CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and materials

The names of chemicals and reagents, and instruments are shown in Appendix A and B, respectively. The detail of reagents and/or buffers used in this study is shown in Appendix C.

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

2.2.1 Cell lines and culture conditions

Human cervical carcinoma cells (HeLa), mammary carcinoma cells (MCF-7), human lung fibroblast (MRC-5), ovarian carcinoma cells (SKOV3), human colorectal carcinoma cells (SW620), and human synovial sarcoma cells (SW982) obtained from Cell Lines Service (CLS, Germany). The cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA.) supplemented with 10% heat inactivated fetal bovine serum (FBS, HyClone, USA), 1xAntibiotic-Antimycotic (Invitrogen, USA) in 5%CO_2, and 37°C. All experiments were initiated with cells in log phase of growth, and designed to be completed before 80% confluence.

2.2.2 Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse Transcription-PCR (RT-PCR) is a method used to amplify cDNA copies of RNA by generating large cDNA libraries from cellular mRNA. This method can measure the strength of gene expression when the amounts of available mRNA are limited and/or when the RNA of the interest is expressed at very low levels.

The first step of RT-PCR is enzymatic conversion of RNA to a single-stranded cDNA template. An oligodeoxynucleotide primer is hybridized to the mRNA and then extended by an RNA-dependent DNA polymerase to create a cDNA copy that can be amplified by PCR. Depending on the purpose of the experiment, the primer for first-strand cDNA synthesis can be specifically designed to hybridize to a particular target gene or it can bind randomly to all mRNAs. Amplification of the desired portion of cDNA can be achieved in PCRs using forward and reverse oligonucleotide primers corresponding to specific sequence in particular cDNAs. The diagram of RT-PCR procedure is shown in Figure 12

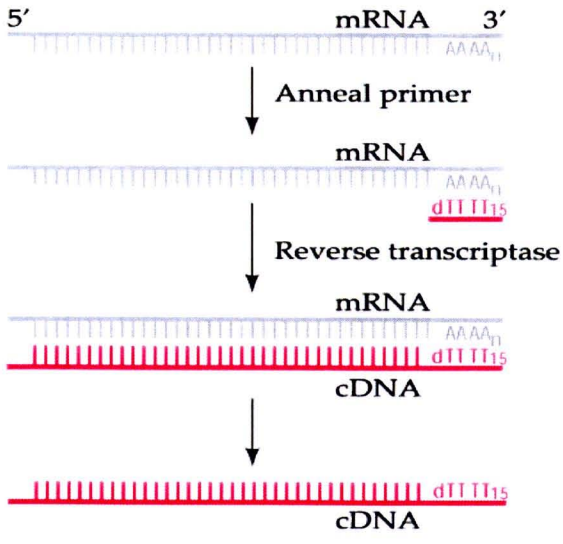

Figure 12 The step of revers transcription.

(http://9e.devbio.com/image.php?id=127)

Maspin expression at the transcriptional level was analyzed using RT-PCR. In brief, the cancer cells (8.0 x 10^5cells) were incubated with IL-1ß (Roche Diagnostics, Germany), TGF-ß1 (Roche Diagnostics, Germany) and TNF-a (Roche Diagnostics, Germany) at 0.1, 1, 10 ng/mL for 24 h. Then, total RNA was extracted using Trizol reagent (Invitrogen, USA). The mRNAs were converted to cDNAs by reverse transcriptase (Fermentus, Germany). The cDNA of Maspin and GAPDH were amplified by PCR using specific primers listed in Table 3. PCR products were separated by agarose gel electrophoresis. The parallel cDNA amplification of GAPDH was also conducted as a normalization control.

