.94% for *CXCL2*). Statistical testing showed, however, that there was no significant difference between gestational groups for any of the genes in the two tissues (Kruskal–Wallis ANOVA and Mann–Whitney *U* test). The variance of *BMP2* and *CXCL2* methylation showed a tendency to increase with gestation in the amnion (Fig. 1A), which reached statistical significance in the case of *CXCL2* (Brown & Forsythe’s robust variance test). The proportion of densely methylated copies differed significantly between genes in both tissues exhibiting the order $PTGS2 = NAMPT < BMP2 = CXCL2$ in the amnion as well as in the decidua with data combined



Key:
 ρ
 n
 Sig. level

<i>PTGS2</i>			
0.7665 22 0.0002	<i>BMP2</i>		
0.7065 23 0.0010	0.6152 23 0.0107	<i>NAMPT</i>	
0.9356 22 0.0000	0.7072 22 0.0014	0.6739 25 0.0025	<i>CXCL2</i>

Figure 2 Correlations of *PTGS2*, *BMP2*, *NAMPT* and *CXCL2* densely methylated copy proportions in the amnion. (Upper panel) Correlation matrix with each dot representing a tissue sample on a logarithmic scale. (Lower panel) Spearman correlation analysis results with significance adjusted for multiple comparisons. ρ and n values and significance levels are as shown in the Key. All correlations are significant (< 0.05 , bold).

**Figure 3**

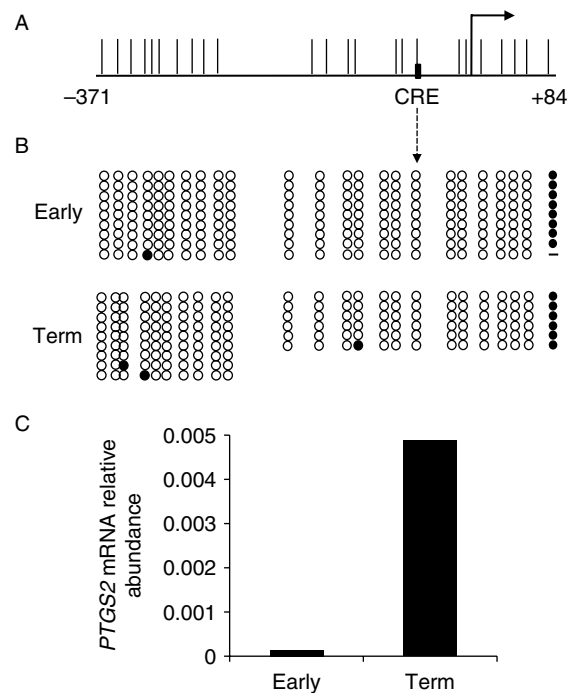
Correlations of *PTGS2*, *BMP2*, *NAMPT* and *CXCL2* densely methylated copy proportions in the decidua. (Upper panel) Correlation matrix with each dot representing a tissue sample on a logarithmic scale. (Lower panel) Spearman correlation analysis results with significance adjusted for multiple comparisons. ρ and n values and significance levels are as shown in the Key. Correlations are significant between *PTGS2* and the other three genes and between *BMP2* and *CXCL2* (<0.05 , bold).

for gestational groups (Kruskal–Wallis ANOVA with multiple comparisons).

As methylated copy proportions varied strongly among individual samples of each tissue, we tested the correlation between the methylation of genes in each individual. Figure 2 shows the scatter plot matrix (with logarithmically transformed data for better visualisation) and the corresponding Spearman correlation data matrix in the amnion sample cohort. Significant positive monotonic relationships were revealed between all combinations of *PTGS2*, *BMP2*, *NAMPT* and *CXCL2* densely methylated copy proportions. Similar analysis with decidua (Fig. 3) indicated positive relationships between *PTGS2* and the other three genes and between *BMP2* and *CXCL2*. There was no significant relationship between *NAMPT* and *BMP2*, or *CXCL2* densely methylated copy proportions in the decidua.

