CHAPTER III

RESULTS

3.1 Effect of IL-1β, TGF-β1 and TNF-α on Maspin gene expression in human cancer cells and human lung fibroblast

The effect of IL-1β, TGF-β1 and TNF-α on Maspin expression was initially explored at the transcriptional level by RT-PCR method in a variety of human cancer cell lines including cervical (HeLa), breast (MCF-7), ovarian (SKOV3), colon (SW620) carcinama and synovial sarcoma (SW982) cells. As shown in Figure 22, only HeLa cells constitutively expressed Maspin while other cancer cell lines and normal fibroblast, MRC5 had no maspin expression. After 24 h treatment, IL-1β had unaffected the levels of Maspin expression in HeLa cells. TGF-β1 clearly increased the amount of Maspin transcript in HeLa cells, but could not stimulate the expression in the other cell lines. In contrast, the expression of Maspin in HeLa cells was slightly reduced by TNF-α.

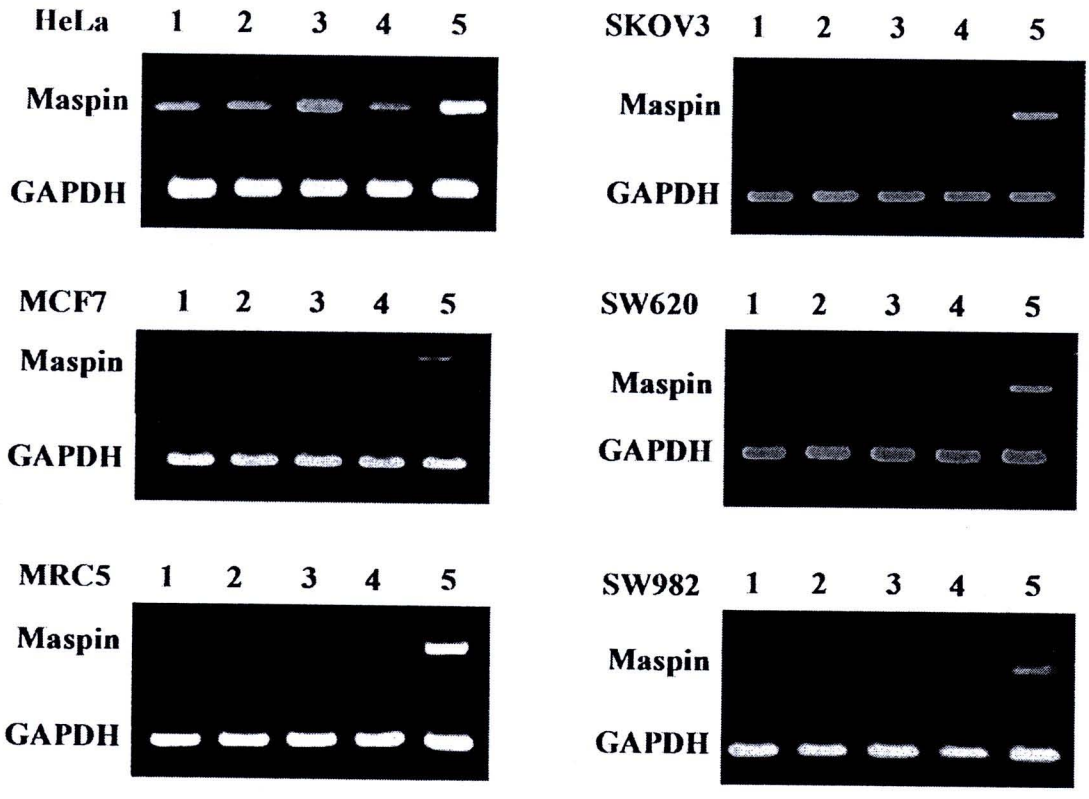

Figure 22 Effect of IL-1β, TGF-β1 and TNF-α on Maspin gene expression in human cancer cell lines and human lung fibroblast. The cells ($8x10^5$cells) were treated with 10 ng/mL IL-1β, TGF-β1 and TNF-α, and incubated for 24 h and then total RNA samples were extracted, reversed transcribed into cDNA, and then subjected to a PCR. A single band was detected in each lane at 614 bp and 452 bp, the predict sizes of fragments given by primers specific for Maspin and GAPDH, respectively.

Lane 1 = Media control

Lane 2 = + IL-1β

Lane 3 = + TGF-β1

Lane 4 = + TNF-α

Lane 5 = PCR positive control

3.2 Effect of IL-1β, TGF-β1 and TNF-α on Maspin gene expression in HeLa cells

The expression of Maspin in HeLa cells was further explored whether the effect of cytokines are dose dependent using 0.1, 1 and 10 ng/ mL of IL-1β, TGF-β1 and TNF-α . The results are shown in Figure 23. Similar to the previous result, IL-1β at all three concentrations did not change the expression of Maspin. After 24 h incubation, the level of Maspin transcript was clearly up-regulated by TGF-β1 at 0.1 ng/mL, and saturated at 10 ng/mL. The down-regulation of Maspin expression by TNF-α was not observed at a low dose (0.1 ng/mL), but apparently achieved at 1 and 10 ng/mL.

The effect of TGF-β1 and TNF-α on Maspin expression was further analyzed by real time qPCR method to quantitatively measure the amount of up- and down-regulation of gene expression after 24 and 48 h incubation. As shown in Figure 24 A, the stimulation of Maspin expression by TGF-β1 was dose dependent at both 24 and 48 h treatment. The level of Maspin transcript was increased more than six fold using 10 ng/mL of the cytokine. Interestingly, at 1 ng/mL, TGF-β1 induced the expression up to six fold at 24 h, but only two-fold increase of Maspin transcript at 48 h.

The effect of TNF-α on Maspin expression was significantly observed in a dose dependent manner only at 24 h after the treatment (Figure 25). TNF-α at 10 ng/mL caused the highest decrease by half of the Maspin expression. Also, the treatment of TNF-α for 48 h could reduce the expression though not significantly when compared to the no treatment control.