Table 3 Primer sequences of Maspin and GAPDH for RT-PCR

Name	Primer Sequence	Product Size (bp.)
Maspin (F)	CTCTCCACCTCTCTGTCACTTGCTC	614
Maspin (R)	TCATCCTCCACATCCTTGGGTAGTA	
GAPDH (F)	CCACAGTCCATGCCATCAC	452
GAPDH (R)	CCACCACCCTGTTGCTGTA	

2.2.2.1 RNA extraction

Total mRNA was extracted using Trizol reagent (Invitrogen, USA), according to the manufacturer's instruction. In brief, the cell monolayer was directly lysed in a culture dish by adding 1 mL of Trizol reagent to a 3.5 cm diameter dish, and passed several times through a pipette. The cleared homogenate solution was transferred to a fresh tube. The homogenized cells were incubated for 5 min at 15 to 30°C to allow the complete dissociation of nucleoprotein complexes. Next, 0.2 mL of chloroform was added per 1 mL of Trizol reagent used in each tube. The tubes were then vigorously shaken for 15 sec and incubated at 15 to 30°C for 2 to 3 min. After centrifugation at 12,000xg for 15 min at 4°C, the mixture was separated into a lower red, phenol-chloroform phase, an inter-phase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The aqueous phase was transferred to a fresh tube. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol, incubated at 15 to 30°C for 10 min and centrifuged at 12,000xg for 10 min at 4°C. After removing the supernatant, the RNA pellet was washed once with 75% ethanol, mixed by a brief vortex and centrifuged at 7,500 × g for 5 min at 4°C. At the end of

the procedure, the RNA pellet was air dried for 5-10 min and then dissolved in diethylpyrocarbonate (DEPC)-treated water. The quantity and quality of total RNA were assessed by the ratio of A_{260} / A_{280}. The concentration of RNA was determined by GeneQuant pro (Amersham Biosciences, Germany). An OD of 1.0 at the wavelength of 260 nm corresponds to approximately 40 μg/mL RNA. The obtained RNA was diluted 50 times in DEPC-treated water before measuring the absorbance. The concentration of RNA is calculated by using the formula below:

$$\text{Concentration of RNA } (\mu g/mL) = OD_{260} \times 40 \times \text{dilution factor}$$

The quality of the RNA is essential to overall success of the analysis. OD_{260} is frequently used to measure RNA concentration and OD_{280} is used to measure protein concentration. For further analysis, it is imperative that the RNA extract should have high purity, displaying ratio of OD_{260}/OD_{280} values between 1.7 and 1.9. Smaller ratio usually indicates contamination of protein or organic chemicals.

2.2.2.2 cDNA synthesis by reverse transcription

In reverse transcription reaction, 1.0 μg of total RNA was reverse-transcribed into cDNA by oligo-$(dT)_{18}$ primer and AMV reverse transcriptase using RevertAid™ First Strand cDNA synthesis kit (Fermentas, Germany), according to the manufacturer's instructions. Briefly, The 20 μL reaction mixture contained 1 μg of extracted RNA, 0.5 μg of oligo-$(dT)_{18}$, 1 mM dNTPs, 20 unit of ribonuclease inhibitor, 200 units of reverse transcriptase, and adjusted volume to 20 μL with DEPC-treated water. The reaction mixture was incubated at 70 °C for 5 min, 4 °C for 1 min, and then 42 °C for 60 min.

2.2.2.3 Polymerase chain reaction (PCR)

A 20 μL PCR reaction mixture contained 2.0 μL of cDNA, 1X buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl and, 2.0 mM $MgCl_2$), 0.2 mM dNTPs, 2 units of *Taq* polymerase, and 0.2 μM of Maspin or GAPDH primers. Each reaction mixture was then placed on an iCycler thermal cycle (Bio-Rad, USA). The temperature profile was as follows: initial denaturation at 94 ℃ for 3 min, followed by 28 cycles of the amplification process, which are denaturation at 94 ℃ for 30 sec, annealing at 58 ℃ for 30 sec and extension at 72 ℃ for 45 sec, and the final extension step at 72 ℃ for 7 min. The optimized conditions results in the efficient amplification of the 614 bp (Maspin) and 452 bp (GAPDH) DNA without non-specific amplified products.

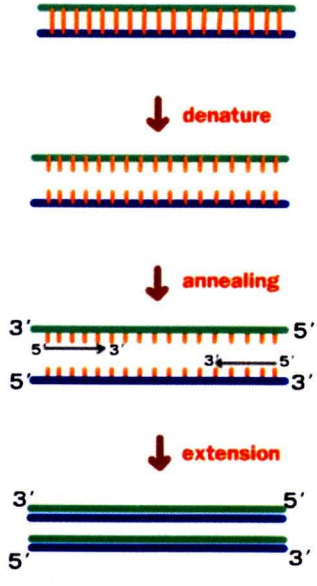

Figure 13 A diagram illustrating the method of PCR

(http://www.il.mahidol.ac.th/e-media/dna/chapter/chapter4application.htm)

After amplification, 13.5 μL from each sample was subjected to 1.5% agarose gel electrophoresis staining with ethidium bromide (EtBr) (Vivantis, USA). The DNA bands were visualized by UV illumination and documented by using a Bio-Rad gel doc 1000 system. The standard 100-base pairs DNA ladder was used as molecular weight marker. Band densities were determined with ImageJ program (National Institutes of Health (NIH), USA). Each Maspin band was normalized to the corresponding GAPDH.

