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Summary Maspin, which belongs to the serine protease inhibitor (serpin) superfamily, has been proposed as a potent tumor suppressor that inhibits cell motility, invasion, angiogenesis, and metastasis. In the present study, we examined the effects of 5-Aza-2'-deoxycytidine (5-Aza-dC), a demethylating agent, and FR901228, a histone deacetylase (HDAC) inhibitor, on maspin expression in oral cancer cell lines. The expression levels of maspin mRNA were divided into two groups, which was the maspin low-expressed and high-expressed cell lines in the 12 oral cancer cell lines. The maspin promoter contained only a few methylated CpG sites in the maspin low-expressed cell lines. Moreover, the methylation status was not altered after 5Aza-dC treatment. However, the transcription of the maspin gene was clearly increased following 5Aza-dC treatment in a number of oral cancer cell lines. These results imply that an action of 5Aza-dC is separate from induction of promoter demethylation. Treatment with FR901228 resulted in a time-dependent stimulation of the re-expression of maspin mRNA as early as 4 hours after treatment in the maspin downregulated cells. The re-expression of the maspin gene may contribute to the recuperation of biological functions linked to FR901228 such as an inhibitory effect on tumor angiogenesis and cell invasion. These results indicate that maspin and its target genes may be excellent leads for future studies on the potential benefits of FR901228, a histone deacetylase inhibitor, in cancer therapy.

Keywords: maspin, methylation, 5-Aza-2'-deoxycytidine, FR901228

1. Introduction

Maspin is a Mr 42000 protein that belongs to the serine protease inhibitor (serpin) superfamily and is expressed in the epithelial cells of the airway, breast, skin, and prostate, but not in skin fibroblasts, lymphocytes, bone marrow, or the heart or kidneys [1, 2]. It is known to be a potent tumor suppressor that inhibits cell motility, invasion, angiogenesis, and metastasis [3-8]. Accumulative evidence demonstrates that maspin inhibits the progression of prostate and breast tumors at the late stages of invasion and metastasis [1, 7, 9-12]. In several mammary carcinoma cell lines, maspin is undetectable or is expressed at very low levels [1]. A loss of maspin expression correlates with increased metastatic potential in breast cancer and other human tumors. Maspin-transfected tumor cells tend to have less necrosis, mitogenesis, and neovascularization, which is associated with better prognosis and lower invasiveness [1, 9, 10, 13]. Domann *et al.* [14] and Futscher *et al.* [2] have shown that the silencing of maspin gene expression is inversely correlated with methylation of the promoter. Although the maspin gene is a potential clinical targeted invention, the regulating mechanism remains to be elucidated.

Domann *et al.* [14] have shown that 5-Aza-2'-deoxycytidine (5-Aza-dC), known to be a demethylating agent, causes maspin mRNA levels to recover and suggests that methylation at CpGs, located in the maspin promoter, is the only impediment to maspin expression in a nonexpressing tissue. The maspin proximal promoter harbors many CpGs. Domann's study offers an intriguing theory of epigenetic silencing of the maspin gene.

Among the new classes of chemotherapeutic agents, histone deacetylase (HDAC) inhibitors have aroused considerable interest in the pursuit of improved anticancer agents. HDAC inhibitors increase or decrease the transcriptional levels of genes by causing hyperacetylation of histone,

which is considered to be a key mechanism for regulating transcription. Various HDAC inhibitors such as sodium butyrate (SB), trichostatin A (TSA), and trapoxin are known to inhibit histone deacetylase activities. FR901288, a novel cyclic peptide inhibitor of HDAC, is isolated from a fermentation broth of Chromobacterium violaceum. FR901228 has a stronger cytotoxic activity than TSA, although only a limited number of genes involved are known to increase or decrease their transcriptional levels followed by hyperacetylation of histone.

In the present study, we examined the regulatory effects of 5-Aza-dC and FR901228 on maspin in favor of its epigenetic regulation through a process such as histone acetylation and promoter methylation in oral cancer cell.