Relationship between *PTGS2* gene methylation and expression

The results in Fig. 1 show that *PTGS2*, *BMP2*, *NAMPT* and *CXCL2* gene methylation does not change significantly in the gestational groups with advancing pregnancy or labour, although increased expression of these genes has been found in the fetal membranes during the labour-associated inflammatory reaction at term. We have examined the relationship between gene expression and promoter methylation density in individuals by correlating methylated *PTGS2* copy proportions with *PTGS2* mRNA abundance measured by qRT-PCR in the same amnion samples. The analysis indicated that mRNA abundance and methylated gene copy

**Figure 4**

Methylation of CpGs in the promoter and 5'-transcribed regions of the *PTGS2* gene in amnion. DNA from an early (Early, 12.7 weeks) and a term gestation (Term) amnion was bisulphite-converted and PCR-amplified using primers recognising bisulphite-modified *PTGS2* gene sequences. The amplification products were cloned, sequenced and compared with the unmodified sequence to determine the methylation status of CpG dinucleotides. (A) Scheme of the analysed *PTGS2* promoter and 5'-transcribed region spanning the CpGs between -371 and $+84$ (relative to the transcriptional start site, $+1$, indicated by the arrow). The approximate location of CpG dinucleotides is shown by the vertical lines. The cyclic AMP response element (CRE) is boxed. (B) The methylation status of CpGs is shown by filled circles (methylated) and empty circles (not methylated). Each horizontal row of circles represents one cloned copy of the *PTGS2* gene. The dashed arrow points to the CpG within the CRE. (C) *PTGS2* mRNA relative abundance, determined by qRT-PCR, in the amnion tissue samples subjected to bisulphite sequencing.

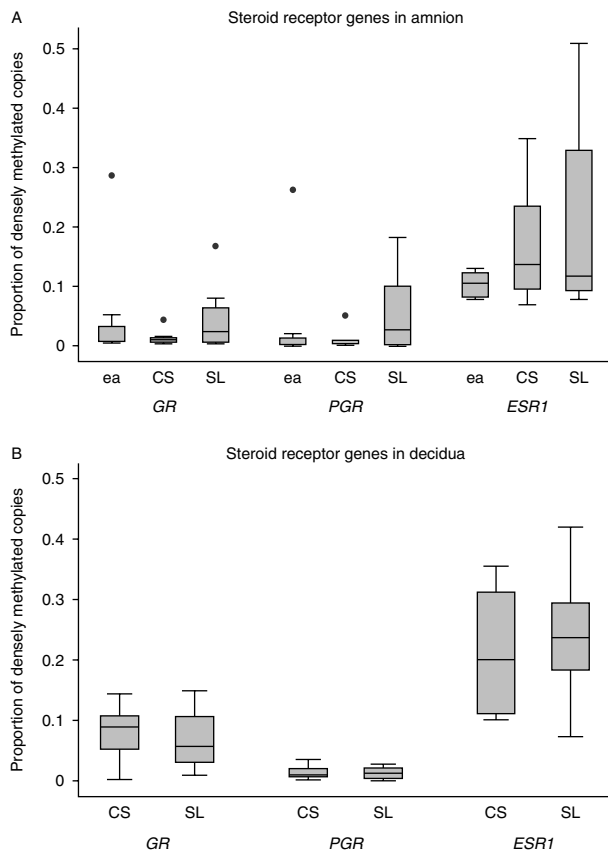
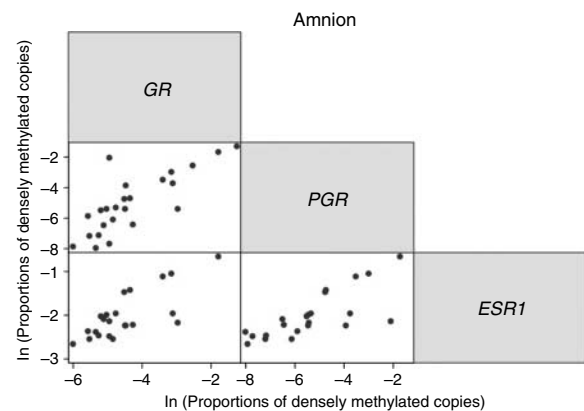


Figure 5 Proportions of densely methylated copies of the *GR*, *PGR* and *ESR1* genes in amnion (A) and decidua (B). Tissues were obtained at 11–13 weeks of pregnancy (ea), after term elective Caesarean delivery (CS) and term spontaneous birth (SL). Boxes span the 25th–75th percentiles with horizontal lines indicating the median, whiskers showing the range of adjacent values and dots showing the outside values. $n=7-8$ per group; there are no significant differences between gestational groups for any gene in both tissues. The variance of *ESR1* methylation increases significantly with advancing gestation in the amnion. Methylated copy proportions are significantly different among the genes in the amnion ($GR=PGR<ESR1$) and the decidua ($PGR<GR<ESR1$).

ratios varied independently of each other showing no significant correlation in individuals (Spearman $\rho: 0.02$, $n=39$, $P=0.23$).