2.2.3 Quantitative real time polymerase chain reaction (Q-PCR/qPCR)

Real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (Q-PCR/qPCR) or kinetic polymerase chain reaction, now become a routine laboratory PCR technique used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of one or more specific sequences in a DNA sample. To confirm the results from RT-PCR experiment, real time qPCR is used to examine the mRNA levels of Maspin. For determination of Maspin gene expression, the qPCR reactions were performed in an ABI 7500 Real-time PCR system (Applied Biosystems, USA) using Maxima^{TM} SYBR Green qPCR Master Mix (Fermentas, Germany). The sequence primers of Maspin and GAPDH are shown in Table 4. Relative expression levels for Maspin were normalized to the expression of GAPDH by the $2^{-\Delta CT}$ method (121).

Table 4 Primer sequences of Maspin and GAPDH for qPCR

Name	Primer Sequence	ProductSize (bp.)
Maspin (F)	CGTAGAAAACTAATCAAGCGGCTCTAG	96
Maspin (R)	CCAATTCCTTTGCATAGGGTCTC	
GAPDH (F)	CGTTGGGTGAAGGTCGGAGTCAAG	92
GAPDH (R)	GGCAACAATATCCACTTTACCAGA	

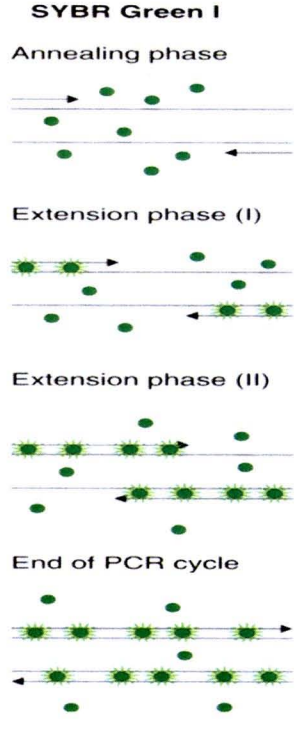

Figure 14 The principle of SYBR Green detection in real-time PCR. (http://www.nature.com/leu/journal/v17/n6/fig_tab/2402922f1.html)

The 25 μL reaction mixture contained 5 μL of cDNA (dilute 1:5), 1X Maxima™ SYBR Green qPCR Master Mix, 10nM ROX Solution and 0.3 μM of Maspin or

GAPDH primers. Each reaction mixture was then placed on an ABI 7500 machine. The temperature profile was as follows: initial denaturation at 95 ℃ for 10 min, followed by 45 cycles of the amplification process, which are denaturation at 95 ℃ for 15 sec, annealing and extension at 60 ℃ for 60 sec.

2.2.4 Western blot analysis

The Western blot analysis is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or cell extract. It uses gel electrophoresis to separate denatured proteins by the size of the polypeptide. The proteins are then transferred to a membrane, where they are probed using antibodies specific to the target protein. In this study, expression of Maspin protein was analyzed by this technique to confirm the results from RT-PCR and qPCR experiments.

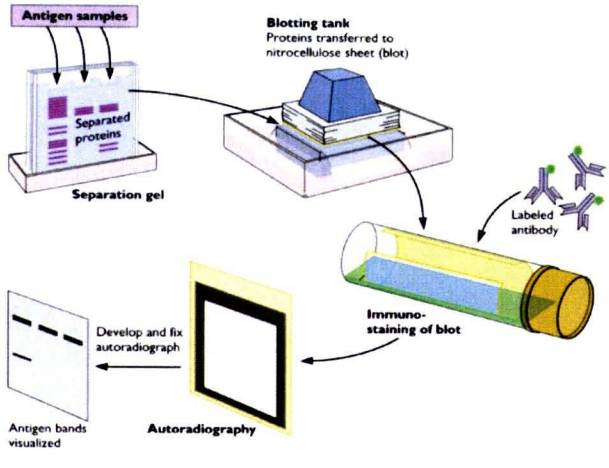

Figure 15 Western blotting workflow

(http://www.virology.ws/2010/07/07/virology-toolbox-the-western-blot/)