2. Materials and methods

2.1. Cell lines and culture

Human oral cancer cell lines (HO-1-u-1, HSC2, HSC3, HSC4, SAS, KB, Hep2, and Ca9-22) were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan), H-O-N-1, KOSC2, and KOSC3 from the Health Science Research Resources Bank (Osaka, Japan), SCC25 from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan), and human breast cancer cell line, MCF-7 obtained from the Cell Resource Center for the Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan). They were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Inc., Logan, UT, USA), 100 units/ml penicillin (Meiji Seika Kaisha, Ltd., Tokyo, Japan) and 100 µg/ml streptomycin (Meiji Seika Kaisha) in a CO2 incubator (Sanyo Electric Co., Ltd., Osaka, Japan) with 95% air plus 5% CO2 at 37℃.

2.2. Chemicals

5-Aza-dC (Sigma Chemical Co., St Louis, MO, USA) and FR901288 (Fujisawa Pharmaceutical Company, Osaka, Japan) diluted in distilled water were added to the DMEM to the final concentrations indicated for each treatment.

2.3. 5-Aza-dC treatment

Stock solutions of 5-Aza-dC were prepared by dissolving the drug at a 10 mM concentration in

distilled water no more than 2 h prior to use in the experiment. Final concentrations were obtained by diluting the stock solution directly into the tissue culture medium. Cells were incubated for 7 days with several different concentrations of 5-Aza-dC ranging from 4.4 to 22 μM.

2.4. FR901228 treatment

First, 1×10^5 cells were seeded in 5 ml MDEM in a petri dish (60 mm in diameter). At 7 days after seeding, the medium was changed to one containing FR901228, and the dish was incubated in a CO_2 incubator with 95% air plus 5% CO2 at 37℃ for an adequate number of hours.

2.5. DNA isolation and bisulfite modification

DNA was isolated by the standard method. Bisulfite treatment was carried out using the CpGenome DNA Modification Kit (Intergen Company, Manhattanville Road, NY) according to the instructions supplied. After bisulfite treatment, the DNA was resuspended in Tris EDTA (*pH* 7.5).

2.6. Bisulfite sequencing

The maspin promoter [15] was amplified from the bisulfite-modified DNA by PCR using primers specific to the bisulfite-modified sequence of the maspin promoter. The amplifications were carried out in 25-μl reaction mixtures containing 1 μl of bisulfite-treated genomic DNA, 2 μl dNTPs, 0.63 μl primers, 0.75 μl MgCl, 2 μl 10×PCR buffer, and 0.25 μl Platinum Taq DNA polymerase (Invitrogen Corp., Carlsbad, CA) under the following conditions: 94℃ for 4 min

followed by 5 cycles of 94℃ for 1 min, 56℃ for 2 min, 72℃ for 3 min, then 35 cycles of 94℃ for 30 sec, 56℃ for 2 min, 72℃ for 1.5 min, and a final extension of 72℃ for 6 min. The first primers were as follows: U2 (nt 673 to nt 703), 5'-AAA AGA ATG GAG ATT AGA GTA TTT TTT GTG-3'; primer D2 (nt 1114 to nt 1141), 5'-CCT AAA ATC ACA ATTATC CTA AAAAATA-3'. The second round primers were as follows: primer U3 (nt 750 to 776), 5'-GAA ATT TGT AGT GTT ATT ATT ATT ATA -3' ; primer D3 (nt 1064 to nt 1090), 5'-AAA AAC ACA AAA ACC TAA ATA TAA AAA -3'. These primer sequences were a generous gift from Dr. B. Futscher. Amplified DNA was ligated into the TA topo cloning vector (Invitrogen, Corp., Carlsbad, CA) and transformed into *E.coli*, Topo 10; at least 5 clones of each fragment were sequenced using a Big Dye terminator sequencing kit (Perkin Elmer, Branchburg, NJ).

2.7. RNA isolation and RT-PCR

Extraction of total cellular RNA was carried out using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was reverse-transcribed with Superscript II Reverse Transcriptase and oligo dT primers (Invitrogen Corp., Carlsbad, CA, USA). Amplification of cDNA was performed under the following PCR conditions: 7 min at 94℃ for 1 cycle; then 28 cycles at 94℃ for 30 s, 55℃ for 30 s, or 72℃ for 30 s; and a final elongation step at 72℃ for 10 min. We used the following primers for amplification, designed by Li *et al.* [16]: Maspin 1, sense: 5'- cac tgg gca atg tcc tct tc -3' (located at 146-165), antisense: 5'- tgg tct ggt cgt tca cac tg -3' (located at 547-528). To confirm our results, additional primer sets for maspin designed by Biliran *et al.* [17] were used in RT-PCR: Maspin 2, sense: 5'- ggg gaa ttc cat gga tgc cct gca act -3', antisense: 5'- ccg gtc tag aca tgg gct atg cca ctt -3'; and

glyceraldehydes-3-phosphate dehydrogenase (GAPDH): sense: 5'-gaa ggt gaa ggt cgg agt c-3', antisense: 5'-caa agt tgt cat gga tga cc-3'. The amplified GAPDH fragment was used as a positive control. The RT-PCR products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and viewed by UV.

