Epigenetic regulation of maspin expression in the human placenta

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Maspin, a tumour suppressor gene, is differentially expressed in the human placenta. Decreased expression of maspin in the first trimester corresponds with the period of maximum trophoblast invasion, suggesting a role in cell invasion and motility. Although methylation of CpG islands regulates maspin expression in cancer cells, the mechanism of maspin regulation in the human placenta is unknown. Our objectives were to determine the role of epigenetic alterations in the regulation of maspin expression in the placenta. Placental samples obtained from 7 to 40 weeks’ gestation were used for bisulphite sequencing and chromatin immunoprecipitation (ChIP) PCR. There was no significant change in the methylation indices in the promoter region of maspin throughout gestation. The levels of histone modifications associated with transcriptionally active chromatin were significantly different in placental tissues from second and third trimester relative to those from first trimester. Addition of trichostatin A (TSA) to placental explants increased the maspin mRNA expression (8-to 20-fold), whereas addition of 5-aza-cytidine (5-AzaC) had no effect on maspin expression. Our data suggest that maspin expression in the human placenta is regulated by changes in histone tail modifications.

Key words: epigenetic regulation/histone modifications/maspin/placenta/promoter methylation

Introduction

Maspin (Serpin B5), a tumour suppressor gene, is differentially expressed during human placental development (Dokras et al., 2002). Very low levels of maspin are detected in the first trimester of pregnancy, a period associated with maximal trophoblast proliferation and subsequent cell invasion into the decidua. Maspin expression levels increase in the early second trimester and remain elevated in the third trimester of pregnancy at both the mRNA and protein levels. Although the exact role of maspin in human gestation is unclear, in vitro data suggest that maspin regulates trophoblast cell invasion (Dokras et al., 2002). The factors that might regulate these intriguing changes in maspin expression in the human placenta are unknown. The anti-estrogen tamoxifen and the anti-androgen flutamide both regulate maspin promoter activity in mammary epithelial and breast cancer cell lines (Khalkhali-Ellis et al., 2004). In addition, gamma linolenic acid (Jiang et al., 1997), nitric oxide (Khalkhali-Ellis and Hendrix, 2003) and manganese containing superoxide dismutase (MnSOD) (Li et al., 1998) have also been shown to up-regulate maspin expression. Reduced signalling through AP-1 and Ets decreases the transcriptional activity of the maspin gene in breast cancer cells (Zhang et al., 1997).

The complexity and the huge diversity of epigenetic modifications (DNA methylation and histone modifications) extend the information potential of the genetic code. It is now established that modification of the epigenetic code affects all biological processes (Jaenisch and Bird, 2003; Rodenhiser and Mann, 2006). Imbalance in cytosine methylation of CpG islands within 5′ promoter regions of genes has been associated with transcriptional inactivation of several genes in human cancer cells (Jones and Baylin, 2002; Laird, 2005; Baylin and Ohm, 2006; Jair et al., 2006). Similarly, modifications of the histones H3 and H4 have been shown to induce a change in chromatin activity (Strahl and Allis, 2000; Zhang and Reinhberg, 2001; Jaskelioff and Peterson, 2003; Kurdistani et al., 2004; Laird, 2005; Zhang et al., 2005b; Shilatifard, 2006). Hypermethylation of CpG islands in the maspin promoter has been shown to be associated with the transcriptional silencing of maspin in breast cancer cells (Domann et al., 2000) and ovarian cancer cell lines (Rose et al., 2006). Hypermethylation and histone deacetylation have both been shown to repress maspin expression in breast cancer cell lines (Maass et al., 2002). Cell-type-specific patterns of CpG island methylation and histone acetylation state of the promoter also correlate with maspin expression in various tissues (Futschek et al., 2002, 2004).