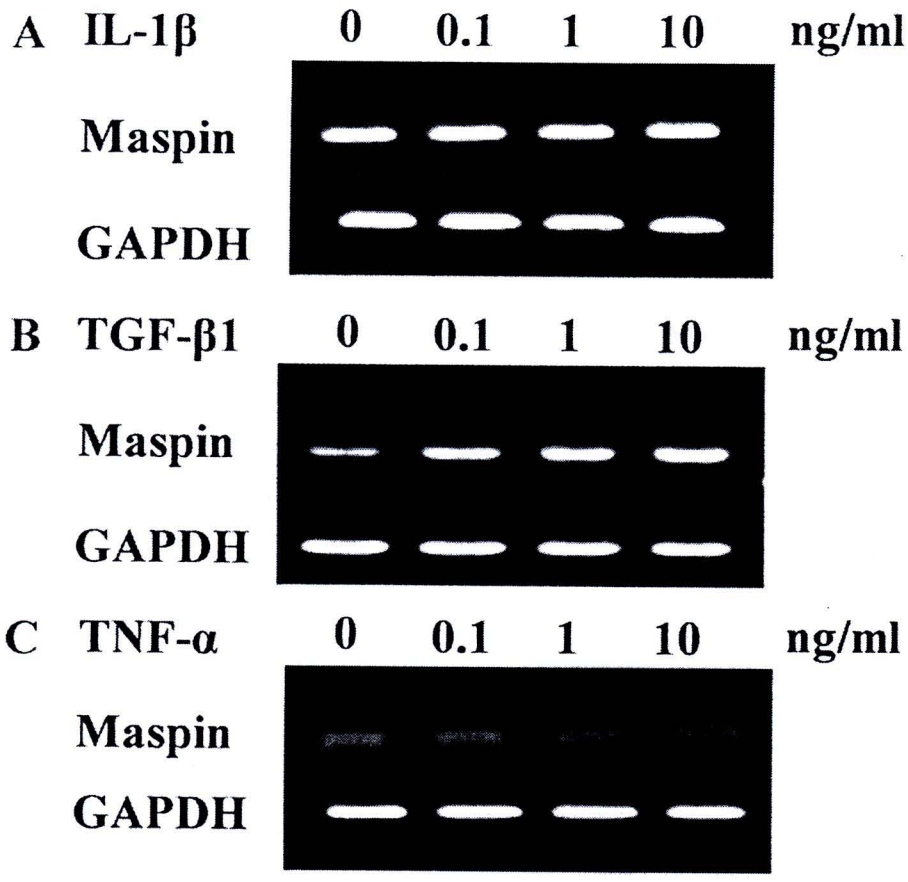

Figure 23 Effect of IL-1β (A), TGF-β1 (B) and TNF-α (C) on Maspin gene expression in HeLa cells. The cells ($8x10^5$cells) were treated with IL-1β, TGF-β1 and TNF-α, (0, 0.1, 1, 10 ng/mL) and incubated for 24 h. Total RNA samples were extracted and converted to cDNA, subjected to PCR using Maspin specific primers, GAPDH was used as normalized control.

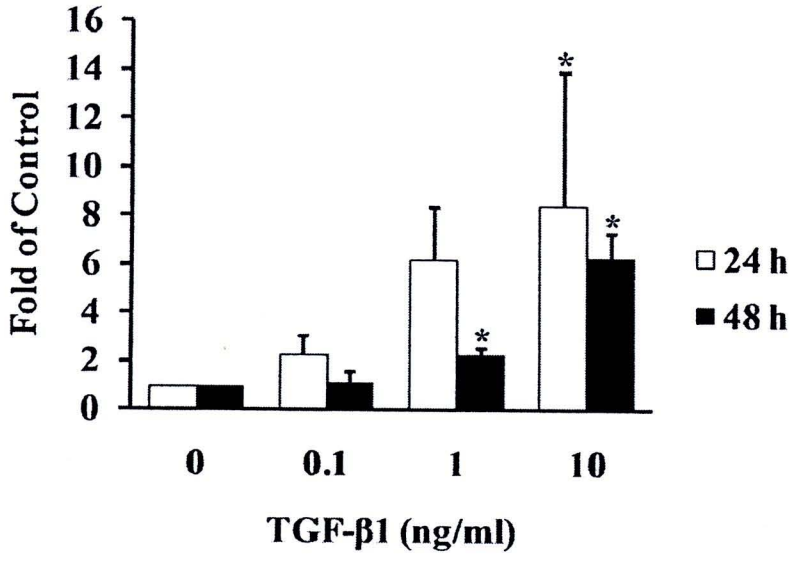

Figure 24 Effect of TGF-β1 on Maspin gene expression in HeLa cells. The cells ($8x10^5$cells) were treated with TGF-β1 and incubated for 24 and 48 h. Total cDNA was synthesized and subjected to a real time PCR, The mRNA expression of Maspin was normalized with that of GAPDH and presented as fold of control values. Data are mean ± SD values from three independent experiments. *P <0.05.

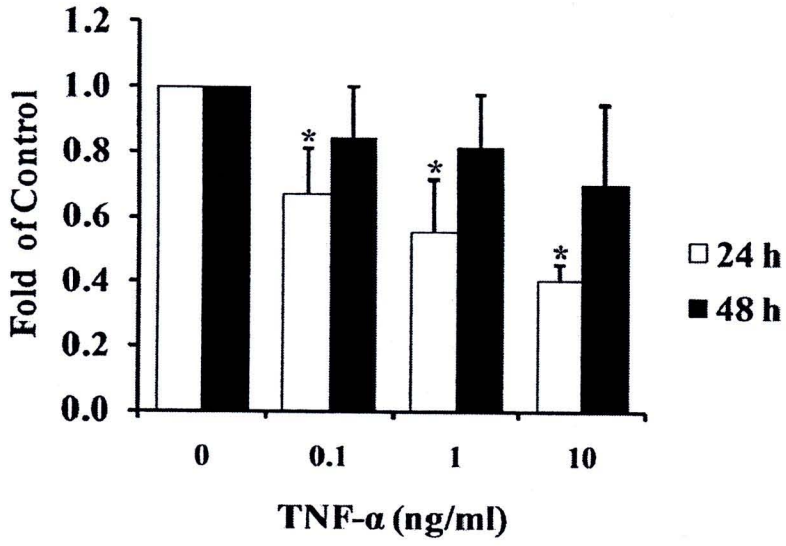

Figure 25 Effect of TNF-α on Maspin gene expression in HeLa cells. The cells ($8x10^5$cells) were treated with TNF-α, and incubated for 24 and 48 h. Total cDNA was synthesized and subjected to a real time PCR, The mRNA expression of Maspin was normalized with that of GAPDH and presented as fold of control values. Data are mean ± SD values from three independent experiments. *P <0.05.