3. Results

3.1. Expression of maspin mRNA in oral cancer cell lines

The expression levels of maspin mRNA in 12 oral cancer cell lines were examined by RT-PCR. The gels clearly showed that the Hep2, SAS, KB, and HO-1-u-1 cell lines contained only trace amounts of maspin mRNA (Fig. 1). The other cell lines produced uniformly large amounts of the maspin mRNA, as indicated by the very dense bands that appear in the relevant lanes (Fig. 1).

3.2. Methylation map of the CpG island in the 5' region of the maspin gene CpGs.

We analyzed the methylation map of the CpG island in the promoter of the *maspin* gene in the three low-expressed cell lines (Hep2, SAS, KB). The maspin promoter contains only a few methylated CpG sites in the maspin low-expressed lines (Fig. 2, upper).

3.3. Effects of 5-Aza-dC treatment on the expression and CpG methylation of the maspin gene

To test whether the maspin gene expression was suppressed by hypermethylation of its promoter, we exposed 5-Aza-dC, an inhibitor that prevents methylation of newly synthesized DNA, to three maspin downregulated cell lines (Hep2, KB, and SAS) and two maspin-expressed cell lines (HSC4 and HSC3). Our results clearly show that the five cell lines regained their ability to produce high levels of maspin mRNA following exposure to graded doses of 5-Aza-dC (Fig. 3). We also analyzed the methylation map in the promoter of the *maspin* after 5-Aza-dC treatment in

the three low-expressed cell lines (Hep2, SAS, and KB). After incubation with 4.4 μM 5-Aza-dC in a flask for 7 days, extracted DNA was bisulfited and sequenced as described in the Materials and Methods section. The methylation status was not altered after 5Aza-dC treatment (Fig. 2 lower).

3.4. Effects of FR901228 treatment on maspin transcription

Three maspin downregulated cell lines that express low levels of maspin mRNA (Hep2, SAS, and KB) were incubated with FR901228 at a concentration of 0.5 or 1.0 μM. Two maspin-expressed cell lines (HSC4 and HSC3) were also analyzed as controls. Maspin expression was induced after 4-16 h of treatment with FR901228 in the maspin downregulated cell lines, while maspin transcriptional levels were not altered after treatment with FR901228 in the maspin-expressed cell lines (Fig. 4a). As a negative control, human breast cancer cell line MCF-7, one of the maspin-negative lines, was incubated with FR901228. Its maspin expression was also induced in a time-dependent manner in MCF-7 (Fig. 4b).

4. Discussion

The expression levels of maspin mRNA were divided into two groups, which was the maspin low-expressed and high-expressed cell lines in the 12 oral cancer cell lines. We first decided to examine the methylation status of the CpG island, one of the well-studied sites of epigenetic regulation in the *maspin* promoter region in the maspin low-expressed cell lines. The maspin promoter contained only a few methylated CpG sites in the maspin low-expressed cell lines. Moreover, the methylation status was not altered after 5Aza-dC treatment. However, transcription of the maspin gene was clearly increased following 5Aza-dC treatment in a number of oral cancer cell lines. These results imply that the action of 5Aza-dC is separate from the induction of promoter demethylation. It is possible that 5Aza-dC disrupts complexes between DNA methyltransferases and histone-modifying proteins [18, 19]. Kondo *et al.* [20] have reported that 5Aza-dC dramatically decreases histone H3 Lys-9 methylation, slightly increases Lys-9 acetylation, and moderately increases histone H3 Lys-4 methylation and reactivated gene expression. These findings could explain the previously reported 5Aza-dC induced activation of the un-methylated gene [21, 22] and could also explain the maspin activation in our results.