We sought to investigate the possible role of epigenetic mechanisms in differential expression of maspin in the human placenta with advancing gestation. In these studies, we investigated the functional relevance of histone modifications and DNA methylation in the regulation of the maspin gene expression. We first characterized the methylation status of CpG islands and the state of various histone tail modifications in the promoter regions of maspin in a panel of placental tissues representing various gestational periods. Next, utilizing the DNA demethylating reagent, 5-aza-cytidine (5-AzaC), and the histone deacetylase (HDAC) inhibitor, trichostatin A (TSA), we explored the mechanisms that might regulate the differential expression of maspin
throughout gestation. Our data indicate that the repressive chromatin associated with methylation of CpG islands in the promoter region of maspin can be altered in the human placenta by histone tail modifications. These modifications result in partial opening of chromatin and correlate with the differential maspin expression with advancing gestation.

Materials and methods

Placental samples

Placental samples before 20 weeks’ gestation were obtained from elective pregnancy terminations performed at the Emma Goldman Clinic, Iowa City. Placental samples were also collected from women with normal pregnancies at term (>37 weeks) delivered by Caesarean section or vaginally at the University of Iowa Hospitals and Clinics. In addition, placental samples were obtained from women with spontaneous preterm delivery (those births which occurred at 24–37 weeks’ gestation after the spontaneous onset of labour or rupture of fetal membranes). In these cases, clinical chorioamnionitis, pre-eclampsia, diabetes and other maternal medical problems were excluded. This project was approved by the University of Iowa Institutional Review Board.

Placental processing for RNA and DNA extraction

The placenta were sampled immediately after extraction from the uterus. To ensure systematic and unbiased sampling of the entire placenta, several placental biopsy specimens of ~0.5 g each were immediately obtained (Rajakumar and Conrad, 2000). After briefly rinsing each sample in three or four changes of saline, we transferred them into RNALater (Ambion, Austin, TX, USA) and stored them at –80°C. Total RNA was extracted using TRIZol (Invitrogen, Carlsbad, CA, USA) and further purified using the RNeasy kit (Qiagen, Valencia, CA, USA) as per manufacturer’s protocol and was treated with DNase I amplification grade (Invitrogen). DNA was extracted using the DNeasy tissue kit (Qiagen).

Human placental explant culture

Villus explant cultures were established from human placenta derived from all trimesters of pregnancy, as described previously (Caniggia et al., 2000). Briefly, placental tissue biopsies were collected in ice-cold phosphate-buffered saline (PBS) and processed immediately after collection. The tissue was dissected to remove decidual tissue and fetal membranes. Small fragments of placental villi (15–25 mg wet weight) were teased apart and placed in 6-well tissue culture plates (Corning Costar, NY, USA) precoated with 0.3 ml of undiluted Matrigel (Collaborative Biomedical Products, Bedford, MA). Explants were cultured in serum-free Dulbecco’s modified Eagle’s medium (DMEM)/

Bisulphite sequencing

Bisulphite sequencing was used to detect methylation in promoter sequences of the maspin gene in DNA extracted from human placental specimens. Sodium bisulphite modification of 4 μg of genomic DNA was carried out using the MethylEasy DNA bisulphite modification kit (Human Genetic Signatures, Sydney, Australia). The promoter region of maspin was amplified using the primer pair F-cctggatgtggaggcagc and R-aactaaactaatcataatcaatactcctaaaaa in the first round and F2-gaattattttttttattgtggataagttt and R2-ctaaaataatcataaaatcacaacacaa in the second round. The PCR mixture contained 25 μM of each primer, 40 ng of bisulphite-treated DNA (or 2 μl of first-round product), 1.25 units of Platinum High Fidelity Taq DNA polymerase, 0.2 mM dNTPs (each) and 2 mM MgSO4 in a final volume of 50 μl. PCR was performed with the following cycling parameters: an activation step of Platinum High Fidelity Taq DNA polymerase at 95°C for 3 min followed by 50 cycles of 94°C for 2 min, 50°C for 2 min and 68°C for 3 min with a final extension step of 68°C for 10 min. PCR products were analysed by agarose gel electrophoresis and cloned using the TOPO TA cloning kit (Invitrogen) as per the manufacturer’s protocol. The transformed bacterial colonies (16 from each sample) were inoculated in 96-well plates in Luria-Bertani (LB) media with appropriate antibiotic. Plasmid DNA was prepared using Sprint prep (Agenecourt Biosciences, Beverly, MA, USA) on a Biomsek robot. The DNA templates were sequenced from both ends with BIG Dye terminators. Sequences were resolved on an Applied Biosystems 3730 XL capillary sequencer, and data were assembled using phred/Phrap and viewed using consed (Ewing and Green, 1998; Gordon et al., 1998, 2001). In-house software written in Perl was used to calculate and visualize the fraction methylation at each CpG site.