3.3 Effect of TGF-β1 and TNF-α on Maspin protein level in HeLa cells

The effect of TGF-β1 and TNF-α on the expression of Maspin protein in HeLa was analyzed by Western blotting as shown in Figure 26 and 27, respectively. Similar to the previous result of RT-PCR, TGF-β1 increased, but TNF-α decreased the level of Maspin protein in HeLa cells. Nonetheless, changes in the expression of Maspin protein by these cytokines were less than that of Maspin transcript. The level of Maspin induction by TGF-β1 was striking, but less than two fold at 48 h. Like the effect on the mRNA level, the reduction of Maspin protein was clearly seen at 24 h after the treatment with 1-10 ng/mL TNF-α.

3.4 Combination effects of TGF-β1 and TNF-α on Maspin gene expression in HeLa cells

Since TGF-β1 and TNF-α had the opposite effect on Maspin expression in cancer cells, whether combination of both cytokines results in antagonist effect in HeLa cells was further studied using real time qPCR analysis. TGF-β1 significantly increased the Maspin gene expression, and the post-treatment of TNF-α could not reduce the effect of TGF-β1 (Figure 28, Lane 2, and 3). In contrast, after 24 h pretreatment with TNF-α, TGF-β1 could overcome the activity of TNF-α (Figure 28, Lane 5), as the level of Maspin transcript was similar to that induced by TGF-β1 alone. Interestingly, the combination of both TGF-β1 and TNF-α treatment resulted in the level of Maspin expression in the middle of those caused by each cytokine treatment (Figure 28, Lane 6). The result suggested that the induction of Maspin expression by TGF-β1 seemed to be antagonized by TNF-α.

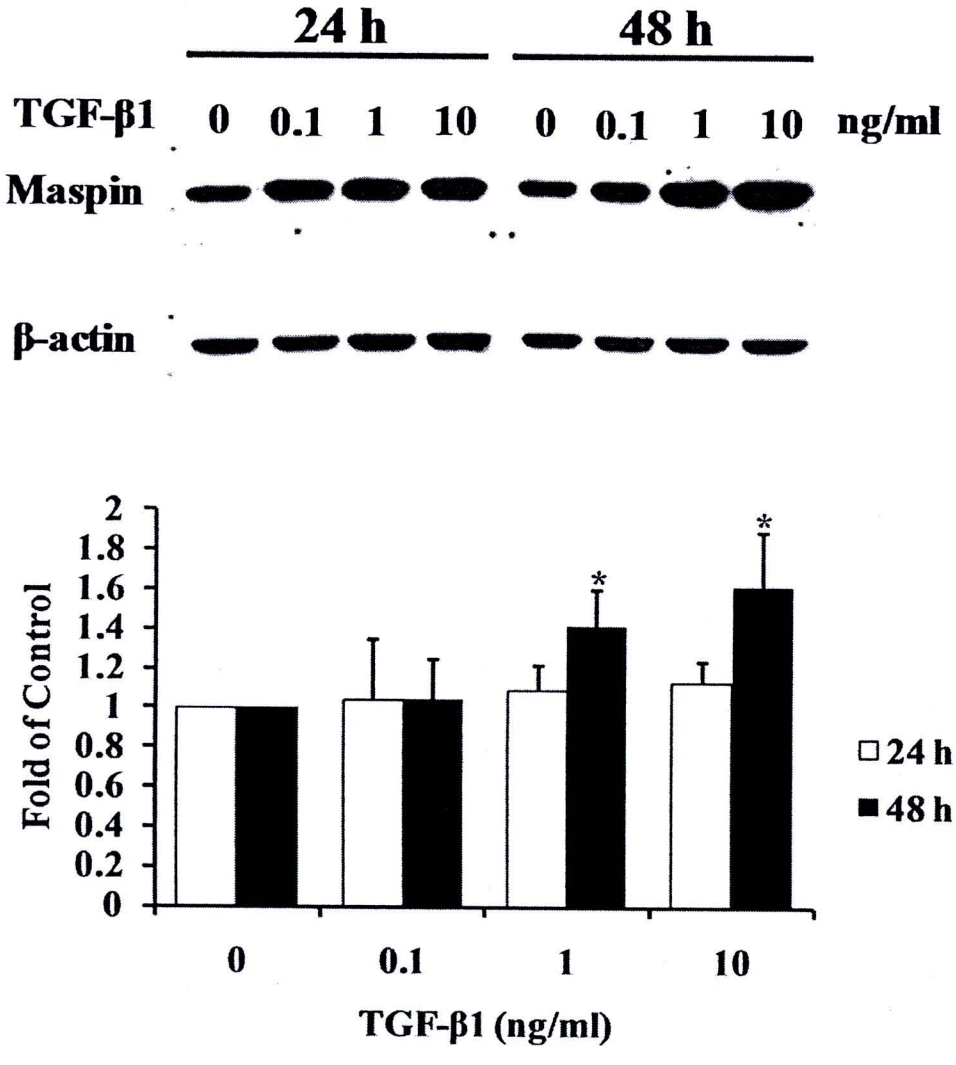

Figure 26 Effect of TGF-β1 on Maspin protein expression in HeLa cells. The cells ($8x10^5$cells) were treated with 0, 0.1, 1, 10 ng/mL TGF-β1 for 24 and 48 h. The total protein samples were extracted and subjected to Western blotting, β-actin was used for normalization. The protein expression of Maspin was presented as fold of control values. Data are mean ± SD values from three independent experiments. *P<0.05

