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Epigenetic Silencing of Maspin Expression Occurs Early in the Conversion of Keratocytes to Fibroblasts

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Abstract

Maspin, a 42 kDa non-classical serpin (serine protease inhibitor) that controls cell migration and invasion, is mainly expressed by epithelial-derived cells but is also expressed in corneal stromal keratocytes. Upon culture of stromal keratocytes in the presence of FBS, maspin is down regulated to nearly undetectable levels by passage two. DNA methylation is one of several processes that controls gene expression during cell differentiation, development, genetic imprinting, and carcinogenesis but has not been studied in corneal stromal cells. The purpose of this study was to determine whether DNA methylation of the maspin promoter and histone H3 dimethylation are involved in the mechanism of down regulation of maspin synthesis in human corneal stromal fibroblasts and myofibroblasts. Human donor corneal stroma cells were immediately placed into serum-free defined medium or cultured in the presence of FBS and passed into serum-free medium or medium containing FBS or FGF-2 to induce the fibroblast phenotype or TGF- β 1 for the myofibroblast phenotype. These cell types are found in wounded corneas. The cells were used to prepare RNA for semi-quantitative or quantitative RT-PCR or to extract protein for western analysis. In addition, P4 FBS cultured fibroblasts were treated with the DNA demethylating agent, 5-aza-2'-deoxycytidine (5-Aza-dC), and the histone deacetylase inhibitor, trichostatin A (TSA). Cells with and without treatment were harvested and assayed for DNA methylation using sodium bisulfite sequencing. The methylation state of histone H3 associated with the maspin gene in the P4 fibroblast cells was determined using a ChIP assay. Freshly harvested corneal stromal cells expressed maspin but upon phenotypic differentiation, maspin mRNA and protein were dramatically down-regulated. Sodium bisulfite sequencing revealed that the maspin promoter in the freshly isolated stromal keratocytes was hypomethylated while both the P0 stromal cells and the P1 cells cultured in the presence of serum free defined medium, FGF-2 and TGF- β 1 were hypermethylated. Down regulation of maspin synthesis was also associated with histone H3 dimethylation at Lysine 9. Both maspin mRNA and protein were reexpressed at low levels with 5-Aza-dC but not TSA treatment. Addition of TSA to 5-Aza-dC treated cells did not increase maspin expression. Treatment with 5-Aza-dC did not significantly alter demethylation of the maspin promoter but did demethylate histone H3. These results show maspin promoter hypermethylation and histone methylation occur with down regulation

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of maspin synthesis in corneal stromal cells and suggest regulation of genes upon conversion of keratocytes to wound healing fibroblasts can involve promoter and histone methylation.

Keywords

Cornea; Stromal Cells; Maspin; DNA Methylation; Stromal Fibroblasts; Stromal Myofibroblasts; Keratocytes

1. Introduction

The cornea serves as a protective barrier to biological or mechanical injury and acts as a transparent material that passes and refracts light. When wounding occurs, the cornea sets into action various mechanisms to repair the injured area with the aim to maintain and/or restore optimum optical properties (Jester et al., 1999a). After wounding, epithelial cells migrate and proliferate to replace damaged cells (Lu et al., 2001). As a consequence of wounding of the epithelium and/or the stroma, the underlying anterior stromal cells undergo apoptosis, while corneal stromal cells distal from the wound undergo phenotypic changes to fibroblasts and myofibroblasts (Jester et al., 1987; Jester et al., 1996; Jester et al., 1999b; Maltseva et al., 2001). The fibroblasts migrate to the wounded area and secrete collagenases and other proteases and extracellular matrix proteins for tissue remodeling (Beales et al., 1999; Ye and Azar, 1998; Ye et al., 2000). In contrast to those in the uninjured cornea, the collagen fibrils are laid down in a random fashion resulting in an opaque region (Kaji et al., 1998). Myofibroblasts, characterized by the expression of α -smooth muscle actin, localize to the edges of the wound and then contract to pull the edges of the wound together (Barry-Lane et al., 1997). Over time, the cells remodel the extracellular matrix and the collagen fibrils become more ordered and regular in size and shape (Davison and Galbavy, 1986).

Maspin (GenBank accession no. NM 002639), a 42 kDa protein, was first identified in normal mammary epithelial cells and is a member of the serpin superfamily (Sheng et al., 1994; Zou et al., 1994). It does not function as a classical serine protease inhibitor but rather as a molecule which inhibits cell motility, invasion, metastasis, and angiogenesis (Maass et al., 2001a; Pemberton et al., 1995; Seftor et al., 1998; Sheng et al., 1996; Shi et al., 2001; Zhang et al., 2000; Zou et al., 1994). Maspin is expressed in cells of epithelial origin, specifically those of the airway, breast, skin, prostate and the cornea but not in most other cell types such as skin fibroblasts, lymphocytes or bone marrow, cardiac or renal cells (Futscher et al., 2002). In the cornea, maspin is expressed not only by the corneal epithelial and endothelial cells but also by the corneal stromal keratocytes in situ (Ngamkitidechakul et al., 2001). When stromal keratocytes directly isolated from the cornea are placed in culture in the presence of FBS, maspin synthesis is down regulated as the cells become more fibroblastic and motile. Exogenously added maspin stimulates adhesion of stromal fibroblasts to corneal extracellular matrix molecules, type I and type IV collagens, fibronectin and vitronectin and inhibits migration. The purpose of this paper is to explore the mechanism of the down regulation of maspin in corneal stromal cells with phenotypic change to fibroblasts and myofibroblasts.

Maspin expression is down regulated upon conversion of many epithelial cells to carcinoma cells as observed in mammary and prostate cancer (Maass et al., 2001a; Seftor et al., 1998; Zou et al., 1994). However, maspin is not expressed in some epithelial cells but is up regulated in pancreatic, ovarian, and gastric carcinoma (Maass et al., 2001b; Sood et al., 2002; Wang et al., 2004). A major breakthrough on maspin regulation was discovered by Domann and Futscher when they showed that maspin expression was inversely correlated with the methylation state of maspin's CpG-rich promoter (Futscher et al., 2002). DNA methylation had long been speculated to be important in establishing and maintaining cell type specific

gene expression during development and in differentiated adult tissues (Holliday and Pugh, 1975; Riggs, 1975). However, it was not proven until 2002 when methylation of the maspin promoter was studied (Costello and Vertino, 2002; Futscher et al., 2002). Subsequent to maspin other genes have been identified whose expression is controlled by methylation in a CpG island including the *MCJ* and the tyrosine hydroxylase genes (Strathdee G et al., 2004; Aranyi et al., 2005). Thus, maspin was the first example of a gene in which cytosine methylation of a promoter, in part, controls normal tissue-specific expression. Paradoxical expression of maspin in certain cancers has now been linked to the aberrant cytosine demethylation of the promoter and an accessible chromatin state (Domann et al., 2000; Fitzgerald et al., 2003; Oshiro et al., 2003).