We have also explored the possibility that methylation at single CpG sites may suppress *PTGS2* gene expression in early gestation amnion potentially by interfering with transcription factor binding (Tate & Bird 1993). Methylation of single CpG sites is not expected to change the overall methylation density of CpG islands, detected by the Methyl-Profiler assay. For this purpose, we have selected an early gestation (12.7 weeks) and a term (not in labour) amnion sample and determined the methylation status of CpG dinucleotides around the transcription start site of the *PTGS2* gene by bisulphite

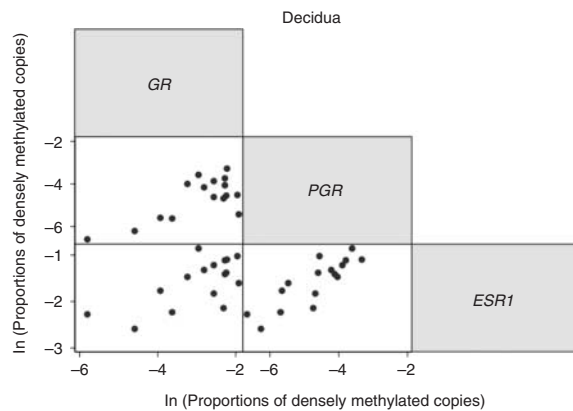
sequencing (Fig. 4). The sequenced region spanned nucleotide positions from the -371 CpG to the $+84$ CpG relative to the transcription initiation site ($+1$), containing 24 potentially methylated CpG dinucleotides (Fig. 4A). One CpG upstream of the transcription initiation site is part of a CRE (cyclic AMP response element), which has been reported critical for *PTGS2* expression in amnion cells (Zhu *et al.* 2009, Guo *et al.* 2012). Methylation of the CpG within the CRE prevents the binding of the cognate transcription factor rendering CREs inactive (Weih *et al.* 1991). The results showed that the analysed gene region was very sparsely methylated in all cloned gene copies (Fig. 4B), which was expected considering the Methyl-Profiler data (densely methylated copy proportions were 0.0023 and 0.0018 in the early and term sample respectively). The CpG within the CRE was



Key:	
ρ	
n	
Sig. level	

<i>GR</i>		
0.7882 24 0.0000	<i>PGR</i>	
0.6736 22 0.0018	0.8060 22 0.0000	<i>ESR1</i>

Figure 6 Correlations of *GR*, *PGR* and *ESR1* densely methylated copy proportions in the amnion. (Upper panel) Correlation matrix with each dot representing a tissue sample on a logarithmic scale. (Lower panel) Spearman correlation analysis results with significance adjusted for multiple comparisons. ρ and n values and significance levels are as shown in the Key. All correlations are significant (<0.05 , bold).



Key:		
ρ		
n		
Sig. level		
GR		
0.4771 16 0.2476	PGR	
0.5588 16 0.0733	0.8735 16 0.0000	ESR1

Figure 7

Correlations of *GR*, *PGR* and *ESR1* densely methylated copy proportions in the decidua. (Upper panel) Correlation matrix with each dot representing a tissue sample on a logarithmic scale. (Lower panel) Spearman correlation analysis results with significance adjusted for multiple comparisons. ρ and n values and significance levels are as shown in the Key. Correlation is significant between *PGR* and *ESR1* (<0.05 , bold).

unmethylated in both tissues, while the CpG at the +84 position was methylated in all gene copies cloned. There was no methylation difference consistent with the difference between the gestational statuses of the two amnion samples. The level of *PTGS2* expression, however, measured by mRNA abundance, was much lower in the early gestation than in the term amnion (Fig. 4C).