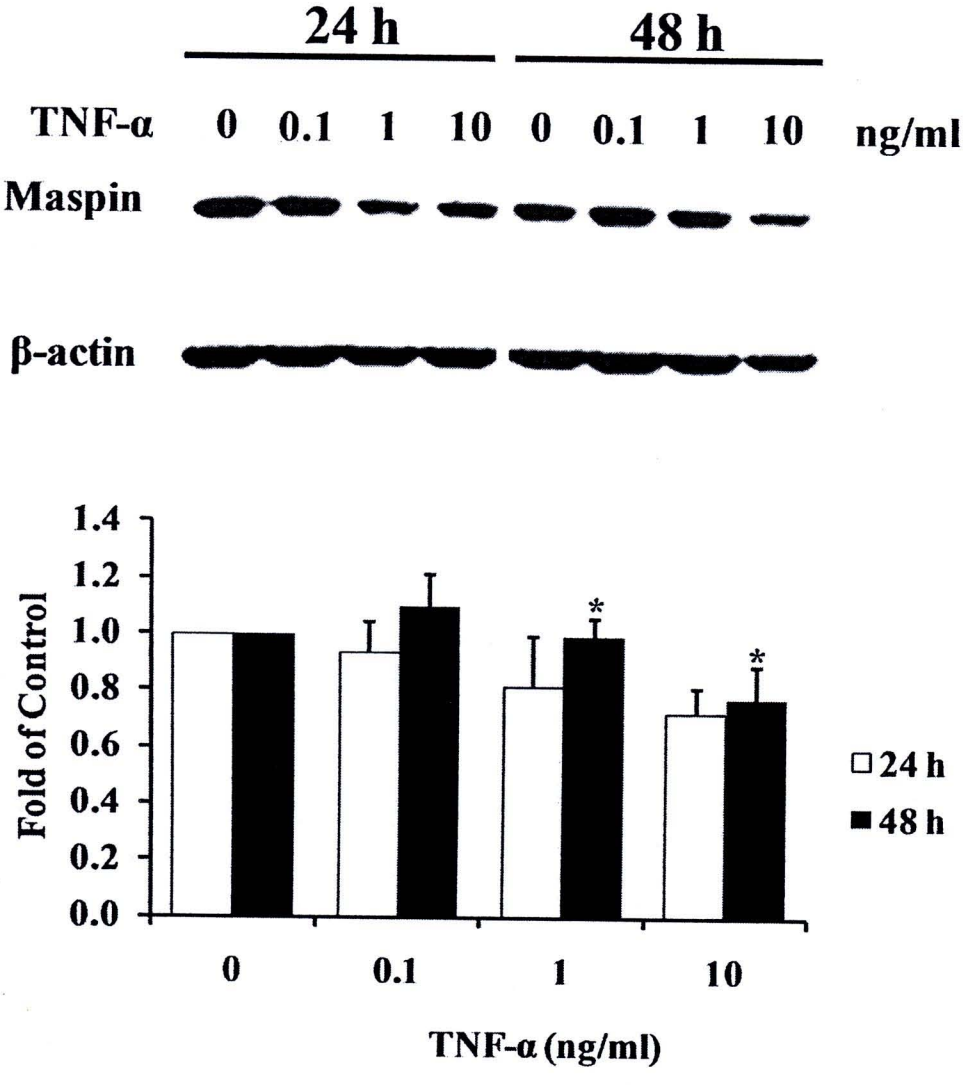

Figure 27 Effect of TNF-α on Maspin protein expression in HeLa cells. The cells ($8x10^5$cells) were treated with 0, 0.1, 1, 10 ng/mL TNF-α for 24 and 48 h. The total protein samples were extracted and subjected to Western blotting. β-actin was used as a normalized control. The protein expression of Maspin was presented as fold of control values. Data are mean ± SD values from three independent experiments. *P<0.05

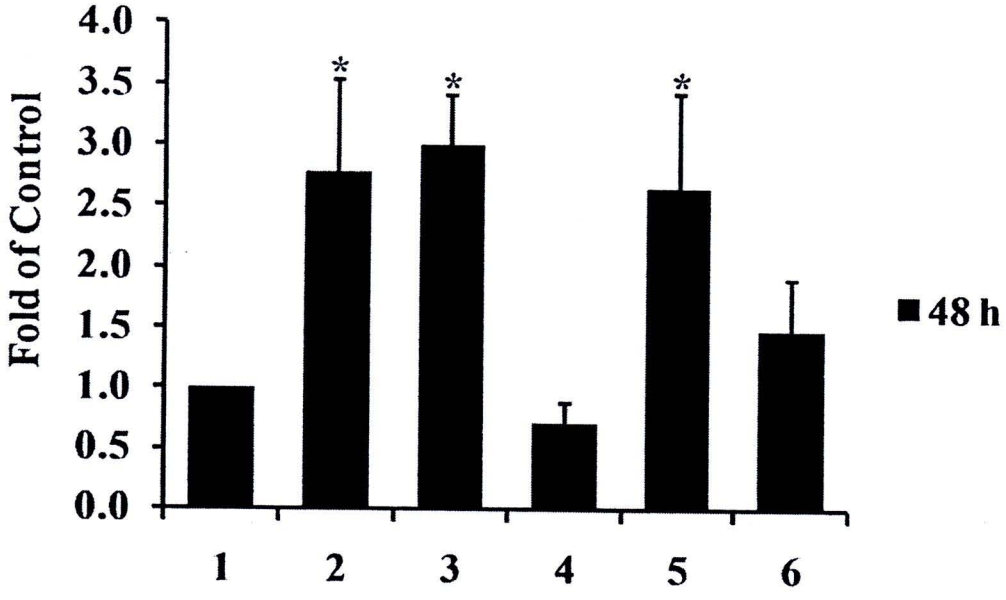

Figure 28 Effect of TGF-β1 and TNF-α on Maspin gene expression in HeLa cells. The cells ($8x10^5$cells) were treated with or without cytokines as Table 5. After 48 h treatment, total RNA samples were extracted and subjected to a qPCR. The mRNA expression of Maspin was normalized to that of GAPDH and presented as fold of control values. Data are mean ± SD values from three independent experiments. *$P<0.05$.

Table 5 The steps of cytokines treatment

Lane / Time	1	2	3	4	5	6
0 h	Media	TGF-β1	TGF-β1	TNF-α	TNF-α	TGF-β1 + TNF-α
24 h	Media	TGF-β1	TNF-α	TNF-α	TGF-β1	TGF-β1 + TNF-α
48 h	RNA extraction					

3.5 Cytotoxic effect of TGF-β1 and TNF-α on HeLa cells

To determine whether TGF-β1 and TNF-α affect the viability of HeLa cells, the cells were treated with various concentrations of cytokines for 24, 48 and 72 h, and then the cell viability determined by Sulforhodamine B (SRB) assay. Dose response cytotoxicity profiles for TGF-β1 and TNF-α were observed as shown in Figure 29 and 30, respectively. TGF-β1 and TNF-α at 0.1 – 10 ng/mL were not cytotoxic to the HeLa cells. Thus, the concentration of TGF-β1 and TNF-α at 10 ng/mL was chosen for a testing the effect of cytokine on cell migration and invasion in the next following experiments.