Chromatin immunoprecipitation

Unlabeled placental explants were incubated with 1% formaldehyde for 15 min to cross-link histones to DNA. After washing the tissues with cold PBS, the samples were homogenized using Medimachine (Beckton Dickinson, Palo Alto, CA, USA). The samples were centrifuged, washed once with cold PBS and resuspended in lysis buffer (Upstate Biotechnology, Lake Placid, NY, USA). Samples were sonicated for 10 s with continuous output using the Branson sonifier, and the lysate was centrifuged for 10 min at 13 600 g at 4°C. The supernatant was incubated with protein A-agarose beads (Upstate Biotechnology) for 2 h. The slurry was removed by centrifugation at 50 g for 1 min, and the supernatant was divided into seven parts. The first part was used as input control, and the other six parts were incubated with either anti-acetylated histone H3-K9, anti-dimethyl H3-K4, anti-dimethyl H3-K9, anti-trimethyl H3-K27 (all from Abcam, Cambridge, MA, USA) and normal rabbit immunoglobulin G (IgG) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or no antibody (negative control) at 4°C overnight. The immunoprecipitated complexes were collected by incubation with protein A-Sepharose beads (Upstate Biotechnology) for 2 h at 4°C. After washing the beads with buffers [low salt, high salt, LiCl and Tris–EDTA (TE)], the cross-links were reversed by heating the samples at 65°C for 4 h with 3.5 M NaCl. The samples were treated with proteinase K overnight, and DNA was extracted by the phenol chloroform method, ethanol precipitated and resuspended in TE. PCRs were carried out using oligonucleotides designed to amplify 250-bp regions in the promoter region. To ensure that PCR amplification was in linear range, we set up each reaction at different dilutions of DNA for varying amplification cycle numbers, and final PCR conditions were selected accordingly. The PCR mixture contained 20 μM of each primer, 1 μl of extracted DNA, 0.5 units of Taq DNA polymerase, 0.2 mM dNTPs (each) and 2 mM MgSO4 in a final volume of 50 μl. PCR was performed with the following cycling parameters: an activation step of 94°C for 3 min followed by 50 cycles of 94°C for 2 min, 50°C for 2 min and 68°C for 3 min with a final extension step of 68°C for 10 min. The promoter region of maspin was amplified using the primer pair F-cctggatgtggaggcagc and R-catgaattcagagccctctcgTC. The PCR products were visualized by 1.5% agarose gel electrophoresis and quantified by densitometry. The assays were done in triplicate, and the changes in level of histone tail modifications with respect to input DNA were calculated.

Real-time PCR confirmation

Purified RNA (1 μg) was reverse transcribed using random primers as per manufacturer’s protocol (High Capacity cDNA Archive Kit, Applied Biosystems, Foster City, CA, USA). The resulting cDNA was diluted 20-fold and used as template. Real-time PCR was performed using Assay on Demand Gene Expression reagents (assay ID: Hs00985283_m1, Applied Biosystems) on the ABI PRISM 7900 HT Sequence Detection System under default conditions: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The expression of human 18S was used as the endogenous control, and comparative Ct method was used for quantification of the transcripts as per manufacturer’s protocol. Measurement of ΔCt was performed in triplicate.