HDAC inhibitors are known to modulate transcription and exert antiproliferative effects on cancer cells. Only a few target genes whose expression is upregulated in response to HDAC inhibitor-mediated growth arrest have been identified, e.g., the cyclin-dependent kinase inhibitors p21/WAF1 [23-25], c-myc [26, 27], and the anti-apoptotic bcl-2 gene [28, 29]. Among the latest reports, Butler *et al.* have found that Suberoylanilide hydroxamic acid (SAHA), one of the HDAC inhibitors, induces expression of the thioredoxin-binding protein-2 (TBP-2) gene in LNCaP prostate cells [30]. However, the gene responsible for inhibition of the proliferation and induction

of cell differentiation or death by HDAC inhibitors remains elusive.

In the present study, we found that transcription of the maspin gene, whose expression has been implicated in apoptosis pathways as another possible candidate gene, is clearly increased following FR901228 treatment in a number of oral cancer cell lines. However, we could not test the oral cancer cell lines for protein expression because of technical limitations with currently available antibodies. It is thus reasonable to speculate that histone hyper- and hypoacetylation are common underlying features in maspin transactivation and repression, and this may be an important aspect of the regulation of maspin expression in oral cancer cells, which are primarily derived from epithelial cells.

Additionally, our data suggest that maspin re-expressed by FR901228 can be an inhibitory regulator of natural tumor growth through its influence on neovascularization because the tumor suppressor activity of maspin may depend primarily on the inhibition of angiogenesis. Zhang *et al.* [10] have previously reported that maspin blocks the growth of human prostate tumor cells and dramatically reduces the density of tumor-associated microvessels in a xenograft mouse model. In the present study, FR901228 was found to induce the gene expression of angiogenic-inhibitor maspin. Supporting our idea, specific HDAC inhibitors have been shown to inhibit angiogenesis [31, 32], although little has been known about the mechanism of HDAC inhibitor in anti-angiogenesis until now. HDAC inhibitor should undergo further examination not only as an anti-angiogenesis agent, but also regarding its role in maspin re-expression to explore its potential as a possible biomarker for relapse risk in cancer therapy, as alterations in maspin expression are known to play an important role in oral tumorigenesis. In oral squamous cell carcinoma, high levels of maspin expression are associated with the absence of lymph node metastasis and with

better rates of overall survival [33].

In conclusion, our research may provide a first suggestion of FR901228-dependent regulation of maspin in oral cancer. Maspin upregulation by FR901228 may be of critical importance in clinical treatment for cancer, and may contribute to the future development of treatment for human oral cancer.

Acknowledgement

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References

[1] Zou Z, Anisowicz A, Hendrix MJC, Thor A, Neveu M, Sheng S, Rafidi K, Seftor E, Sager R. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. Science (Washington DC) 1994; 263: 526-9.

[2] Futscher BW, Oshiro MM, Wnzniak RJ, Holtan N, Hanigan CL, Duan H, Domann FE. Role for DNA methylation in the control of cell type-specific maspin expression. Nat Genet 2002; 31(2):175-9.

[3] Hendrix MJ. De-mystifying the mechanism(s) of maspin. Nat Med 2000; 6: 374-6.

[4] Sheng S, Pemberton PA, Sager R. Production, purification, and characterization of recombinant maspin proteins. J Biol Chem 1994; 269: 30988-93.

[5] Sager R, Sheng S, Anisowicz A, Sotiropoulou G, Zou Z, Stenman G, Swisshelm K, Chen Z, Hendrix MJ, Pemberton P. RNA genetics of breast cancer: maspin as paradigm. Cold Spring Harbor Symp Quant Biol 1994; 59: 537-46.

[6] Zhang M, Magit D, Sager R. Expression of maspin in prostate cells is regulated by a positive ets element and a negative hormonal responsive element site recognized by androgen receptor. Proc Natl Acad Sci USA 1997; 94: 5673-8.

[7] Seftor RE, Seftor EA, Sheng S, Pemberton PA, Sager R, Hendrix MJ. Maspin suppresses the invasive phenotype of human breast carcinoma. Cancer Res 1998; 58: 5681-5.

[8] Pemberton PA, Wong DT, Gibson HL, Kiefer MC, Fitzpatrick PA, Sager R, Barr PJ. The tumor suppressor maspin does not undergo the stressed to relaxed transition or inhibit trypsin-like serine proteases. Evidence that maspin is not a protease inhibitory serpin. J Biol Chem 1995; 270: 15832-7.