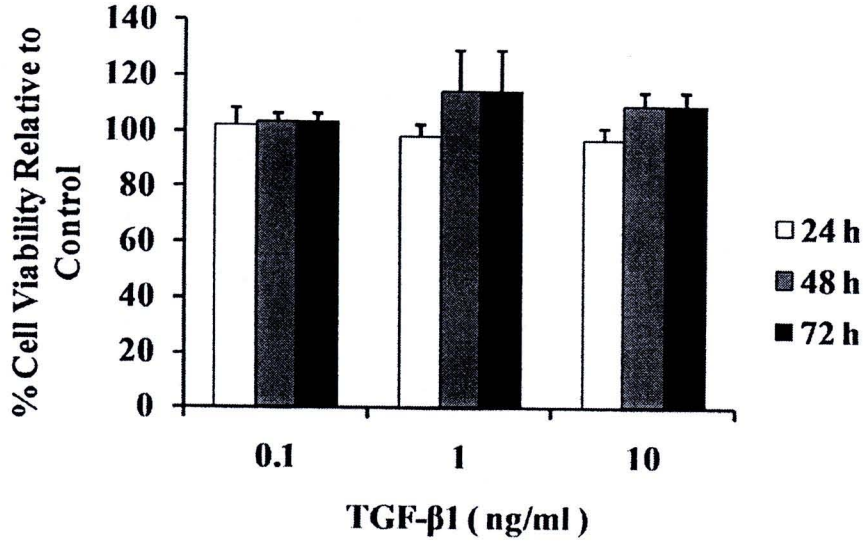

Figure 29 Effect of TGF-β1on viability of HeLa cells. The cells ($1x10^4$cells) were seeded into 96 well plates and cultured overnight. Various concentrations of TGF-β1 were then added and incubated further for 24, 48 and 72 h in a 37°C. The number of viable cells was determined by SRB assay as described in the *Materials and Methods*. The Y-axis shows the percent of cell survival relative to no treatment control. Each bar represents the mean of three independent experiments performed in triplicate.

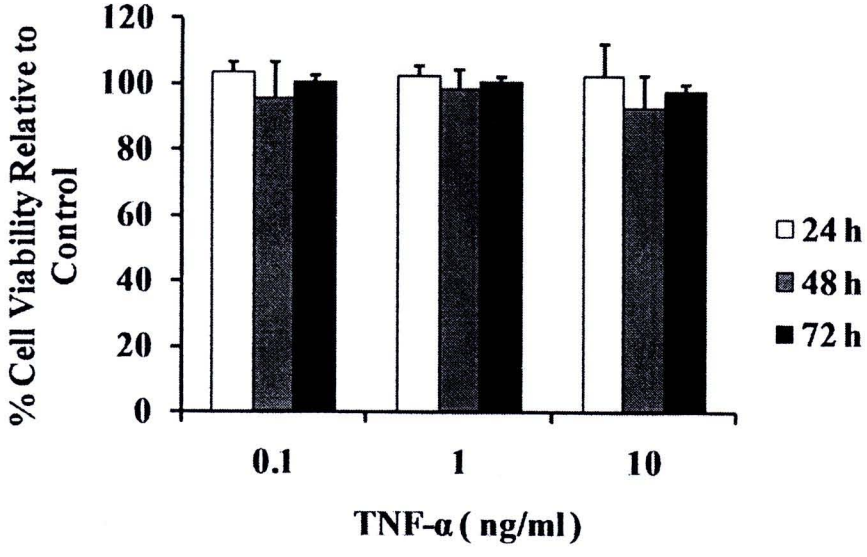

Figure 30 Effect of TNF-α on viability of HeLa cells. The cells ($1x10^4$cells) were seeded into 96 well plates and cultured overnight. Various concentrations of TNF-α were then added and incubated further for 24, 48 and 72 h in a 37°C. The number of viable cells was determined by SRB assay as described in the *Materials and Methods.* The Y-axis shows the percent of cell survival relative to no treatment control. Each bar represents the mean of three independent experiments performed in triplicate.

3.6 Effect of TGF-β1 and TNF-α on migration and invasion of HeLa cells

The function of Maspin has been shown to inhibit migration and invasion of carcinoma cells (128). Because the expression of Maspin in HeLa cells was altered by either TGF-β1 or TNF-α, the effect of these cytokines on migration and invasion of HeLa cells were studied to determine whether alteration of Maspin levels by these cytokines can affect the properties of the cells. HeLa cells were pretreated with 10ng/mL TGF-β1 or TNF-α for 24 h to allow the stimulation or reduction of Maspin expression, respectively. Next, migratory and invasive properties of the cells were determined as described in the *Materials and Methods*. The results were shown as in Figure 31 and 32. TGF-β1 significantly increased both cell migration and invasion by approximately 40 percent . Likewise, TNF-α at 10 ng/mL induced the migration to some extent. However, the effect of TNF-α on cell invasion was not significantly different from the untreated control.