Western blot analysis

Placental explants were lysed in 150 μl of RIPA buffer [50 mM Tris, 150 mM NaCl, 0.5% deoxycholate, 1% Triton X-100 and 0.1% sodium dodecyl sulphate (SDS)] plus 2 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml of aprotinin, 10 μg/ml of leupeptin and 2 mM sodium vanadate. After sonication, the lysates were centrifuged at 13 000 g for 30 min at 4°C. Protein concentrations
were determined with the bicinechonic acid assay kit (Pierce, Rockford, IL, USA), using albumin as standards. Laemml sample buffer was then added to 50 µg of protein and heated in a boiling water bath for 5 min. Equal amounts of protein from each sample were fractionated in a 10% SDS–polyacrylamide gel electrophoresis (PAGE). The fractionated protein samples were transblotted onto a nitrocellulose membrane (Midwest Scientific, Valley Park, MO, USA), and non-specific binding was blocked for 1 h in 5% non-fat dry milk. After extensive washing with Tris-buffered saline (TBS), the blots were probed with a 1:500 dilution of maspin monoclonal antibody (Pharmingen, San Deigo, CA, USA), a 1:40 000 dilution of acetyl-histone H3 (Upstate Biotechnology) or actin antibody (Chemicon International, Temecula, CA, USA) in TBSTB [TBS/1% bovine serum albumin (BSA)/0.1% Tween 20] for 1 h at room temperature with gentle shaking. After extensive washing, the blots were probed with a 1:5000 dilution of goat anti-mouse IgG conjugated to horse-radish peroxidase (Jackson Immuno Laboratories, West Grove, PA, USA). The blots were washed extensively and the protein detected using enhanced chemiluminescence (ECL System, Amersham, Arlington Heights, IL, USA). The expression of maspin, HIF 1α and actin was further quantified using densitometric analysis (Scion Image J).

**Statistical analyses**

Methylation and real-time expression data were analysed with the JMP suite of programs (Version 6.0, SAS Institute, NC, USA) using the Kruskal–Wallis one-way analysis of variance (ANOVA) test as indicated. Values of \( P < 0.05 \) were considered significant.

**Results**

**Methylation status of CpG islands in the promoter region of maspin in human placental tissues**

A few studies have shown that changes in methylation status of CpG islands in the promoter region of maspin play an important role in regulating its expression (Domann et al., 2000; Futscher et al., 2002, 2004). Owing to these findings, we utilized bisulphite sequencing to assess the methylation status of the CpG islands in the promoter region of maspin in a panel of human placental tissues obtained from various gestational ages. These placental tissues (\( n = 28 \)) showed distinctive expression of maspin mRNA at various gestational ages (Figure 1) with 6-fold higher levels in second trimester placental samples (\( P < 0.001 \)) and 8-fold higher levels in third trimester placental samples (\( P < 0.001 \)) relative to tissue samples from first trimester. These results provide an appropriate setting for correlating the state of histone and DNA modifications with its expression. Using methylation-sensitive PCR and bisulphite-treated genomic DNA from 30 placental tissues representing 7–39 weeks’ gestational periods, a CpG-rich region between nt –239 and –26 relative to the ATG start site containing 12 CpG sites was amplified. All cytosines at non-CpG sites in various samples were converted to uracils by bisulphite treatment, and those present at CpG sites either remained as cytosines or were converted to uracil. DNA sequence analysis of 16 individual clones from each PCR product was performed to determine the methylation status of individual CpG sites (Supplementary Figures S1–S3). The fractional methylation for each sample was determined by calculating the percentage of methylated cytosines at each CpG site (Figure 2). The total methylation represented as methylation index (MI) for each sample was determined by averaging the fractional methylation of CpG sites over the entire PCR product. The Wilcoxon one-way ANOVA test demonstrated that, although maspin expression levels are significantly higher in second and third trimesters relative to first trimester, there were statistically no significant differences in the methylation indices of the CpG islands in its promoter region at various gestational ages. The median (interquartile range) values of methylation along with maspin expression levels for different trimesters are listed in Table I. We also analysed the methylation level of each individual CpG site in the promoter region of maspin and found no correlation between methylation levels of target CpG sites with gestational age (Supplementary material, Table S1). These results suggest that the gestation-specific expression of maspin in placental tissue is unlikely to be regulated by differential methylation of CpG islands in its promoter region.