The current study focused on the methylation states of the maspin promoter and its associated histone H3 of corneal stromal cells undergoing phenotypic change to cells involved in corneal wound healing. For these studies, we used the widely accepted corneal wound healing model cells, FBS and FGF-2 converted fibroblasts and TGF- β 1 converted myofibroblasts (Jester et al., 1996; Maltseva et al., 2001). We showed the maspin promoter in freshly isolated maspin expressing corneal keratocytes and epithelial cells was hypomethylated. However, hypermethylation was observed as the corneal keratocytes were placed into culture and differentiated into fibroblasts and myofibroblasts. Maspin synthesis was down regulated in SFDM and decreased with phenotypic change. Maspin mRNA and protein were re-expressed in passage four FBS converted stromal fibroblasts using 5-Aza-2'deoxyctidine-dC (5-Aza-dC), a DNA methyl transferase inhibitor with histone demethylase activity. Our study suggests that during the conversion of stromal keratocytes to corneal wound healing cells, hypermethylation and histone H3 dimethylation are involved in the mechanism by which the maspin gene is down-regulated.

2. Methods and Materials

2.1 Isolation and Culture of Human Corneal Stromal Cells

Human corneas (obtained from Wisconsin Lion Eye Bank, Madison, WI or National Disease Research Interchange, Philadelphia, PA) were isolated by collagenase digestion, as previously described by Taylor et al. (Taylor et al., 1989). Some cells were directly plated and cultured for three days in serum free defined medium (SFDM) composed of DMEM containing 1% RPMI vitamin mix, 100 μ M non-essential amino acids, 1 mM pyruvate, 0.5 mM ascorbic acid and 10 μ g/ml ciprofloxacin (Bayer, Kankakee, IL) (Jester et al., 1996). For passage and expansion of the corneal stromal cells, the cells were cultured in high-glucose DMEM with L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 5% FBS defined (HyClone, Logan, UT), 0.1% Mito⁺ serum extender (Collaborative Research), and 10 μ g/ml ciprofloxacin (Bayer) at 37°C in a 5% CO₂ humidified atmosphere. For generation of defined fibroblasts and myofibroblasts, corneal stromal cells were cultured on vessels coated with type I collagen (Inamed, Los Angeles, CA) in SFDM containing 10 ng/ml FGF-2 (Sigma-Aldrich, St. Louis, MO) for defined fibroblasts or 1 ng/ml TGF- β 1 (Sigma-Aldrich) for myofibroblasts for 7 days with a change of medium every 2 days (Jester et al., 1996). As a control, cells were grown in SFDM with no additives. All experiments were confirmed using cells from two to three different donors.

2.2 RT-PCR and Real Time RT-PCR

Total RNA was extracted from corneal stromal cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. One μ g corneal stromal RNA was reverse-transcribed using SuperScript III reverse transcriptase and random hexamers (Invitrogen). For PCR, the cDNA was amplified under the following PCR conditions: 2 min at 94°C for 1 cycle; then 30 cycles at 94°C for 30 sec, 53°C for 30 sec and 72°C for 60 sec; final elongation step at 72°C

for 5 min. The following primers which hybridized to sequences in different exons were used: maspin sense: (nucleotides +727–784) 5'-AAG CTT TTT CGT GGA TGC CAC AGG ACT-3', maspin antisense: (nucleotides +1212–1234) 5'-ACA GAA AAG TCA GGG AGG-3' and β -actin (Accession Number BC016045) sense: (nucleotides 755–775) 5'-TGG CCA CGG CTG GCT TCC AGC T-3', β -actin antisense: (nucleotides 904–884) 5'-TTT CGT GGA ATG CCA CAG GAC T-3'. The amplified β -actin fragment was used as an internal control. The amplified products were electrophoresed on a 1.5% agarose TAE gel, stained with ethidium bromide (Sigma-Aldrich) and viewed by UV light.

For real time PCR, the following LUX primers to sequences in different exons were labeled with FAM: Maspin (Accession Number NM_002639) sense: 5' –CGT AGA AAA CTA ATC AAG CGG CTC TA[FAM] G -3' (nucleotides +320 to 345), maspin antisense 5' –CCA ATT CCT TTG CAT AGG GTC TC -3' (nucleotides +374 to 416) and GAPDH (Accession Number NM_002046) sense: 5' –CGT TGG GTG AAG GTC GGA GTC AA[FAM] G -3' (nucleotides +106 to 128), GAPDH antisense 5' –GGC AAC AAT ATC CAC TTT ACC AGA -3' (nucleotides +175 to 198). The cDNA was amplified using Brilliant II QPCR Master Mix per manufacturer's protocol (Stratagene, La Jolla, CA) under the following Two-step PCR conditions: 10 min at 95°C for 1 cycle; then 40 cycles at 95°C for 30 sec, 60°C for 60 sec using the Mx3005P system (Stratagene). Maspin mRNA quantification was normalized using GAPDH,

2.3 Western Blot Analysis

Total protein extraction was carried out by using modified RIPA lysis buffer (Cella et al., 2006): 50 mM Tris pH 7.4, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM Na_3VO_4 , 10 mM NaF, 10 mM β -glycerol phosphate, 1 mM EDTA pH 8.0, 1 mM EGTA, 100 mM lactose, 1 mM PMSF, 5 $\mu\text{g}/\text{ml}$ aprotinin, and 5 $\mu\text{g}/\text{ml}$ leupeptin (Sigma-Aldrich). Twenty μg of total protein was electrophoresed on a 10% SDS-PAGE gel under reducing conditions and electrotransferred to nitrocellulose membrane (Bio-Rad). The membrane was incubated with a primary mouse monoclonal antibody to human maspin (16 ng/ml, BD Pharmingen, San Jose, CA) or a rabbit anti-human GAPDH antibody (66 ng/ml; Chemicon, Temecula, CA) diluted in 1% BSA in TBS-T. The blot was incubated with the secondary monoclonal antibody; goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) (BioRad, Hercules, CA) diluted 1:5,000 in 1% BSA TBS-T. Maspin and GAPDH protein was detected by chemiluminescence using ECL (GE Healthcare, Piscataway, NJ).