2.2.4.1 Protein determination

The protein concentration of cell pellets was quantified by the Bio-Rad Protein Assay (Bio-Rad). When Coomassie dye in the protein assay kit binds to proteins in acidic medium, there is an immediate shift in absorption maximum occurs from 465 nm to 595 nm with a concomitant color change from brown to blue. Protein concentrations are estimated by reference to absorbance obtained for a series of standard protein dilutions, which are assayed alongside the unknown samples.

The procedure of protein determination by Bio-Rad protein assay reagent was as follows: 250 µL of the assay reagent was added to each well of a 96-well microplate which contains bovine serum albumin (BSA) standard solution in various concentrations (187.5 – 500 µg/mL) and samples. Then, the absorbance was measured at 620 nm with a microplate reader; Biotech K-40 (BioTEK, USA). The absorbance values of all individual standard and sample replicates were subtracted by that of the blank. The standard curve of protein was plotted as shown in Figure 16. and used to determine the protein concentration of each sample

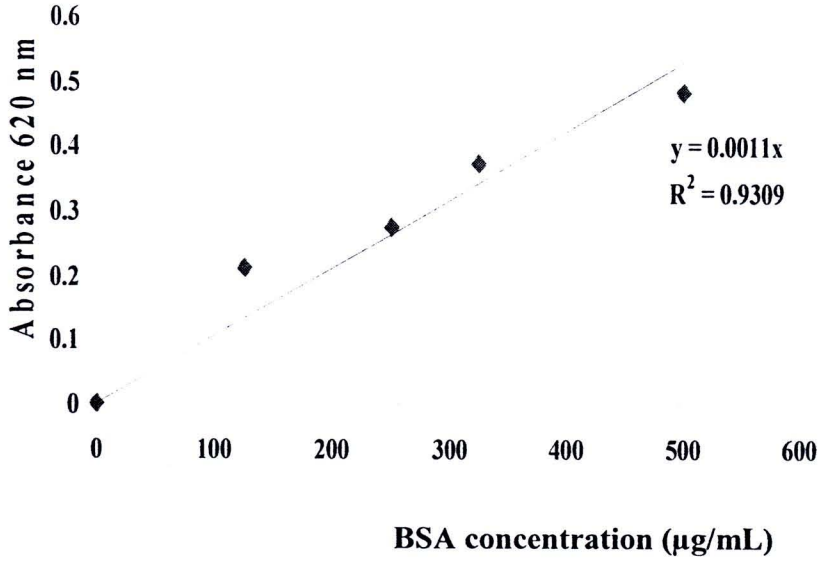

Figure 16 The standard curve of bovine serum albumin (BSA)

2.2.4.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

SDS-PAGE maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (e.g. disulfide bonds [S-S] to sulfhydryl groups [SH and SH]) and thus allows separation of proteins by their molecular weight. Protein samples become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of the gel. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size.

The equal amount of protein from treated cell homogenate were loaded on a 12% SDS-polyacrylamide gel and electrophoresed at 40 mA for 1.5 h. The proteins in the gel were transferred to a nitrocellulose membrane by Mini Trans-Blot® (Bio-Rad, USA) at 30 Volt for 16 h. The membrane was then incubated sequentially with 5%

skim milk in PBS (blocking solution) for 2 h at room temperature or overnight at 4 °C for blocking non-specific binding, and probed with primary mouse anti-human Maspin (BD Biosciences, USA) at 1:2,000 in 1% blocking solution for 2 h at room temperature. Next, the nitrocellulose membrane was washed 5 times by 0.1% Tween 20-phosphate buffer saline (TPBS) buffer to remove excess antibodies. The membrane was then incubated with goat anti-mouse IgG linked to horseradish peroxidase (Bio-Rad, USA) at a 1: 8,000 dilution in 1% skim milk-TPBS and washed again with the buffer 5 times. The protein band was visualized by VisiGlo™ /VisiGlo PLUS™ HRP chemiluminescent Substrates (Ameresco, USA), and captured on Kodak X-ray film. The detected band was quantified by a scanning densitometry.