**Differential maspin expression in human placenta correlates with changes in histone tail modifications**

Covalent modifications to histones are key epigenetic marks that control gene transcription. The possibility that site-specific histone modifications might regulate maspin expression with gestational age was next investigated by chromatin immunoprecipitation (ChIP) assays in placental tissues using primers that encompass the maspin promoter region. ChIP PCR analysis was performed using primary antibodies to anti-acetylated histone H3-K9, anti-dimethyl H3-K4, anti-dimethyl H3-K9 and anti-trimethyl H3-K27. The level of acetylated H3-K9 and methylated H3-K4 in the promoter region of maspin was significantly higher (\( P < 0.001 \)) in placental tissue samples from both second (12–28 weeks) and third trimesters (29–40 weeks) relative to those from first trimester (<12 weeks) (Figure 3). Similarly, histone H3 at the K9 and K27 positions was less methylated in placental tissue samples from second and third trimesters relative to those from first trimester. These epigenetic marks at histone H3 are indicative of an increase in transcriptional activity of maspin during both second and third trimesters. These results suggest that maspin expression might be regulated at the chromatin level in the placenta during human pregnancy.

**Effect of epigenetic inhibition on maspin gene expression in human placental explants**

To investigate the direct effect of epigenetic silencing on the expression of maspin in human placenta, we established four study treatments. The groups consisted of treatment of placental explants with a
HDAC inhibitor (TSA), a demethylating agent (5-AzaC) or culture media containing DMSO or PBS (controls). Treatment of placental tissues with 0.3 μM TSA for 24 h resulted in an increase in the expression levels of maspin mRNA (8–20 fold, \(P < 0.001\)). The relative change in maspin expression level upon treatment with TSA compared with baseline expression was more pronounced in placental tissues from early gestation (Figure 4). On the contrary, treatment of placental explants with 5-AzaC ranging from 0.2 to 5 μM failed

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**Figure 2.** Bisulphite sequencing reveals methylated CpG island in the promoter region of maspin in placental tissues. A 214-bp region containing 12 CpG sites in the promoter region of maspin gene (between nt –239 and –26) was amplified using methylation-specific PCR on bisulphite-treated DNA from placental tissues (7–39 weeks’ gestation, \(n = 30\)). PCR products were cloned into TOPO TA (Invitrogen). Plasmid DNA from 16 independent clones was prepared and sequenced from both ends with BIG Dye terminators V3.1. Each square represents an individual CpG site with the degree of shading representing fractional methylation.

**Table I.** Distribution of methylation index (MI) and maspin expression levels at different trimesters

<table>
<thead>
<tr>
<th></th>
<th>Median (IQR)</th>
<th>First trimester</th>
<th>Second trimester</th>
<th>Third trimester</th>
<th>(P) value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylation index</td>
<td>30.21 (22.39–38.28)</td>
<td>24.22 (19.53–38.54)</td>
<td>34.64 (17.45–42.71)</td>
<td>&gt;0.10</td>
<td></td>
</tr>
<tr>
<td>Maspin RNA expression</td>
<td>0.3 (0.2–0.6)</td>
<td>1.9 (0.9–2.3)</td>
<td>2.4 (2.0–3.1)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

IQR, interquartile range.