2.4 Immunofluorescence Microscopy

Human corneal stromal cells (1×10^4) were plated on glass coverslips (1-mm thickness) that were precoated with type I collagen (Inamed) and grown in defined medium in the presence or absence of 5% FBS, 10 ng/ml FGF-2 or 1 ng/ml TGF- β 1 under conditions described above. To localize endogenous maspin, cells were fixed in 3.7% formaldehyde in PBS and permeabilized with 0.1% Triton X-100. Fixed monolayers were blocked by treatment with 1% chicken serum, incubated with primary monoclonal antibody for maspin (1:100; BD Pharmingen) and then with AlexaFluor 594 chicken anti-mouse IgG (1:200; Invitrogen). The cells were also reacted with bis-benzimide (Hoechst 33258, Sigma-Aldrich) for nuclear staining and/or FITC-phalloidin (Sigma-Aldrich) for F-actin staining. Cells treated with TGF- β 1 for myofibroblasts were incubated with a rabbit polyclonal anti-human maspin (1:100; Oncogene Research Products), AlexaFluor 594 chicken anti-rabbit IgG antibody (1:100; Invitrogen), a mouse monoclonal antibody to α -smooth muscle actin (1:400; Sigma), AlexaFluor 488 chicken anti-mouse IgG antibody (1:100; Invitrogen) and bis-benzimide (1:100). Control slides were stained with AlexaFluor 594 Chicken Anti-Mouse IgG (1:100) but not the primary mouse antibody to maspin. Coverslips were mounted on slides with

Fluorogard (Vector Laboratories, Burlingame, CA) and examined and photographed with Nikon Eclipse 80i fluorescence microscope (Nikon Instruments, Melville, NY).

2.5 Sodium Bisulfite Sequencing

Total genomic DNA from corneal stromal cells directly isolated from corneas, cultured keratocytes, passage 1 (P1) and P4 FBS cultured corneal stromal cells, FGF-2 or TGF- β 1 treated P1 cells or defined medium cultured cells was isolated using TRIzol (Invitrogen) according to manufacturer's protocol. Two μ g genomic DNA was denatured and sodium bisulfite (Sigma-Aldrich) treated at 55°C overnight in dark (Murakami et al., 2004). The treated DNA was desalted using Wizard DNA Clean Up System (Promega, Madison, WI) and desulfonated with NaOH. Genomic DNA was precipitated and resuspended in 100 μ l Tris (100 mM, pH 8.0).

The maspin promoter was amplified in the region that includes CpG sites from -254 to +152 relative to the transcription start site under conditions used by Murakami et al. (Murakami et al., 2004). This region was chosen based on the region most often studied in previous papers and is methylated under the same as two downstream CpG islands (Futscher et al., 2002). The amplification was performed using bisulfite-modified DNA in 50 μ l reaction using 1 μ M U2 primer (-284 to -255 nt of maspin promoter), 5'-AAAAGAATGGAGATTAGAGTATTTTGTG-3', D2 primer (+180 to +153 nt of maspin promoter), 5'-CCTAAAATCACAATTATCCTAAAAAATA-3' (Fig 1) under the following conditions: 94°C for 4 min followed by 5 cycles of 94°C for 1 min, 56°C for 2 min, 72°C for 3 min, then 35 cycles of 94°C for 30 sec, 56°C for 2 min, 72°C for 1.5 min and final extension of 72°C for 6 min.

The PCR products were gel purified using Qiaex II Gel Extraction kit (Qiagen, Valencia, CA), A/T cloned into pGEM-T Easy vector (Promega) and transformed into the E. coli strain DH5 α . DNA from ten positive clones per original set of cells was sent to the Protein Nucleic Acid Facility at MCW for sequencing. The sequences were compared to the published human maspin promoter sequence (Zhang et al., 1997a).

The bisulfite reaction for each clone was validated by determination of the percentage of cytosines converted to thymidines that are not in the CpG motif. The overall conversion efficiency was 99.8% with the individual sequences being 98 to 100% converted. Of the sequences, 82% had 100% conversion. The patterns of methylation were evaluated using SigmaStat (SYSTAT).

2.6 5-Aza-dC and TSA Treatment

Corneal stromal cells at P4 were plated at 2.5×10^5 cells/well on a 6-well plate (Corning Life Sciences, Corning, NY) in defined medium. The next day, the medium was removed and the cells were treated with SFDM containing 5-Aza-dC (Sigma-Aldrich) dissolved in acetic acid (10 mM stock) at various concentrations between 10 nM and 1 μ M and TSA (Sigma-Aldrich) dissolved in DMSO (10 mM stock) between 10 nM and 1 μ M. Controls contained the medium plus solvents only.

2.7 ChIP assay for histone methylation

P4 human corneal stromal fibroblasts were cultured with and without 5-Aza-dC and/or TSA treatment. At 75% confluence, 1% formaldehyde (Sigma-Aldrich) was added to crosslink associated proteins to DNA. The cells were lysed and the DNA sheared by sonication to 200–1000 bp. After preclearing the samples by incubation with protein G-Sepharose (Sigma-Aldrich), the DNA-protein complexes were reacted with mouse anti-human histone H3-dimethyl K9 (Abcam), an antibody used routinely for ChIP assays and equivalent to other

antibodies used for this purpose (Lawrence et al., 2004). The antibody-histone-DNA complexes were pulled down with protein A/G-Sepharose (Sigma-Aldrich). The protein-DNA crosslinks in the isolated complexes were reversed using 5 mM NaCl at 65°C. The proteins were digested with proteinase K (CalBiochem) and the genomic DNA isolated. PCR was performed using 1 μ M U2 primer (−284 to −256 nt of maspin promoter), 5'-AAAAGAATGGAGATTAGAGTATTTTTGTG-3' and D2 primer (+180 to +153 nt of maspin promoter), 5'-CCTAAAATCACAATTATCCTAAAAAATA-3' primers specific for the promoter of the maspin. Controls were the input DNA and DNA recovered without antibody added to the pull down step.