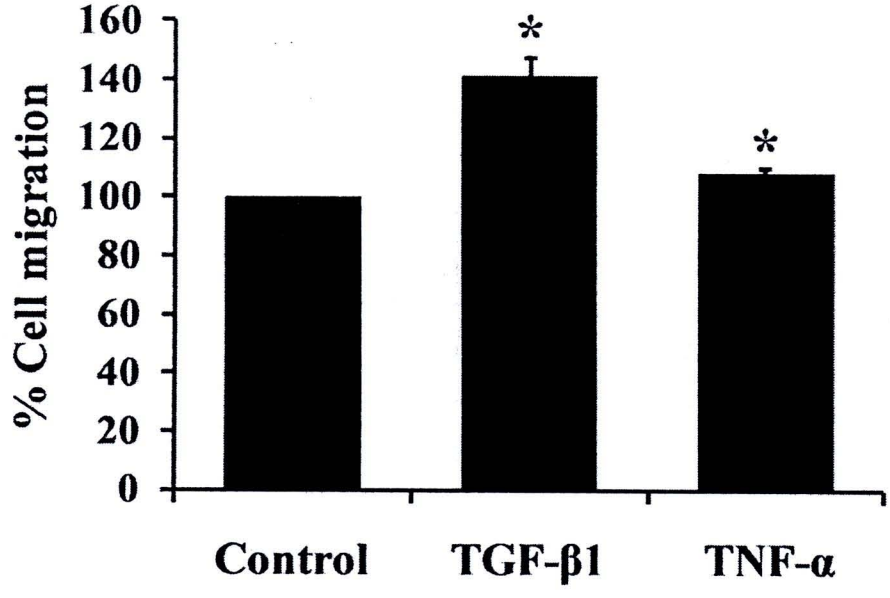

Figure 31 Effect of TGF-β1 and TNF-α on migrationof HeLa cells. The cells were pretreated with 0 and 10 ng/mL of TGF-β1 or TNF-α for 24 h prior to the assays. Migration assay was performed in 96-transwell cell culture chambers using 10% fetal bovine serum as a chemoattractant for 24 h. The numbers of migrating cells were quantified using CyQuant GR fluorescent staining. The results were expressed as percentages of cells relative to the untreated control. Data are mean ± SD values from three independent experiments. *P<0.05.

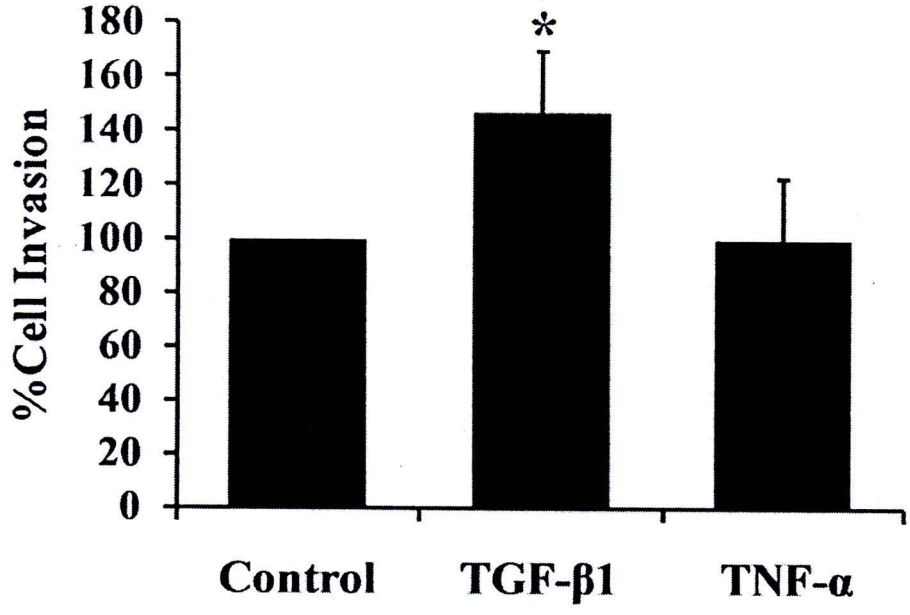

Figure 32 Effect of TGF-β1 and TNF-α on HeLa cell invasion. The cells were pretreated with 0 and 10 ng/mL of TGF-β1 or TNF-α for 24 h prior to Matrigel invasion assay. The assay was performed in 96-transwell cell culture chambers using 10% fetal bovine serum as a chemoattractant for 48 h, respectively. The numbers of invasive cells were quantified using CyQuant GR fluorescent staining. The results were expressed as percentages of cells relative to the untreated control. Data are mean ± SD values from three independent experiments. *$P<0.05$.

3.7 Characterization of Maspin promoter methylation

To investigate a mechanism by which TGF-β1 and TNF-α modulate Maspin expression, the epigenetic regulation of Maspin gene was chosen as it affects at the transcription level of the gene. The Maspin promoter methylation status in HeLa cells was examined between -254 to +152 relative to the transcription start site, a region previously studied for other cell types (50). First, genomic DNA was extracted and analyzed as described in the *Materials and Methods*. As shown in Figure 33, the PCR product was amplified at the expected size using 200, 500, 1,000 ng genomic DNA template with MP primers specific to the normal sequence of Maspin promoter. Using U2D2 primers, there was no PCR product because the sequences of U2D2 primers were designed to match with sequences in which the cytosine bases are converted to uracil following the bisulfite treatment. A complete bisulfite reaction was confirmed by a specific amplification of the bisulfite-treated Maspin promoter using U2D2, but not MP primers as shown in Figure 34. Then, the PCR condition of annealing temperature was optimized to approximately 60°C (Figure 35). Next, the PCR was performed using the bisulfite-treated genomic DNA of HeLa cells after incubation with 10ng/mL TGF-β1 and TNF-α for 24 h, The amplified PCR products were cloned and sequenced as shown in Figure 36. The result indicated that either TGF-β1 or TNF-α has no effect on CpG methylation sites of Maspin promoter.