\(P\)-values represent comparisons between second or third trimester samples relative to placental tissues from first trimesters.

*Wilcoxon/Kruskal-Wallis Tests.
Histone modifications regulate maspin expression in placenta

Figure 3. Chromatin immunoprecipitation (ChIP) assays on DNA harvested from human placental tissues representing each trimester of pregnancy (n = 4). The changes in the level of histone H3-K9 acetylation, K4-dimethylation, K9-methylation and K27-trimethylation with changes in gestation were evaluated using antibodies specific for these modifications. Normal rabbit immunoglobulin G (IgG) or no antibodies were used as negative controls. PCR products were analysed by agarose gel electrophoresis (A) and quantified by densitometry. The changes in histone tail modifications were measured relative to input DNA (B). The assays were done in triplicate.

Figure 4. Western blot analysis of maspin protein expression in placental explants treated with TSA or 5-AzaC. Although an increase in the levels of maspin protein expression was detected in TSA-treated explants, there was no change in maspin protein expression in 5-AzaC-treated explants (data not shown). We then determined whether TSA treatment resulted in histone hyperacetylation in the human placenta. Western blot analysis using antibodies against acetylated core histone H3 revealed a substantial increase in acetylated histone H3 in placental tissues from first trimester relative to that observed at third trimester (Figure 5B). The results of the western blot analysis are concordant with the results of quantitative real-time PCR analysis and consistent with our earlier finding that histone tail modifications at the maspin gene promoter region serve as histone code for maspin gene expression.
Figure 4. Changes in maspin expression in human placental explants on epigenetic inhibition. Total RNA was extracted from placental explants (n = 3 from each trimester) after treatment with 0.3 μM trichostatin A (TSA) [histone deacetylase (HDAC) inhibitor] or a corresponding volume of dimethylsulphoxide (DMSO) (final DMSO concentration not exceeding 0.1% v/v, controls) for 24 h and 0.2–5 μM 5-aza-cytidine (5-AzaC, DNA methyl transferase inhibitor) or a corresponding volume of phosphate-buffered saline (PBS) containing 1 μM acetic acid (control) for 48 h. Real-time PCR was used to evaluate relative fold change in response to TSA or 5-AzaC treatment relative to DMSO or PBS-treated explants, respectively, in triplicate using comparative ΔCt method. y-Axis units are arbitrary expression units used to calculate fold change.

**Discussion**

Maspin is a member of the non-inhibitory serpin family and does not exhibit protease inhibitor activity (Bass et al., 2002). Maspin has been shown to play a role in the regulation of cell motility, invasion, apoptosis, and angiogenesis (Zou et al., 1994; Sheng et al., 1996; Zhang et al., 2000, 2005a). Some studies have reported the potential role of epigenetic mechanisms in regulating the expression of maspin in various cancer cell lines (Domann et al., 2000; Futscher et al., 2002, 2004; Rose et al., 2006). Initial studies have demonstrated hypermethylation of the maspin promoter in seven of nine breast cancer cell lines compared with normal human mammary cells (Domann et al., 2000). This methylation pattern corresponds to the maspin expression profiles in these cell lines. Loss of maspin expression also appears to be an early event in breast cancer development, with ~50% of ductal carcinoma in situ specimens demonstrating the absence of maspin expression (Futscher et al., 2004). However, the loss of maspin expression was not linked to aberrant DNA methylation in all these cases. These findings suggest that other mechanisms also influence the expression of maspin in mammary cells. Another group of investigators has shown hypermethylation and histone deacetylation to reactivate maspin expression in breast cancer cell lines (Maass et al., 2002). Increased unmethylated maspin concentrations in plasma from women with pre-eclampsia compared with healthy pregnant controls have been recently reported (Chim et al., 2005). Pre-eclampsia is a common obstetric complication diagnosed after 20 weeks’ gestation and is associated with significant morbidity and mortality. We have detected elevated expression of maspin in placental samples from women with severe pre-eclampsia as compared with gestation-matched controls (unpublished data). Although the exact function of maspin in the placenta is unclear, a better understanding of the regulation of maspin in the human placenta may provide insights into the pathophysiology of conditions such as pre-eclampsia.