3. Results

3.1 Maspin mRNA levels in various types of corneal stromal cells

Maspin mRNA levels in stromal keratocytes directly isolated from human corneas was about three fold less than those of epithelial cells also directly isolated from human corneas (Fig 2A, Epi and Stroma in situ). P0 stromal cells cultured in the presence of 5% FBS contained about the same amount of maspin mRNA relative to GAPDH than the freshly isolated in situ cells (Fig 2A Stroma P0 vs. in situ). In contrast, P0 stromal cells directly cultured in serum-free defined medium (SFDM) lost maspin mRNA expression within three days (Data not shown). Maspin mRNA expression was reduced to about 1% in P1 stromal cells cultured in the presence of medium additives (10 ng/ml FGF-2 or 5% FBS for fibroblastic type cells or 1 ng/ml TGF- β 1 for myofibroblasts) relative to the P0 cells (Fig 2A Stroma P0 vs P1). No maspin mRNA was detected in P1 stromal cells cultured in the presence of SFDM.

3.2 Maspin protein levels in various types of corneal stromal cells

Maspin was present in and secreted from corneal stromal cells (Fig 2B and Fig. 3). Maspin (42 kDa) levels within freshly isolated human corneal stromal keratocytes were less than those present in freshly isolated human corneal epithelial cells when the relation to the GAPDH levels was considered (Fig 2B, Epi and Stroma, in situ). Relative to the freshly isolated keratocytes, maspin was decreased in P0 stromal cells cultured in 5% FBS containing medium (Fig 2B, Stroma, in situ vs. P0) and was not present in P0 cells cultured in SFDM (Data not shown). Maspin was further reduced within P1 stroma cells cultured in the presence of 5% FBS or 10 ng/ml FGF-2 and was not detected on western blots in cells grown in SFDM nor 1 ng/ml TGF- β 1 (Fig 2B, Stroma, P1). Additional maspin forms, probably due to post translational modifications, are observed in the epithelial and stromal samples including degradation products (Fig 2 B, asterisks). P0 stromal cells in 5% serum secreted a larger 43 kDa maspin form into the conditioned medium which was the same size as maspin secreted by human corneal epithelial cells (Fig 3). The larger size of secreted maspin relative to that found in cells suggests the presence of posttranslational modifications on the secreted form. Maspin secretion decreased over the culture period despite the increase in cell number. Neither P0 cells cultured in SFDM nor P1 stromal cells secreted maspin (Data not shown). Thus, maspin protein levels, as well as, mRNA levels are reduced with cell passage and phenotypic change for cells established in 5% serum and are not present in SFDM cultured cells.

3.3 Maspin localization in different types of corneal stromal cells

Maspin was observed at highest levels in the cytoplasm and nucleus of P0 FBS cultured stromal cells that still express maspin. Maspin expression varied from cell to cell. For example, only one of the two overlapping cells shown in Fig 4A contained significant amounts of maspin. Variation in maspin present in the cells is not surprising since the overall level of extracted maspin from the P0 cultures is less than that observed in stromal keratocytes freshly isolated from the cornea (Fig 2B). The greatest amount of maspin in P1 stromal cells was observed in the FBS and FGF-2 treated fibroblastic cells (Fig. 4B-C vs. D and E). Maspin was observed

at a low level in the nucleus of many of both sets of fibroblast cells (Fig 4B and C). This staining was specific for maspin (Fig 4B vs. 4F). Only cells treated with FGF-2 retained a low level of cytoplasmic staining (Fig 4C vs. 4B, D and E). Very little maspin was observed in cells treated with TGF- β 1 or SFDM alone (Fig 4D-E). Not all P1 cells were converted by TGF- β 1 to myoblastic type cells which assembled α -smooth muscle actin fibers (Fig 4D). In contrast, the same conditions converted nearly 100% of P4 cells to myofibroblasts (Data not shown).

3.4 DNA methylation of the maspin promoter of various types of corneal stromal cells

To explore the mechanism of maspin down regulation with culture and phenotypic change in human corneal stromal cells, the maspin promoter methylation status was examined between -254 to +152 relative to the transcription start site, a region previously studied for other cell types (Futscher et al., 2002). As expected, based on the levels of maspin mRNA and protein (Fig. 2A and B), the maspin promoter of stromal cells freshly isolated from donor corneas was hypomethylated (Fig. 5A). Although maspin mRNA and protein were still present in P0 stromal cells cultured with 5% FBS (Fig. 2A and B), the maspin promoter was hypermethylated (Fig. 5B). The maspin DNA in the non-expressing SFDM cultured P0 cells was also hypermethylated (Fig 5C). In contrast, the promoter in the maspin synthesizing P1 corneal epithelial cells (Fig. 2) was hypomethylated (Fig 5D). The P0 epithelial cells contained maspin at similar levels to freshly isolated epithelial cells (data not shown). Passage of the P0 cells into SFDM (Fig 5E) or this medium with the addition of 5% FBS (Fig. 5F) or 10 ng/ml FGF-2 (Fig. 5G) for the fibroblast phenotype or 1 ng/ml TGF- β 1 (Fig. 5H) for the myofibroblast phenotype did not significantly alter the methylation patterns relative to that for the P0 stromal cells (Fig. 5B and 5C). Examples of the sequences obtained following bisulfite treatment are given in Fig 5I. In these cells, 81–85% of the potential CpG methylation sites modified in the analyzed region. These results show that the maspin promoter is methylated soon after the human stromal cells were removed from the intact cornea and placed in culture. For a given condition, the levels of mRNA (Fig 2) were not correlated with the total methylation of the promoter (Fig 5) as determined by linear regression.