2.3 The Cytotoxicity effect of TGF-β1 and TNF-α in HeLa cells

Cell survival is determined by using the colorimetric Sulforhodamine B (SRB) assay. Sulforhodamine B (Sigma-Aldrich, USA) is bright-pink aminoxanthane dye with two sulfonic groups that bind to basic amino-basic residues under mild acidic conditions, and dissociate under basic conditions. The assay relies on the ability of SRB dye to bind to protein components of cells that have been fixed to tissue-culture plates by trichloroacetic acid (TCA). As the binding of SRB is stoichimetric, the amount of dye extracted from stained cell is directly proportional to the cell mass (122).

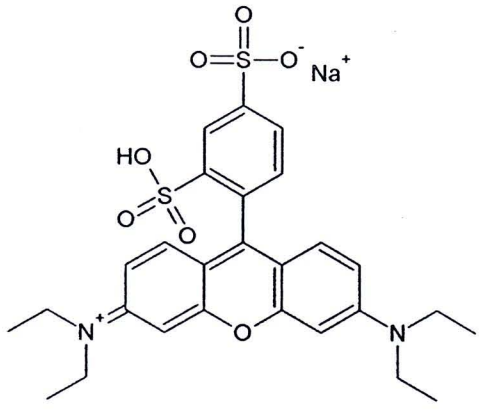

Figure 17 Structure of Sulforhodamine B

2.3.1 Cell preparation

After removing medium, the cells were washed with sterilized phosphate-buffered saline (PBS), and then trypsinised with 0.25% (w/v) trypsin in versene-EDTA to evenly cover the cell-growth surface. When the cells were started to dissociate, they were dispersed from the culture surface by culture medium containing FBS using a sterilized plastic or glass pipette with and mixed to obtain a homogeneous cell suspension. After transferring the cell suspension to sterilized tube, the cells were centrifuged at 200xg for 5 min. The cell concentration were determined by counting in a hematocytometer chamber under a microscope, using a 1:1 mixture of cell suspension and 0.4% (w/v) trypan blue solution and calculate by using the formulae below:

$$\text{Number of cells (cells/}\mu\text{L)} = N \times Cv \times Cd$$

N = number of cells

Cv = correction factor of volume

Cd = correction factor of dilution

2.3.2 Sulforhodamine B assay protocol

Viable cells treated with different concentration of TGF-β1 and TNF-α were evaluated before and after treatment by Sulforhodamine B (SRB) assay in triplicate. Briefly, after adjusted the cell concentration with growth medium to obtain density at 1.0×10^4 cells/well, the cell suspensions were seeded in 96-well tissue-culture plates. They were occasionally mixed during plating to ensure an even distribution of the cells and incubate at 37°C in a humidified incubator with 5%CO_2 over night. Next, cells were incubated with TGF-β1 and TNF-α at 0.1, 1, 10 ng/mL for 24, 48 and 72 h. At the end of each treatment, the cells were fixed by gentle addition of 100 µl of cold 10% TCA to each well, followed by incubate at 4°C for 1 h. Plates were washed four times with deionized water. Then, the plates are left air dry at room temperature (25-30°C). Fixed cells were stained with 100 ul of SRB solution (0.057% SRB in 1% acetic acid) added to each well for 30 min at room temperature. Then, the plates were quickly rinsed four times with 1% (v/v) acetic acid to remove unbound dye and allowed to dry at room temperature. Next, bound dye was solubilized with 10 mM Tris-base solution (pH 10.5). The optical density (OD) was read at 510 nm using Biotech K-40 (BioTEK, USA). The absorbance values of treated samples were calculated as the percentage of cell-growth inhibition using the equation below:

$$\% \text{ of control cell growth} = \frac{\text{mean OD}_{\text{sample}} - \text{mean OD}_{\text{day0}} \times 100}{\text{mean OD}_{\text{neg.control}} - \text{mean OD}_{\text{day0}}}$$