[9] Sheng S, Carey J, Seftor E, Dias L, Hendrix MJC, Sager R. Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. Proc Natl Acad Sci USA 1996; 93: 11669-74.

[10] Zhang M, Volpert O, Shi YH, Bouck N. Maspin is an angiogenesis inhibitor. Nat Med 2000; 6: 196-9.

[11] Zhang M, Shi Y, Magit D, Furth PA, Sager R. Reduced mammary tumor progression in WAP-TAg/WAP-maspin bitransgenic mice. Oncogene 2000; 19: 6053-8.

[12] Zhang M, Sheng S, Mass N, Sager R. mMaspin, the mouse homolog of a human tumor suppressor gene, inhibits tumor invasion and motility. Mol Med 1997; 3: 49-59.

[13] Sager R, Sheng S, Pemberton P, Hendrix MJC. Maspin, a tumor suppressing serpin. Adv Exp Med Biol 1997; 425: 77-88.

[14] Domann FE, Rice JC, Hendrix MJC, Futscher BW. Epigenetic silencing of maspin gene expression in human breast cancers. Int J Cancer 2000; 85 (6): 805-10.

[15] Zhang M, Mass N, Magit D, Sager R. Transactivation through Ets and Ap1 transcription sites determines the expression of the tumor-suppressing gene *Maspin*. Cell Growth & Differ 1997; 8: 179-86.

[16] Li JJ, Colburn NH, Oberley LW. Maspin gene expression in tumor suppression induced by overexpressing manganese-containing superoxide dismutase cDNA in human breast cancer cells. Carcinogenesis 1998; 19 (5): 833-9.

[17] Biliran H Jr, Sheng S. Pleiotrophic inhibition of pericellular urokinase-type plasminogen activator system by endogenous tumor suppressive maspin. Cancer Res 2001; 61 (24): 8676-82.

[18] Fuks F, Burgers WA, Brehm A, Hughes-Davies L, Kouzarides T. DNA methyltransferase

dnmt1 associates with histone deacetylase activity. Nat Genet 2000; 24: 88-91.

[19] Rountree MR, Bachman KE, Baylin SB. DNMT1 binds HDAC2 and a new co-repressor DMAP1 to for a complex at replication foci. Nat Genet 2000; 25: 269-77.

[20] Kondo Y, Shen LL, Issa JP. Critical role of histone methylation in tumor suppressor gene silencing in colorectal cancer. Mol Cell Biol 2003; 23 (1): 206-15.

[21] Milutinovic S, Knox JD, Szyf M. DNA methyltransferase inhibition induces the transcription of the tumor suppressor p21 (WAF1/CIP1/sdi1). J Biol Chem 2000; 275:6353-9.

[22] Soengas MS, Capodieci P, Polsky D, Mora J, Esteller M, Opitz-Araya X, McCombie R, Herman JG, Gerald WL, Lazebni YA, Cordon-Cardo C, Lowe SW. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. Nature 2001; 409: 207-11.

[23] Archer SY, Meng S, Shei A, Hodin RA. p21(WAF1) is required for butyrate-mediated growth inhibition of human colon cancer cells. Proc Natl Acad Sci U S A 1998; 95(12): 6791-6.

[24] Nakano K, Mizuno T, Sowa Y, Orita T, Yoshino T, Okuyama Y, Fujita T, Ohtani-Fujita N, Matsukawa Y, Tokino T, Yamagishi H, Oka T, Nomura H, Sakai T. Butyrate activates the WAF1/Cip1 gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. J Biol Chem 1997; 272(35): 22199-206.

[25] Janson W, Brandner G, Siegel J. Butyrate modulates DNA-damage-induced p53 response by induction of p53-independent differentiation and apoptosis. Oncogene 1997; 15(12):1395-406.

[26] Heruth DP, Zirnstein GW, Bradley JF, Rothberg PG. Sodium butyrate causes an increase in the block to transcriptional elongation in the c-myc gene in SW837 rectal carcinoma cells. J Biol Chem 1993; 268: 20466-72.

[27] Buckley AR, Leff MA, Buckley DJ, Magnuson NS, De Jong G, Gout PW. Alterations in

pim-1 and c-myc expression associated with butyrate-induced growth factor dependence in autonomous rat NB2 lymphoma cells. Cell Growth & Differ 1996; 7: 1713-21.