3.5 Effect of 5-Aza-dC and TSA on maspin reexpression

To further establish a relationship between promoter methylation and maspin gene expression, maspin-negative P4 stromal cells cultured in the presence of FBS were treated with the DNA methyltransferase (DNMT) inhibitor, 5-Aza-dC (5'-Aza-dC) and/or the histone deacetylase inhibitor, trichostatin A (TSA). Treatment with increasing concentrations of 5-Aza-dC showed increased expression of maspin mRNA (Fig. 6A) with highest expression at 500 nM, whereas, reexpression was not observed with treatment of TSA alone (Fig. 5B). At 1,000 nM 5-Aza-dC, maspin mRNA levels decreased and most of the cells detached from the plates (data not shown). The maspin mRNA reexpression levels in the presence of 5-Aza-dC were low (<0.5%) relative to that for P0 cultures (Fig. 6C). The amount of mRNA was increased at 72 hrs relative to that at 48 hrs. Addition of TSA alone does not increase maspin expression but acetylation secondary to demethylation may increase maspin expression. To test if TSA can synergistically increase maspin expression, both 5-Aza-dC and TSA were added to the P4 stromal cells. Maspin mRNA levels were increased in the presence of 10 nM TSA at 48 but unchanged at 72 hr post treatment compared to 5-Aza-dC alone (Fig. 6C) but the maspin protein levels were decreased at both time points (Fig. 6E and F). With higher levels of TSA (100 and 500 nM) added, both mRNA and protein levels decreased at 48 and 72 hrs (Fig. 6C-E). These results would suggest that maspin gene activation is due to demethylation of DNA and/or histones and that histone acetylation does not enhance maspin expression.

To determine whether the low level of maspin reexpression in the presence of 5-Aza-dC correlated with promoter demethylation, sodium bisulfite sequence analysis was performed. The maspin promoter methylation state of the 5-Aza-dC treated P4 stromal cells was similar

to that for the non-treated and TSA treated cells (Fig 7A-C). This suggests that the low level of reexpression of maspin is not due to DNA demethylation of the maspin promoter. In the presence of 5-Aza-dC and TSA, a greater amount of maspin promoter demethylation was observed (Fig. 7D) relative to 5-Aza-dC or TSA alone (6B and C). This was surprising since the amount of maspin mRNA and protein was not greatly increased over that for 5-Aza-dC alone (Fig. 6C). This result suggests that the presence of TSA induces an accessible chromatin state which increases the efficiency of the DNMT inhibitor 5-Aza-dC and thus reduces cytosine methylation of the maspin promoter. However, the reduced cytosine methylation does not increase transcription of the maspin gene by the corneal stromal cells relative to that for the 5-Aza-dC or TSA treated cells. For a given condition, the levels of mRNA (Fig 6) were not correlated with the total methylation of the promoter (Fig 7) as determined by linear regression.

Since the low level of maspin reexpression observed (Fig 6C-F) may result from histone demethylation rather than DNA demethylation, a ChIP assay was carried out using an antibody to the K9-dimethyl form of histone H3 for immunoprecipitation. Histone H3 of untreated P4 human corneal stromal cells was methylated while histone H3 of TSA, 5-Aza-dC and TSA plus 5-Aza-dC treated cells were not methylated (Fig 8). This suggests not only the DNMT inhibitor 5-Aza-dC but also the histone deacetylase inhibitor TSA can disrupt histone methylation and allow low levels of transcription of the maspin gene.

4. Discussion

In this study, maspin was down regulated during the conversion of human corneal stromal keratocytes to phenotypically distinct fibroblast and myofibroblast cells and cells in the presence of SFDM. Down regulation of gene expression and protein synthesis was progressive with assumption of the typical fibroblastic morphology. Maspin was observed in the nucleus and in the cytoplasm of P0 cells, some in both locations in P1 stromal cells treated with FGF-2 and only nuclear maspin in P1 cells treated with FBS. The role of cytoplasmic and nuclear maspin is not clearly understood, however some hints can be obtained from the effect of maspin interaction with proteins in these two parts of cells. In the nucleus, maspin binds to and inhibits histone deacetylase (Li et al., 2006) and binds to interferon regulatory protein-6 (Bailey et al., 2005). The presence of maspin prevents interferon regulatory protein-6 induced increases in N-cadherin expression in the invasive MDA-MB-231 mammary carcinoma cells. In the cytoplasm, maspin interacts with glutathione-S-transferase and enhances its activity thus playing a regulatory role in oxidative stress (Yin et al., 2005). The presence of maspin in the nucleus of some carcinoma cells indicates a better prognosis than if maspin is only found in the cytoplasm (Sood et al., 2002). The implications of maspin in the nucleus vs the cytoplasm await further research.

In the present study, we demonstrated that the overall methylation state of the maspin promoter is altered in primary human corneal stromal cells in culture. This is consistent with the function of DNA methylation which is important in X-chromosome-inactivation, development, and tumorigenesis (Holliday and Pugh, 1975; Riggs, 1975; Tycko, 2000). Recently, promoter methylation of maspin was shown to regulate tissue-specific gene expression and changes in gene expression upon transformation of normal cells to carcinoma cells (Domann et al., 2000; Domann and Futscher, 2003; Fujisawa et al., 2005; Murakami et al., 2004; Ohike et al., 2003; Sato et al., 2004; Yatabe et al., 2004). Conversion of the maspin promoter from the hypomethylated state observed in freshly isolated (non-cultured) stromal cells to a hypermethylated state occurred between the time corneal keratocytes were released from the matrix of the cornea and when the non-dividing P0 stromal cells in SFDM were harvested at three days or the P0 cells cultured in the presence of 5% FBS became confluent. This *de novo* methylation of the maspin gene in the P0 cells probably is carried out by DNMT-3a and b which do not require a methylation template and do not require cell division for activity (Okano

et al., 1999). The phenotype of these P0 cells cultured in the presence of FBS retained some of the characteristics of keratocytes in that they have not yet assumed the characteristic elongated shape of fibroblasts (Ngamkitidechakul et al., 2001). Hypermethylation of the maspin promoter also occurred early in the conversion of mammary epithelial cells to carcinoma cells (Futscher et al., 2004). The maspin promoter in ductal carcinoma in situ cells (DCIS), an intermediate cell type between normal mammary epithelial cells and carcinoma cells, is often hypermethylated when maspin mRNA and protein are still observed. Maspin mRNA and protein were also observed at high levels for the P0 corneal stromal cells cultured in the presence of FBS and at low concentrations for the P1 corneal stromal cells cultured in the presence of FBS, FGF-2 or TGF- β 1 despite promoter hypermethylation. This may reflect a long half life for the message and protein, or residual transcription of the maspin gene. Alternatively, since RNA polymerase II can bind and initiate transcription of methylated DNA (D'Alessio et al., 2007), the observed mRNA may be newly transcribed.