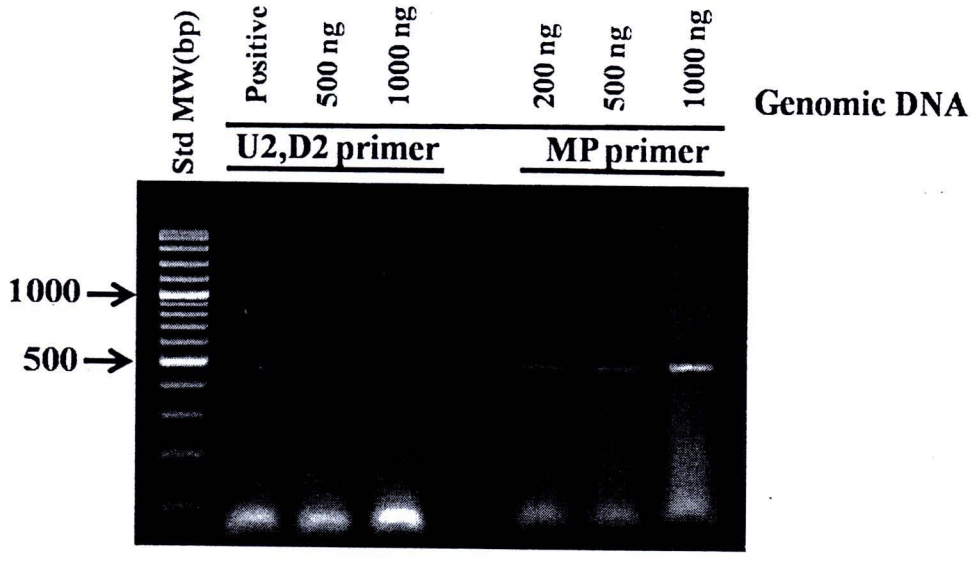

Figure 33 PCR amplification of Maspin promoter before bisulfite conversion of DNA. Total genomic DNA was extracted from HeLa cells. Three different concentrations (200, 500 and 1,000 ng) of the extracted DNA was subjected to PCR amplification using either U2,D2 primers or MP primers specific to the 464 bp region of Maspin promoter.

Lane 1 = Positive control

Lane 2 = 500 ng Genomic DNA

Lane 3 = 1,000 ng Genomic DNA

Lane 4 = 200 ng Genomic DNA

Lane 5 = 500 ng Genomic DNA

Lane 6 = 1,000 ng Genomic DNA

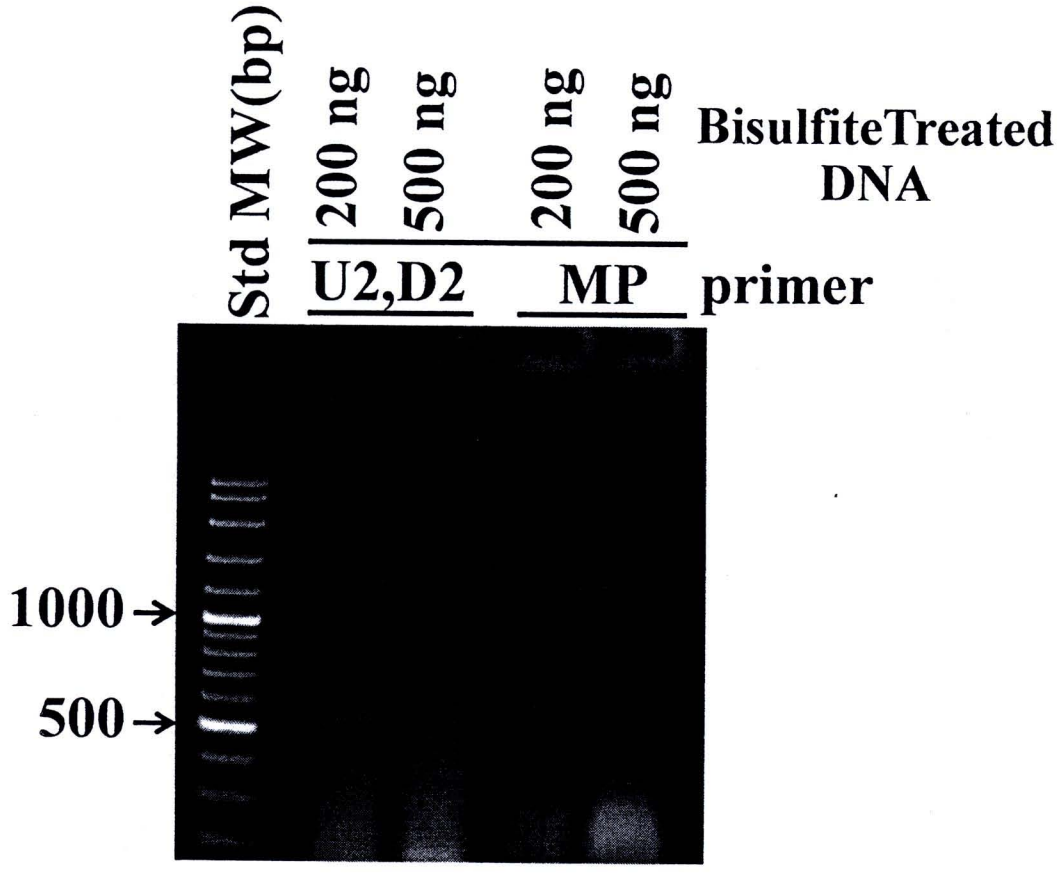

Figure 34 PCR amplification of Maspin promoter after bisulfite conversion of DNA. Total genomic DNA was extracted from HeLa cells and then 200 or 500 ng of the DNA were treated with sodium bisulfate. The bisulfite treated DNA was subjected to PCR amplification using either U2,D2 primers or MP primers specific to the 464 bp region of Maspin promoter.