Methylation of steroid receptor genes

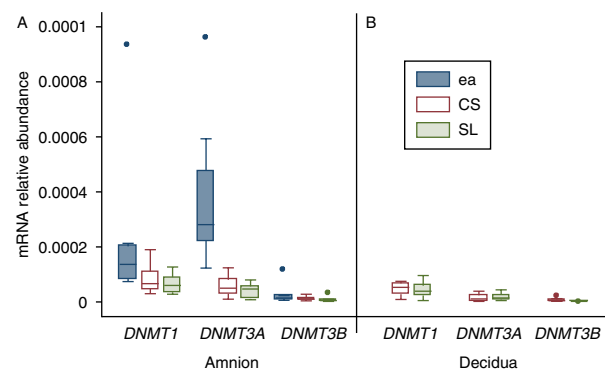
The proportions of methylated copies of the *GR*, *PGR* and *ESR1* genes are presented in Fig. 5. Variable proportions were detected both in the amnion (Fig. 5A; *GR*, 0.25–28.58%; *PGR*, 0.03–26.18%; *ESR1*, 6.9–50.935%) and in the decidua (Fig. 5B; *GR*, 0.3–14.82%; *PGR*, 0.13–3.61%; *ESR1*, 7.41–41.94%). There were no significant differences between the gestational groups;

however, the variance of methylated *ESR1* copy proportions increased significantly with advancing pregnancy in the amnion. Methylated copy proportions were significantly different between the steroid receptor genes in the order of $GR = PGR < ESR1$ in amnion and $PGR < GR < ESR1$ in the decidua (with gestational groups pooled).

All combinations of *GR*, *PGR* and *ESR1* densely methylated copy proportions correlated significantly in individual amnion samples (Fig. 6). In individual decidua samples, only *PGR*- and *ESR1*-methylated copy proportions showed significant correlation (Fig. 7).

DNMT expression in amnion and decidua

The methyltransferase isoenzymes DNMT3A and -3B perform *de novo* CpG methylation of DNA, while cell-specific meCpG patterns are maintained during DNA replication by DNMT1, which methylates the newly synthesised DNA strand at hemi-methylated CpG sites. Results in Fig. 8 show that mRNAs encoding these enzymes are present in the amnion and the decidua. In the amnion, *DNMT1* mRNA levels were significantly different between the gestational groups being highest in early pregnancy and lowest after term labour (Kruskal–Wallis ANOVA corrected for multiple comparisons). *DNMT3A* mRNA showed relatively robust levels in the amnion in early pregnancy and significantly lower abundance in the term amnion groups. *DNMT1*

**Figure 8**

Relative abundance of *DNMT1*, *DNMT3A* and *DNMT3B* mRNAs in amnion (A) and decidua (B). Gestational groups ($n=8$, each) are 11–13 weeks of pregnancy (ea, blue), term elective Caesarean delivery (CS, maroon) and term spontaneous birth (SL, green). Boxes span the 25th–75th percentiles with horizontal lines indicating the median, whiskers showing the range of adjacent values and dots showing the outside values. *DNMT1* mRNA levels are significantly higher in ea vs SL and *DNMT3A* mRNA levels are significantly higher in ea vs SL and CS in the amnion. There are no significant differences in the decidua. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-12-0211>.

and -3A mRNA levels showed no change with labour in the decidua. *DNMT3B* mRNA abundance values were close to or below the detection limit of our qRT-PCR assay in both tissues.

Discussion

Understanding the regulation of inflammatory genes in the gestational tissues is critical for unravelling the mechanisms that determine gestational length and underlie pregnancy complications such as preterm birth or post-term pregnancy (Doherty & Norwitz 2008, MacIntyre *et al.* 2012). DNA (CpG) methylation has been proposed to participate in this process (Mitchell 2006, Sato & Mitchell 2006, Wang *et al.* 2008, Menon *et al.* 2012, Mitchell *et al.* 2012), because this epigenetic chromatin modification is involved in establishing, maintaining and altering gene expression patterns during development. In this study, we have analysed the methylation status of four prototypic 'acute inflammatory gene expression signature' genes in the amnion and decidua, which are gestational tissues of fetal and maternal origin respectively. The expression of these genes, *PTGS2*, *BMP2*, *NAMPT* and *CXCL2*, increases during fetal membrane activation at labour (Haddad *et al.* 2006, Johnson *et al.* 2006, Mazaki-Tovi *et al.* 2008, Li *et al.* 2011, Lim *et al.* 2012). Their promoters are rich in CpG motifs, which form clusters called CpG islands. Genome-wide studies have revealed that CpG island-containing promoters are generally unmethylated with the exception of a few specialised groups of genes (e.g. germ cell-, pluripotency- and developmental-specific genes) that are repressed by CpG island methylation in a tissue- and developmental stage-specific manner (Shen *et al.* 2007, Weber *et al.* 2007, Illingworth *et al.* 2008, Straussman *et al.* 2009). Promoter hypermethylation has been shown to suppress *PTGS2* and *BMP2* expression in cancer cells (Song *et al.* 2001, Wen *et al.* 2006), suggesting that this chromatin modification may contribute to the suppression of labour-associated inflammatory genes in the gestational tissues during pregnancy. Indeed, we found that variable copy proportions of the studied inflammatory genes were densely methylated in both tissues. It is reasonable to conjecture, therefore, that these gene copies were transcriptionally inactive. Notably, the methylated copy proportions varied but did not exceed 50% in any tissue sample, suggesting that at least one allele per cell remained unmethylated at the tissue level overall. It must be pointed out that the various cell types present in the tissue samples may have different methylation densities at the studied genes and