$$\% \text{ growth inhibition} = 100 - \% \text{ of control cell growth}$$

2.4 The effect of TGF-β1 and TNF-α on cancer cell migration and invasion related to maspin expression

2.4.1 Migration assay

The migration assay was performed using polycarbonate 8μm pore-sized Multiscreen MIC 96-well. HeLa cells ($5x10^4$ cells/well) were pre-treated 24 h with 10 ng/mL TGF-β1 and TNF-α in serum-free Dulbecco's modified Eagle's medium (DMEM). Next, the cells were harvested using PBS containing 10mM EDTA, resuspended in serum-free DMEM, and transferred to the upper wells of the filter plate. DMEM culture medium containing 10% FBS is added to each well of the lower chambers. After 24 h of incubation at 37 °C in 5% CO_2 incubator, the medium in the second plate was carefully removed by aspirating. The wells were gently washed with PBS to remove nonadherent cells. The adherent cells in both microplates were freezed at -70 °C. Later, they were all labeled with fluorescent dye CyQuant (Molecular Probes, USA) according to the manufacturer's protocol.

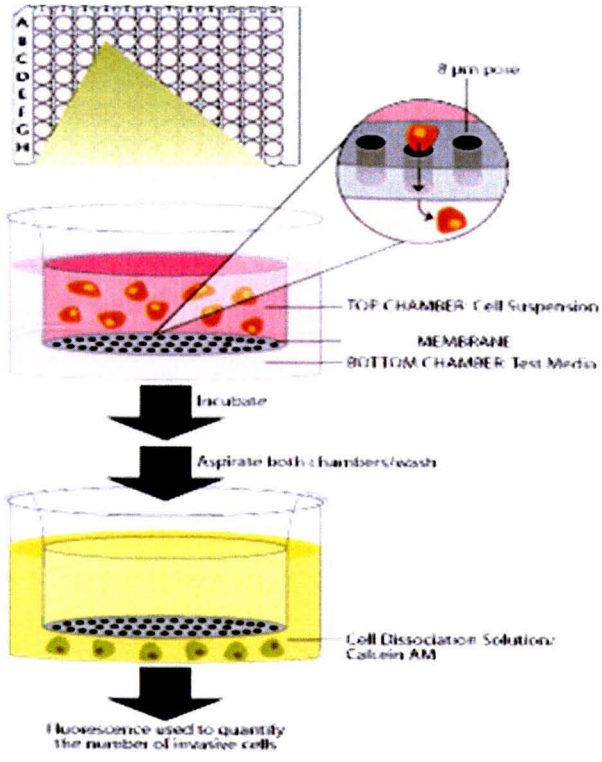

Figure 18 Experimental setup to study cell Migration *in vitro*

(http://www.rndsystems.com/product_detail_objectname_cell_invasion_assays.aspx)

The fluorescence was measured using a Biotech K-40 (BioTEK, USA) with Ex:480 nm and Em: 530 nm filters. Cells in triplicate wells without Transwell inserts serve as controls for cell proliferation and/or death during the incubation period. Migration is calculated by dividing the relative fluorescence value of migration cells with that of total cells plated in triplicate wells without Transwell inserts. Migration of the control is set at 100%. The experiments were repeated 3 times with at least triplicate per experiment.

2.4.2 Invasion assay

Cell invasion is one of the fundamental processes required during tumor progression and metastasis. If TGF-β1 and TNF-α have effect on Maspin mRNA and protein level of cancer cells, this leads us to hypothesize that TGF-β1 and TNF-α may control the invasion properties of human cancer cells.

The invasion assay was performed using the method as described elsewhere with several modifications (123). Polycarbonate 8μm pore-sized Multiscreen MIC 96-well were precoated with 5 μg/mL Matrigel (BD Bioscience, USA). HeLa cells (5 x 10^4 cells/well) pretreated with TGF-β1 and TNF-α for 24 h in serum-free DMEM were added to the upper wells of the filter plate in the absence of cytokines. DMEM culture medium containing 10% FBS was added to the lower chambers. After 48 h of incubation at 37 °C in 5% CO_2 incubator, the medium from the second plate was carefully removed by aspirating. The wells were gently washed with PBS to remove nonadherent cells. The adherent cells in both microplates were freezed at -70 °C. Later, they were all labeled with fluorescent dye CyQuant (Molecular Probes, USA) according to the manufacturer's protocol.