[28] Mandal M, ande Kumar R. Bcl-2 expression regulates sodium butyrate- induced apoptosis in human MCF-7 breast cancer cells. Cell Growth & Differ 1996; 7: 311-8.

[29] Hague A, Diaz GD, Hicks DJ, Krajewski S, Reed JC, Paraskeva C. Bcl-2 and bak may play a pivotal role in sodium butyrate-induced apoptosis in colonic epithelial cells; however, overexpression of bcl-2 does not protect against bak-mediated apoptosis. Int J Cancer 1997; 72: 898-905.

[30] Butler LM, Zhou X, Xu WS, Scher HI, Rifkind RA, Marks PA, Richon VM.

The histone deacetylase inhibitor SAHA arrests cancer cell growth, up-regulates thioredoxin-binding protein-2, and down-regulates thioredoxin. Proc Natl Acad Sci U S A 2002; 99(18): 11700-5.

[31] Oikawa T, Onozawa C, Inose M, Sasaki M. Depudecin, a microbial metabolite containing two epoxide groups, exhibits anti-angiogenic activity in vivo. Biol Pharm Bull 1995; 18(9):1305-7.

[32] Kim MS, Kwon HJ, Lee YM, Baek JH, Jang JE, Lee SW, Moon EJ, Kim HS, Lee SK, Chung HY, Kim CW, Kim KW. Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. Nat Med 2001; 7(4):437-43.

[33] Xia W, Lau YK, Hu MC, Li L, Johnston DA, Sheng S, El-Naggar A, Hung MC. High tumoral maspin expression is associated with improved survival of patients with oral squamous cell carcinoma. Oncogene 2000; 19: 2398-403.

Figure legends

Fig.1 Expression of maspin mRNA in oral cancer cell lines.

The expression levels of maspin mRNA in 12 oral cancer cell lines were examined by RT-PCR. The gels clearly show that the Hep2, SAS, KB, and HO-1-u-1 cell lines contained only trace amounts of maspin mRNA. The other cell lines produced uniformly large amounts of the maspin mRNA, as indicated by the very dense bands appearing in the relevant lanes.

Fig. 2 Methylation map of the CpG island in the 5' region of the *maspin* gene CpGs

We analyzed the methylation map of the CpG island in the promoter of the *maspin* gene in the three low-expressed cell lines (upper). | bars represent CpG dinucleotides in the *maspin* promoter. Closed circles on the bar represent methylated cytosine. The numbers below the map represent the methylation frequency for each site, calculated as the percentage of clones with methylated CpG cytosines at that site. Bars with open circles indicate no methylation of the CpG cytosine. At least five clones of each fragment were sequenced. Interestingly, we found that the maspin promoter contains only a few methylated CpG sites in the maspin low-expressed lines. We also analyzed the methylation map in the promoter of *maspin* after 5-Aza-dC treatment in the three low-expressed cell lines (lower). After incubation with 4.4 μM of 5-Aza-2'-deoxycytidine in a flask for 7 days, extracted DNA was bisulfited and sequenced as described in the Materials and Methods section. The methylation status was not altered after 5Aza-dC treatment.

Fig. 3 Effects of 5-Aza-dC treatment on expression of maspin gene. To test whether or not expression of the maspin gene had indeed been altered by hypermethylation, leading to its partial or

complete demethylation, we exposed a demethylating agent, 5-Aza-dC, to the three maspin downregulated cell lines (Hep2, SAS, and KB) and two maspin-expressed cell lines (HSC4 and HSC3). The RT-PCR results clearly show that the five cell lines regained their ability to produce high levels of maspin mRNA following exposure to graded doses of 5-Aza-dC.

Fig. 4 Effects of FR901228 treatment on maspin transcription.

(a) Three maspin downregulated cell lines that expressed low levels of maspin mRNA (Hep2, SAS, and KB) were incubated with FR901228 at a concentration of 0.5 or 1.0 μM. Two maspin-expressed cell lines (HSC4 and HSC3) were also analyzed as controls. Maspin expression was induced after 4 h-16 h of treatment with FR901228 in the maspin downregulated cell lines, while maspin transcriptional levels were not altered after treatment with FR901228 in the maspin-expressed cell lines. (b) As a negative control, human breast cancer cell line MCF-7, one of the maspin negative lines, was incubated with FR901228. RT-PCR resulted in the induction of maspin expression in a time-dependent manner in MCF-7.