our Methyl-Profiler analysis reflects the tissue level sum of methylated and unmethylated copy proportions. These unmethylated copies appear responsible for the regulated expression of the genes, as densely methylated copy proportions did not decrease with the increasing levels of gene expression at term and did not correlate with expression in individuals, as demonstrated in the case of the *PTGS2*. The high resolution analysis of *PTGS2* promoter CpG methylation by bisulphite sequencing also supported the conclusion that the hypomethylated gene copies participate in the gestational control of *PTGS2* expression in amnion. The methylation analysis of steroid receptor genes yielded similar results. The *GR*, *PGR* and *ESR1* genes possess CpG island-containing promoters, and CpG methylation has been shown to reduce steroid receptor expression in cancer cells and in response to environmental conditions (Sasaki *et al.* 2001, O'Doherty *et al.* 2002, Champagne *et al.* 2006, Wu *et al.* 2008, McGowan *et al.* 2009). Nuclear steroid receptor levels have not been reported to change in the gestational membranes during pregnancy; however, expression shows tissue-specific differences with decidua expressing all three receptors while amnion lacks detectable *PGR* and *ESR1* mRNA or protein (Padayachi *et al.* 1987, 1990, Merlino *et al.* 2009). These tissue-specific differences were not reflected by the methylation status of the receptor genes, which showed variable densely methylated copy proportions of up to 50.9% and overall highest methylation of the *ESR1* in both tissues.

The absence of significant gestational differences in the overall methylation status (median densely methylated copy proportions) suggests that the methylation levels of the studied genes is established before the gestational age of the early pregnancy group of our amnion sample cohort (ea, 11–17 weeks). This conclusion is supported by the lack in any sample of intermediately methylated gene copies representing the transition between the sparsely and densely methylated states (Holemon *et al.* 2007). Further, DNA methyltransferase (*DNMT1* and -3A) expression was most pronounced in the early gestation amnions, which was the sample group nearest to the proposed methylation event. Methylation is influenced by DNA sequence context, as indicated by the significant differences between the methylated copy proportions of genes in both tissues. The wide variation of densely methylated gene copy proportions among individuals suggests that individual circumstances have major influence on methyltransferase action at the time when methylation patterns are established in the embryos. Strong support for this notion is provided by

the correlation of densely methylated copy proportions between all inflammatory and steroid receptor genes in individual amnions (Figs 2 and 6). At the same time, the tendency for increased variance of methylation during pregnancy, reaching statistical significance in the case of *CXCL2* and *ESR1* in the amnion (Figs 1A and 5A), is suggestive of plasticity resulting in increased methylated copy proportions in some individuals during later stages of the pregnancy. Correlation of methylation levels was not observed between all genes in the decidua, which is a maternal tissue with a potentially long history of exposure to modifying factors.

In conclusion, we have determined by DNA methylation density analysis that individually varying proportions of CpG island-containing inflammatory and steroid receptor gene copies are methylated in the human amnion and decidua at high levels known to repress gene activity. Methylation in the amnion happens predominantly in the first trimester influenced by sequence context and affected strongly by individual circumstances. The proportion of densely methylated gene copies has no influence on either the gestational changes or the tissue specificity of gene expression. It must be noted, however, that all tissue samples in our study were obtained from uncomplicated, normal pregnancies. Further studies are needed to establish whether changing methylation of inflammatory, steroid receptor or other disease-associated genes contributes to aberrant gene expression in pathological pregnancies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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