Hypomethylation of the maspin promoter was concordant with high levels of maspin in the in situ cells and hypermethylation of the promoter was concordant with low levels of maspin expression in the P0 cells cultured in SFDM and in the P1 cells. However, this did not hold for the P0 stromal cells cultured in the presence of 5% serum which were hypermethylated yet expressed maspin mRNA at the same levels as that observed for the hypomethylated in situ stromal cells. DNA methylation is only one component in the downregulation of gene transcription. Methyl CpG binding proteins can bind methylated DNA and repress transcription (Bird and Wolffe, 1999; Yoon et al. 2003; Kuzmichev et al. 2004). In addition, specific modification of histones and binding of proteins that recognize these modifications can repress gene expression (Reviewed by Ruthenburg et al. 2007 and Taverna et al. 2007). The present study would predict that the rate of downregulation of maspin expression through these mechanisms is prolonged by the presence of FBS in the stromal cell cultures. The interaction of proteins with the methylated maspin DNA or associated histones has not been studied.

Analysis of the patterns of methylation in the region of -247 to 155 of the promoter relative to the mRNA levels in the P0 and P1 cells showed total methylation in non-expressing SFDM cells at positions 12 and 32 while at least one of the clones from the maspin expressing P0 and P1 cells were demethylated at these sites. This pattern did not hold for the P4 cells. The non-expressing cells produced clones that were demethylated at one or more of these sites. Position 194 was methylated in the maspin non-expressing P4 cells (untreated and TSA treated) and was demethylated in at least one clone from the maspin expressing 5-Aza-dC and 5-Aza-dC plus TSA treated cells. This difference in methylation was true for the maspin non-expressing P0 but not the P1 cells cultured in SFDM. Analysis of the methylation patterns at the individual sites between the low and non-expressing cells by the Kruskal-Wallis One Way Analysis of Variance on Ranks showed no significant differences between these two groups. Thus maspin methylation patterns in the corneal stromal P0, P1 and P4 cells are probably random as previously found for the maspin promoter in other tissues (Futscher et al., 2002).

The DNA methyl transferase inhibitor 5-Aza-dC at 500 nM induced low levels of maspin reexpression without significant demethylation of the maspin promoter. However, this treatment was able to demethylate the H3 histones associated with the maspin promoter. This probably resulted in alteration of the chromatin allowing a very low level of transcription by RNA polymerase II, which binds and initiates transcription of methylated DNA (D'Alessio et al., 2007). DNA demethylation of the maspin gene probably follows this low level of transcription as suggested by D'Alessio et al. 2007.

The observation that 5-Aza-dC at 500 nM induced low levels of maspin reexpression without significant demethylation of the maspin promoter is not unique to the corneal stromal cells. Low levels of maspin reexpression were observed with 5-Aza-dC in mammary carcinoma cell

lines without demethylation of the maspin promoter (Wozniak et al., 2007). Use of small interfering RNA to the DNA methyltransferase, DNMT1 increased the expression of maspin in MDA-MB-231 breast mammary carcinoma cells supporting DNA methylation in the regulation of maspin expression.

Even though the primary function of 5-Aza-dC at 10 μ M or less is to inhibit the DNA methyltransferase, DNMT-1, it could have another role as a histone methyltransferase inhibitor (Kondo et al., 2003; Murakami et al., 2004; Wozniak et al., 2007; Zhu et al., 2001). This role has been demonstrated for histone H3 associated with the maspin gene. Treatment of P4 corneal stromal cells with 5-Aza-dC demethylated histone H3 at K9 in the same manner as was observed in mammary carcinoma cells (Wozniak et al., 2007).

Reexpression of maspin may depend on factors other than demethylation of histone H3. Despite demethylation of this histone by TSA, maspin transcription was not stimulated by this HDAC1 inhibitor in the P4 corneal stromal cells. Demethylation of histone H3 associated with the genes for Ikaros and FGFR2 occurs upon TSA treatment of pituitary cells (Zhu et al., 2007a; Zhu et al., 2007b). In contrast to the maspin gene in corneal stromal fibroblasts, FGFR2 and Ikaros were re-expressed in pituitary cells with TSA treatment alone. The inability of TSA to induce the reexpression of maspin is a characteristic of the human corneal stromal fibroblasts since this reagent can also induce maspin transcription in hepato-biliary tract carcinomas and in normal pancreatic cells (Fujisawa et al., 2005; Ohike et al., 2003).

As suggested by Wozniak et al. (Wozniak et al., 2007), the lack of demethylation of the maspin promoter when maspin is re-expressed in response to 5-Aza-dC could be due to gene reactivation from a few alleles that become demethylated in the cell population. This might account for the really low maspin levels (mRNA and protein) observed in the presence of 5-Aza-dC. Another possibility is that a non-epigenetic factor is at play here. There may be a transcription factor necessary for maspin gene transcription or a RNA regulatory molecule required for stability that is down-regulated during the phenotypic change from human stromal keratocytes to fibroblastic and myofibroblastic cells that is up regulated at low levels in the presence of 5-Aza-dC but not TSA.

The role of methylation of the maspin promoter across species is not known. The only species besides humans for which the distribution of maspin synthesis across tissues has been reported are rat and mouse (Zhang et al., 1997c; Umekita et al., 1997). The distribution is similar for all three species. This would suggest that regulation of expression, including regulation by DNA methylation is conserved. Using the Vista Genome Browser (pipeline.lbl.gov/), multiple areas of high identity were found when 50 kb 5' and 3' of the maspin transcription start site were examined for human vs chimpanzee, dog and mouse (Fig 9A, arrows). These areas represent potential important binding sites for transcription factors that may regulate maspin synthesis. When any one of these areas was queried with the transcription binding site programs, TFBIND (<http://tfbind.hgc.jp/>) and PATCH (<http://www.gene-regulation.com/pub/programs.html#patch>) hundreds of potential sites were identified. Future studies will identify the importance of these sites.

Because some transcriptional binding sites have been identified in the region of the transcription start site, this area was selected for more in depth *in silico* exploration. Comparison of multiple species using the Vertebrate Multiz Alignment of the UCSC Genome Browser (<http://genome.ucsc.edu/cgi-bin/hgGateway>) revealed considerable identity with the human maspin promoter between -284 and +184 (Ch18: 59,294,792-59,295,260) (Fig 9B). The greatest identity is among human, chimpanzee and rhesus, less with dog, cow and rabbit and even less with mouse and rat. The chimpanzee and rabbit contain 20 and 24 potential CpG methylation sites, respectively, in comparison to the 22 CpG sites that are methylated in the

human maspin promoter. Rhesus, cow, mouse and rat have 15 or less sites in this region. If all species use CpG methylation of this region for control, the most important methylation sites for silencing the gene may be -170, -103 and 32 which are conserved in 6 or 7 of the 8 species compared. There is no consistent pattern for these sites between the low expressing (0.1–5%) cells and those that do not express maspin. The importance of individual CpG sites awaits further experimentation. Use of reporter constructs is problematic to study regulation of gene transcription by CpG methylation because chromatin associated with plasmids is abnormal (Jeong et al., 1994).