Figure 1

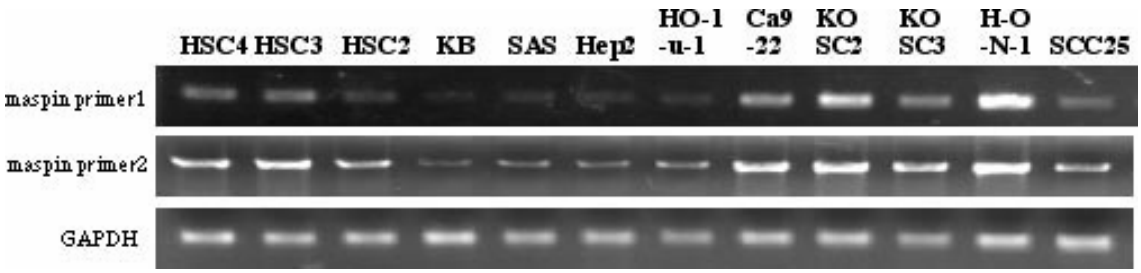


Figure 2

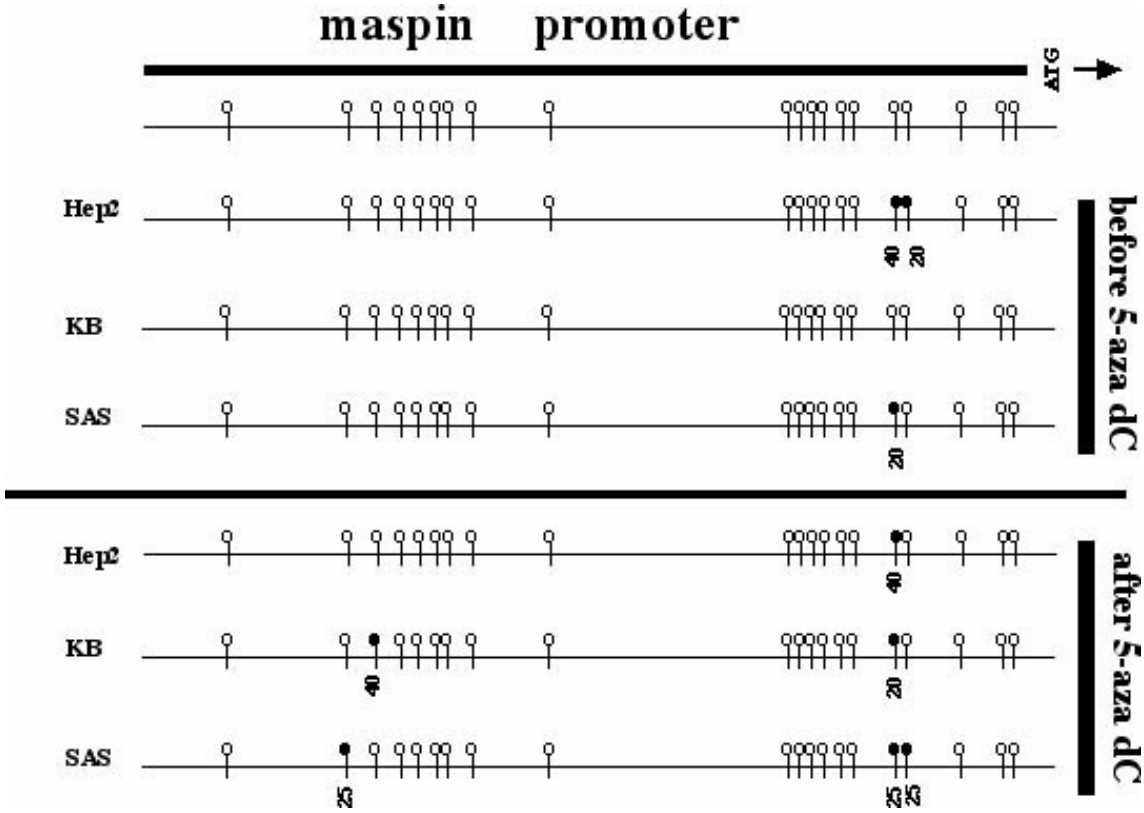


Figure 3

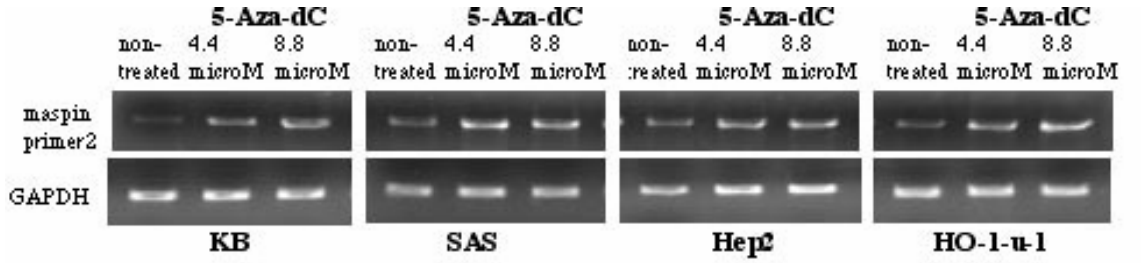