The goal of this study was to determine whether differential expression of maspin in the human placenta is epigenetically regulated. Our previous studies have shown that maspin expression is predominantly restricted to the cytotrophoblast and syncytiotrophoblast layers of the placenta (Dokras et al., 2002). By quantitative real-time PCR, we detected a 6-fold increase in maspin expression between the first and second trimester and an 8-fold increase between the first and third trimesters. We identified multiple histone modifications that correlate with the increased expression of maspin in human placenta with advancing gestational age. Our data suggest that an increase in the expression of maspin is achieved as a result of the switch from methylation to acetylation of H3 at K9, increase in methylation of H3 at K27 and decrease in methylation of H3 at K4 and decrease in methylation of H3 at K27. It has been shown that these changes are associated with a relaxed and active chromatin (Jaenisch and Bird, 2003; Kurdistani et al., 2004; Zhang et al., 2005b). Significantly, however, our data suggest that DNA methylation of maspin promoter is not involved in the differential expression of maspin in human placenta with advancing gestation. The region examined for methylation was 214 bp, containing 12 Cpg sites, with a C + G content of 60.5% and an observed-to-expected CpG ratio of 0.641. Our bisulphite sequence data indicated that there was no change in the methylation levels of any of these 12 CpG sites with increasing gestation. The extent of methylation observed was similar to that reported earlier in human placental tissues from both first trimester and third trimester placentas (Chim et al., 2005).

To investigate a potential link between maspin expression and methylation of its promoter, we quantified the relative expression of
maspin in placental tissues with human embryonic kidney cell line (HEK 293) and normal prostate epithelium cells. The promoter region of maspin in HEK293 cells is hypermethylated and has been associated with decreased maspin expression relative to normal prostate epithelium cells (Futschier et al., 2002). We found maspin to be expressed at similar levels in the placental tissues from various gestational ages as in HEK293 cells and significantly down-regulated relative to normal prostate epithelium cells (Supplementary Figure S4). This suggests that despite transcriptional repression of maspin by DNA methylation, changes in histone modifications assembled at the maspin promoter region induce a more accessible chromatin structure and contribute to its differential expression in human placenta. However, these changes are not sufficient to completely overcome repressive effects of promoter methylation of maspin. Consistent with this notion, expression of maspin changed upon HDAC inhibition by TSA. However, doses of the demethylating agent, 5-AzaC, previously shown to increase maspin expression in other cell lines, had no effect on placentals explants (Figure 4 and Supplementary Figure S5). We also looked at the synergistic activation of maspin by TSA and 5-AzaC and found that TSA was solely responsible for resetting the histone code during this process (data not shown).

We acknowledge that second trimester placental tissues obtained after delivery may not entirely represent in vivo changes in maspin gene expression. We were very particular about including placentas from subjects with no medical problems or clinical infection. Most of our cases represent women with cervical incompetence or unexplained preterm labour with no chorioamnionitis. Despite the limitations, second trimester placental samples showed similar changes to third trimester samples after real-time PCR analysis, bisulphite sequencing and ChIP analysis. As the results corroborated for methylation indices and H3 acetylation and methylations changes, these findings suggest that our second trimester placental samples were appropriately selected. Our findings expand earlier observations of epigenetic control of maspin expression in various human placental tissues. Further investigations will dissect mechanisms that act cooperatively to stimulate changes in histone side-chains and enhance chromatin accessibility resulting in increased transcriptional activity of maspin at later gestational ages.

Supplementary material
Supplementary data are available at http://humrep.oxfordjournals.org/.

References

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