The identified transcription factor binding sites in this region experimentally elucidated by previous studies include the sequences in the negative regulator site HRE and the positive regulator binding sites, ETS, AP1, ATF-126 and ATF-2 sites (Maekawa et al., 2007; Zhang et al., 1997 a and b; Beltran et al., 2007). These sites are 60–83% identical with human suggesting these transcription factors may be active across the different species (Fig 9B). Although p53 is known to stimulate maspin transcription, the exact sites that are important for p53 binding have not been determined. There are two p53 binding sites between -103 and -1 with at least 50% and 75% identity with human (Fig 9B). The first site has at least one CpG site associated with the exception of dog. The most homologous region within the -284 to 181 region is -119 to 23 (Fig 9B). Within this region, there are multiple potential transcription binding sites including SP1, CAP, GC, ELK 1, HSF 1, HSF2, E47, GATA1, CETSIP54, NRF 2, LYF 1, E2F, CP2, YY1, NYCMA X, STAF, OLF 1, CEBPB, SRY, NF 1, MYO D, AP2, PAX 5, XFD 3, AHRARNT, RFX1, MYB, AP4, SOX 5, T3R, CJUN, NRF, GFI, IRF 2, YM, P300, AP 1, HOX 13, OCT 1, SRF, MZF 1, USF, NFkB, EGR 1 and SEF 1 as determined by the TFBIND program. This region of the maspin gene is only one of many in the 50 kb on the 5' and 3' side of the transcription site that is highly conserved across several species (Fig 9A).

One of the open questions is whether DNA methylation regulates corneal wound healing *in vivo*. For this analysis, an animal model is needed, preferably one that has CpG islands and CpG sites in similar regions to the human. Based on the presence of 24 CpGs (Fig 9B) and an identified CpG island using MethPrimer (<http://www.urogene.org/methprimer/>) in the same area as those for the human, the overall identity of the sequence and the size of the cornea, the rabbit model of corneal wound healing probably is the best. These studies are being initiated in our laboratory.

Genes other than maspin may be controlled by DNA methylation upon conversion of corneal stromal keratocytes to fibroblasts. Examples of molecules down-regulated to undetectable levels upon this phenotypic change include the keratin sulfate proteoglycans, lumican and keratocan, and the corneal crystallins, aldehyde dehydrogenase-1, aldehyde dehydrogenase-3 and α -transketolase (Saghizadeh et al., 2005). CpG islands in the 5' UTR and 1000 bp upstream of the start site were found in aldehyde dehydrogenase-3 and α -transketolase using MethPrimer. In addition, DNA demethylation may play a role in the keratocyte to fibroblast conversion. The promoter for tenascin-C, a gene that is not observed in keratocytes but is expressed by corneal fibroblasts, has two CpG islands. Thus, DNA methylation may be pertinent to the changes in gene expression observed upon cornea wound healing.

The elucidation of corneal wound healing mechanisms is important based on the number of refractive surgeries performed. Analysis of corneas following LASIK and PRK by non-invasive techniques and post mortem has revealed altered structure of the cornea even 7–10 years following these procedures (Kramer et al., 2005). The role of maspin in corneal wound healing is an area of intense study in our laboratory. Our hypothesis is that downregulation of maspin synthesis upon phenotypic change of stromal keratocytes to wound healing fibroblasts and myofibroblasts allows migration of these cells into the wounded area. Upon reestablishment of the epithelial sheet, maspin is secreted from the epithelial cells and inhibits

migration of the stromal cells. Exogenously added maspin inhibits migration of cultured human corneal stromal fibroblasts and the closure of epithelial wounds in organ culture (Unpublished Data). Maspin also upregulates the synthesis and secretion of plasminogen activators involved in extracellular matrix remodeling (Unpublished Results) indicating that maspin may stimulate the turnover of proteins that contribute to corneal opacities.

This is the first study to show down regulation of a gene is epigenetically controlled during the conversion of corneal keratocytes to fibroblasts. Down regulation of maspin synthesis was shown to involve methylation of the maspin promoter on CpG islands and dimethylation of K9 of the histones associated with the maspin gene. Future research will determine the universality of this mechanism for turning off genes during the phenotypic change of corneal keratocytes to fibroblasts.

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Fig. 1.

Diagram of the maspin gene promoter region. The numbers refer to the sequence of the maspin promoter as defined by Sager (Zhang et al., 1997) and colors red and blue refer to the 5'UTR and promoter, respectively. The asterisks denote location of CpG positions at -247, -194, -178, -170, -163, -151, -147, -135, -103, 9, 12, 17, 22, 27, 32, 55, 60, 62, 101, 105, 134 and 155. The bent arrow indicates the transcriptional start site. The location of the PCR primers are indicated by U2 and D2 primers at positions -284 to -256 and +184 to +157, respectively.

Fig. 2.

Down regulation of maspin expression upon phenotypic change of corneal cells. In situ human epithelial and stromal cells were both directly harvested from human corneas and extracted for protein and mRNA or placed in culture in 5% FBS supplemented defined medium and cultured until confluent (P0). Stromal cells (P1) were cultured for seven days in serum free defined medium (SFDM) alone or treated with 5% FBS or 10 ng/ml FGF-2 for fibroblastic-like cells or 1 ng/ml TGF- β 1 for the myofibroblast phenotype. The cells were extracted for protein or mRNA. (A) Maspin and GAPDH cDNA was generated by the RT reaction and real time PCR was used to determine the cDNA levels of maspin and GAPDH. Data was normalized for GAPDH and then expressed relative to the in situ stromal cell maspin mRNA levels. Insert: Melting curves for maspin and GAPDH cDNA. Only one peak was observed for each, maspin (left) and GAPDH (right), with each giving the calculated t_m of the amplified DNA (B) Western blots of extracted maspin relative to GAPDH. Note: Because of the differences in maspin levels in the samples, the loading levels of protein were different as observed in the GAPDH levels. The exposure time for the maspin blots was much longer than that for the GAPDH blot.