Lane 1 = 200 ng treated DNA

Lane 2 = 500 ng treated DNA

Lane 3 = 200 ng treated DNA

Lane 4 = 500 ng treated DNA

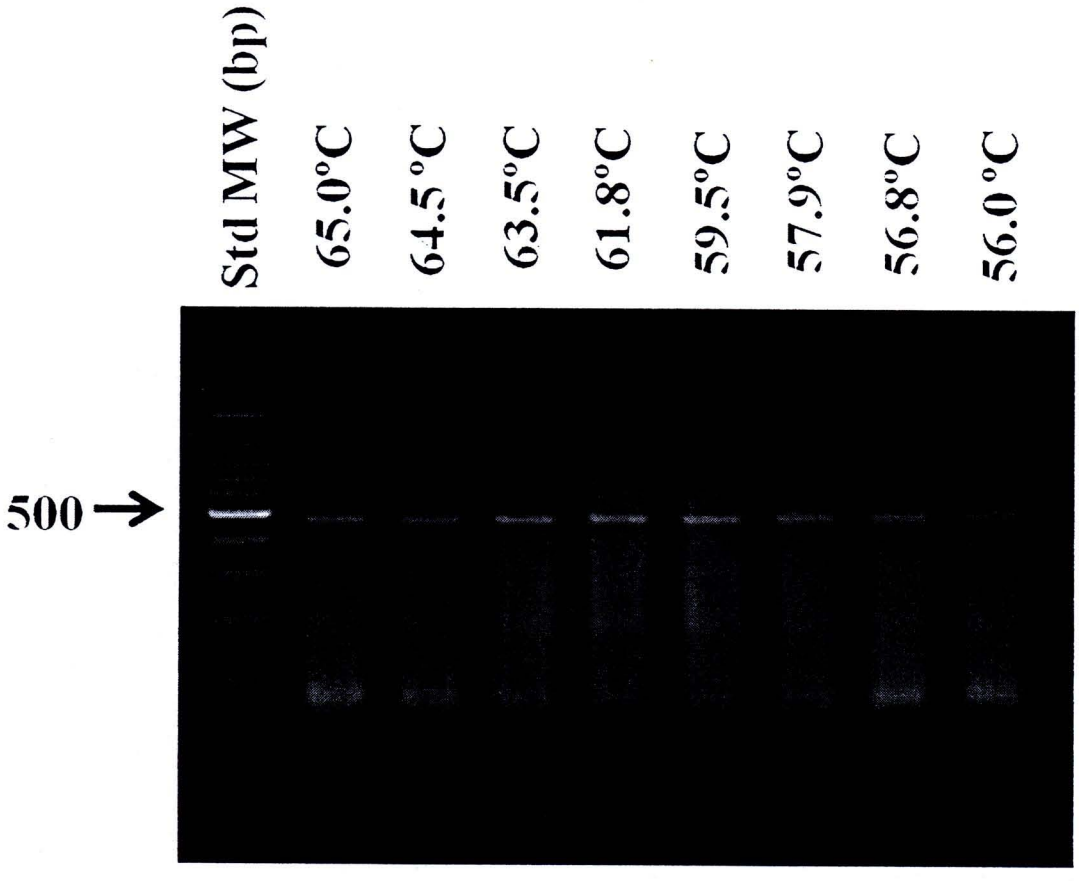

Figure 35 Optimization of PCR annealing condition using U2,D2 primer. Genomic DNA (200 ng) were treated with sodium bisulfate and then subjected to PCR using U2,D2 primers at annealing temperatures from 56.0°C to 65°C.

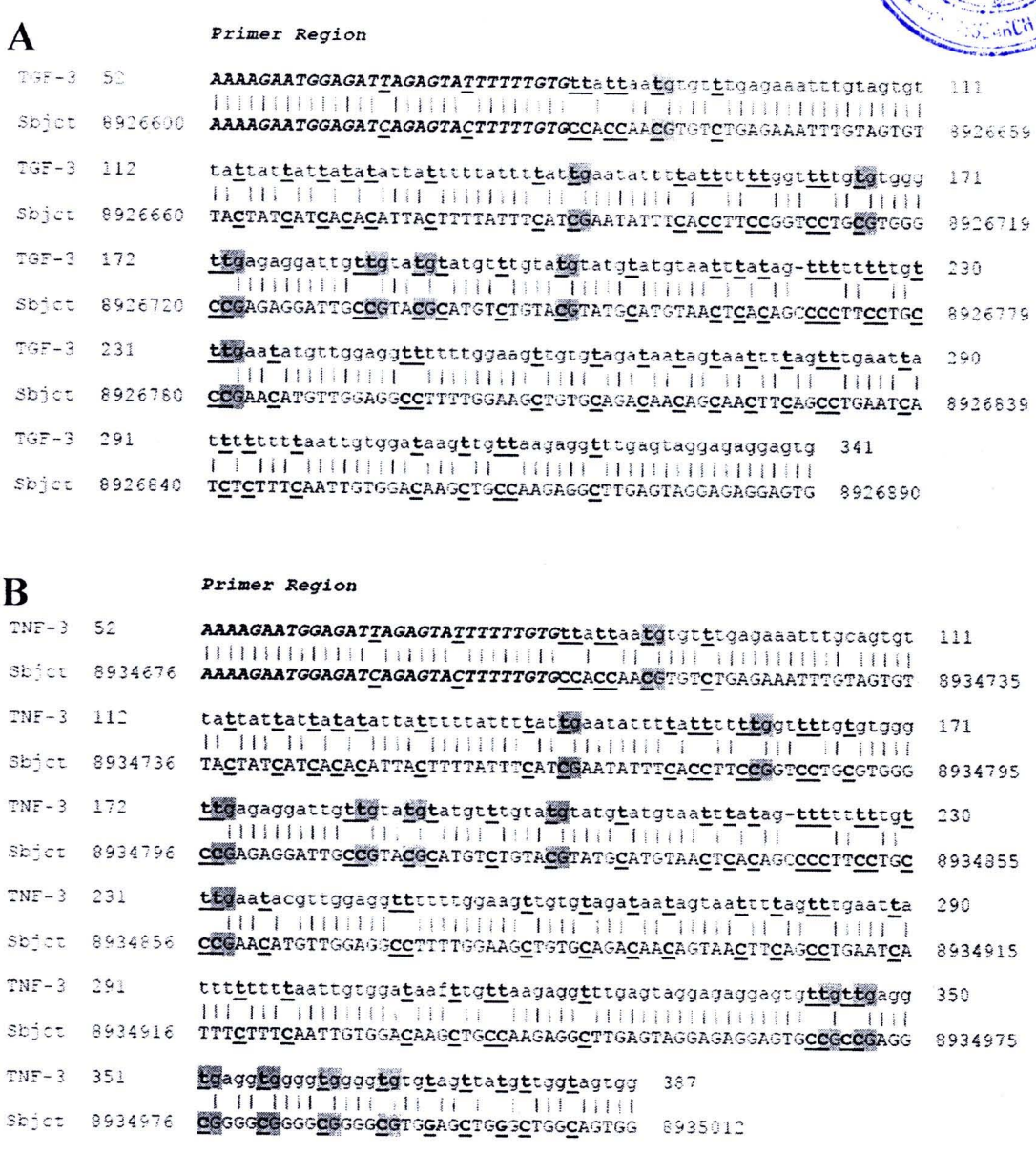

Figure 36 Examples of the Maspin promoter sequences obtained following bisulfite treatment in HeLa cells. The cells were treated with 10 ng/mL of TGF-β1(A) and TNF-α(B) for 24 h and then methylation state of cytosines was obtained by bisulfite-modified genomic DNA sequencing of the Maspin promoter (- 254 to + 152). In these cells, 100% of the potential CpG methylation sites were modified in the analyzed region (highlight and over all conversion efficiency of cytosines to thymines was 98%.