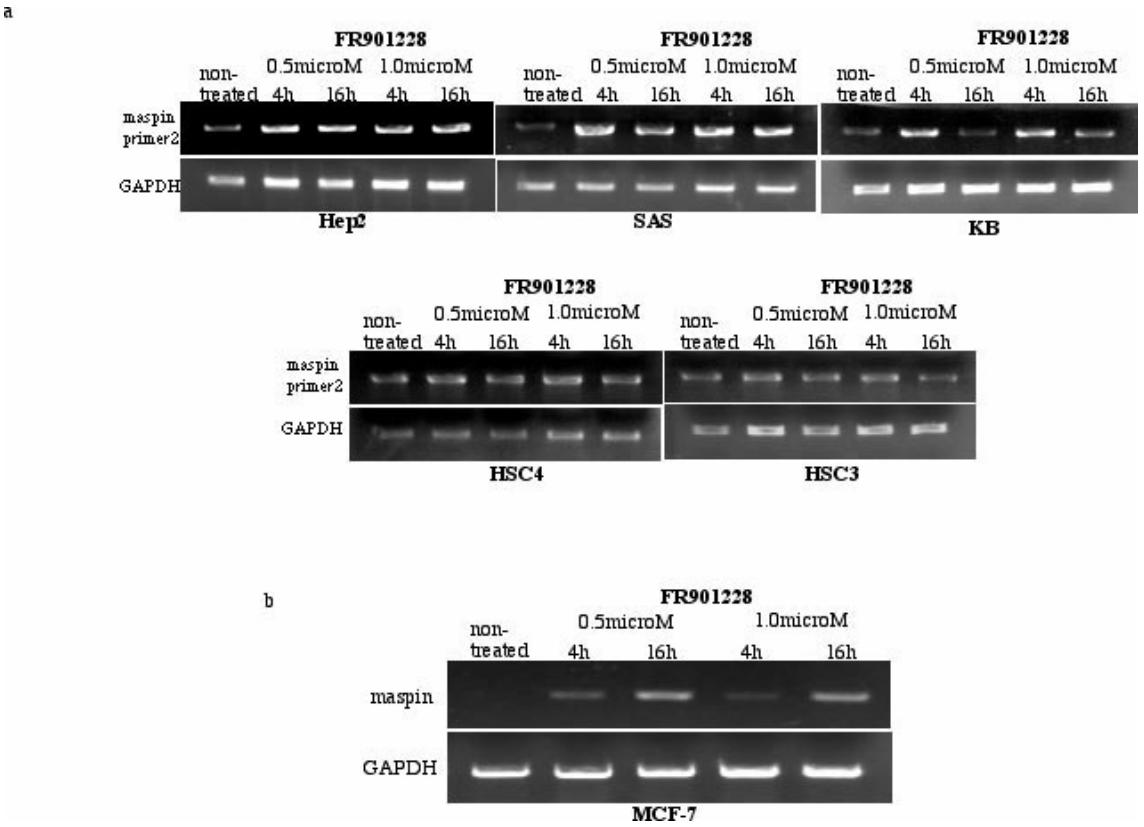


Figure 4


a
FR901228
non-treated
0.5microM 4h
0.5microM 16h
1.0microM 4h
1.0microM 16h
maspin primer2
GAPDH
Hep2
SAS
KB
HSC4
HSC3
b
MCF-7