*Denotes post transcriptionally modified forms of maspin.

Fig. 3.

Decreased secretion of maspin from stromal P0 cultured cells with time in culture. Epithelial and stromal cells were harvested from human corneas. The epithelial cells were cultured in keratinocyte medium and the stromal cells were cultured in 5% FBS supplemented defined medium (P0) until confluent. The medium was collected and changed every two days. The proteins in equal volumes of medium were separated by SDS-PAGE, electrotransferred to nitrocellulose and the blot probed with mouse monoclonal antibodies to human maspin and a goat anti-mouse IgG coupled to horseradish peroxidase.

Fig. 4.

Down regulation of maspin expression and alteration of maspin localization upon phenotypic change of corneal stromal cells. Stromal cells were harvested from human corneas and cultured in 5% FBS supplemented defined medium (P0) (A). Stromal cells were passed (P1) and cultured for seven days in serum free defined medium (SFDM) alone (E) or treated in the same medium with 5% FBS (B, F) or 10 ng/ml FGF-2 (C) for fibroblastic like cells or 1 ng/ml TGF- β 1 (D) for the myofibroblast phenotype. The cells were fixed, permeabilized and incubated with mouse monoclonal maspin antibodies and AlexaFluor-594 chicken anti-mouse IgG (A–C, E, red), FITC-phalloidin for F-actin (A–C, E, F, green) and bis-benzimide for nuclear DNA (A–F, blue). Cells treated with TGF- β 1 were stained with rabbit polyclonal antibodies to maspin, AlexaFluor 594 chicken-anti rabbit IgG (D, red), mouse monoclonal antibodies to α -smooth muscle actin, AlexaFluor 488 chicken-anti mouse IgG (D, green). The control (F) was not incubated with the maspin antibodies. Magnification bar = 50 μ m

Fig. 5. Hypomethylation of the maspin promoter in human corneal stromal cells in situ and hypermethylation of the maspin promoter in cultured human corneal stromal cells. The methylation state of cytosines was obtained by bisulfite-modified genomic DNA sequencing of the maspin promoter [-254 to +152] obtained from human corneal stroma cells freshly isolated from a donor cornea (A, in situ), P0 stromal cells cultured in 5% FBS (B), P0 stromal cells cultured in serum free defined medium alone (SFDM) (C), P0 epithelial cells cultured in defined keratinocyte medium (DKM) (D), and P1 stromal cells cultured in SFDM (E), 5% FBS (F), 10 ng/ml FGF-2 in SFDM (G), and 1 ng/ml TGF- β 1 in SFDM (H). Each row of squares represents the methylation state of an individual clone of the *maspin* promoter. Each square represents a CpG dinucleotide site. The filled squares are methylation-positive and the open squares are methylation-negative. The number at the top indicates position of each CpG site from the transcription start site. (I) cDNA sequences of clones from sodium bisulfite treated Stroma In Situ (I: ^{a+} plasmid a in A) and P1 Stromal Cells SFDM (I: ^{b+} plasmid b in E) and without treatment (I: -). The CpG methylation sites are numbered relative to the major transcription start site (Bent arrow). The overall conversion efficiency of non-CpG cytosines to thymidines was 99.8 %.

Fig. 6.

Reexpression of maspin in FBS converted fibroblasts using 5-Aza-dC. P4 stroma cells were cultured in 5% FBS and treated with increasing amounts of 5-Aza-dC (A) or TSA (B) for 48 hrs or with 5-Aza-dC plus increasing amounts of TSA for 48 hrs or 72 hrs (C and D). The cells were extracted for RNA and protein. Maspin and β -actin mRNA were amplified by RT-PCR (A and B). Maspin and GAPDH mRNA were amplified by quantitative RT-PCR was used to amplify maspin and GAPDH (C). The data was normalized to GAPDH and then expressed relative to maspin mRNA in P0 cells cultured in the presence of 5% FBS. The proteins were transferred to nitrocellulose and probed with mouse monoclonal antibodies to maspin or GAPDH followed by a goat anti-mouse IgG coupled to horseradish peroxidase (D). The exposure time for the maspin blots was much longer than that for the GAPDH blot resulting in more non-specific bands.

Fig. 7.

Lack of demethylation of the maspin promoter upon 5-Aza-dC/TSA treatment. Aberrant hypermethylation of the maspin promoter was observed in P4 stromal cells cultured in the presence of 5% FBS (A), 500 nM 5-Aza-dC (B), and 10 nM TSA (C) treated at 48 hr. However, the combined treatment of 5-Aza-dC and TSA (D) reduced the methylation state of maspin promoter after 48 hrs. Each row of squares represents the methylation state of an individual clone of the maspin promoter. Each square represents a CpG dinucleotide site. The filled squares are methylation-positive and the open squares are methylation-negative. The number at the top indicates position of each CpG site from the transcription start site.

Fig. 8.

Demethylation of histone H3 with TSA and 5-Aza-dC treatment. P4 human corneal stromal fibroblasts converted with FBS were cultured with or without TSA, 5-Aza-dC (Aza) or Aza plus TSA treatment for 48 hrs. At 75% confluence, the DNA was crosslinked with associated proteins using 1% formaldehyde to DNA and prepared for ChIP assays. Sheered DNA-protein complexes were reacted with mouse anti- human histone H3-dimethyl K9 and pulled down with protein A/G-Sepharose. The crosslinks were reversed, the proteins digested with proteinase K and the genomic DNA isolated. PCR was performed using primers specific for the promoters of the maspin. Controls were the input DNA (Input) and DNA recovered with only protein A/G-Sepharose.

Figure 9.

In silico analysis of the maspin gene across species. Areas of high identity between human and chimpanzee, dog and mouse maspin genes were calculated using the Vista Genome Browser (pipeline.lbl.gov/) for Ch18:59,244,500–59,344,500 and are given in pink (A). Arrows denote regions of high identity across the three species. * Denotes the transcription start site and the area expanded in B. The coding sequence for maspin is given in blue. Alignment of human chimpanzee, rhesus, dog, cow, rabbit, mouse and rat calculated by the UCSC Genome Browser (<http://genome.ucsc.edu/cgi-bin/hgGateway>) for Ch18:59,294,792–59,295,260 (B). CpG methylation sites are in italics and underlined, and numbered relative to the major transcription start site (Bent Arrow).