The fluorescence was measured using a Biotech K-40 (BioTEK, USA) with Ex:480 nm and Em: 530 nm filters. Cells in triplicate wells without Transwell inserts serve as controls for cell proliferation and/or death during the incubation period. Invasion is calculated by dividing the relative fluorescence value of invading cells with that of total cells plated in triplicate wells without Transwell inserts. Invasion of the control is set at 100%. The experiments were repeated 3 times with at least triplicate per experiment.

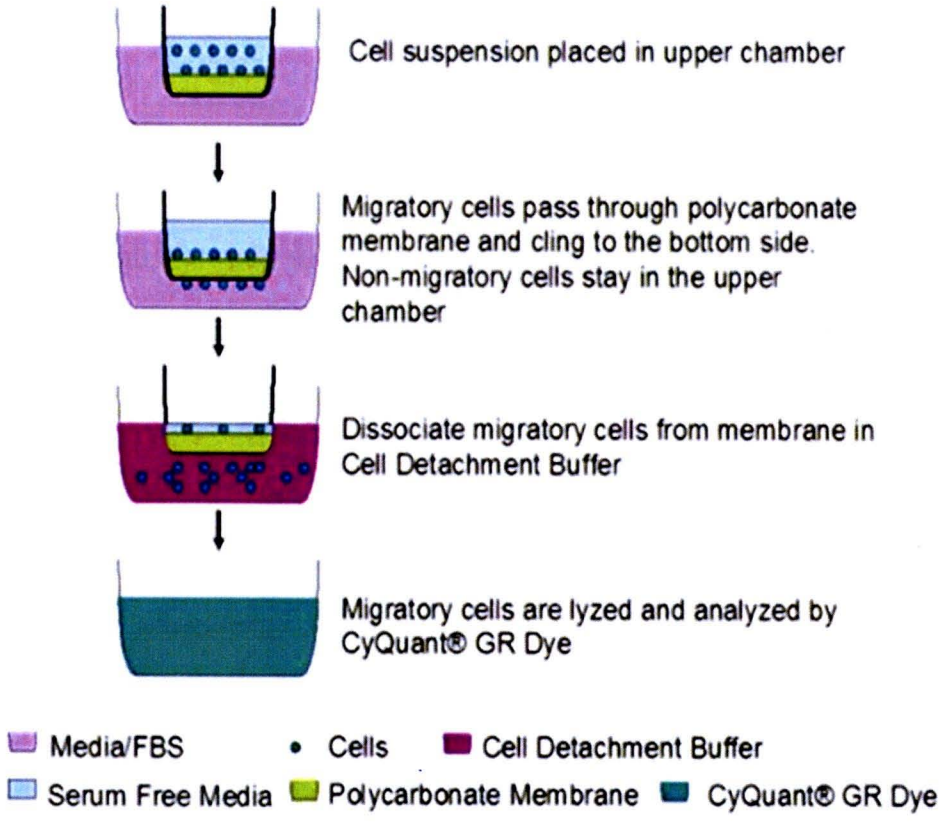

Figure 19 Illustration of *in vitro* Invasion

(http://www.biocat.com/cgibin/page/sub2.pl?main_group=cell_biology&sub1=cell_migration&sub2=fluorometric_cell_migration_assays)

2.5 Alteration of Maspin promoter methylation by TGF-β1 and TNF-α

Epigenetics is the study of heritable changes in gene regulation that do not involve a change in the DNA sequence or the sequence of the proteins associated with DNA. DNA methylation is an essential epigenetic signal involved in development, gene regulation, imprinting, and preserving genome integrity. In mammals, it mainly occurs at cytosine in the context of the CpG-dinucleotide. The bisulfite genomic-sequencing method is based on the selective deamination of cytosine residues, but not 5-methylcytosines, by treatment with sodium bisulfite and the sequencing results from the subsequently generated PCR products as shown in Figure 20 (124, 125).

Currently, bisulfite sequencing of single clones is regarded as the gold standard of DNA methylation analysis because sequencing of subcloned individual DNA molecules provides the most reliable and detailed information on the methylation pattern for every single CpG site in a relatively long stretch of sequence (300–500 bp). Furthermore, it provides unambiguous methylation information for haplotypes of DNA molecules in a qualitative and quantitative manner and is able to show correlations between the DNA methylation states of different CpG sites.

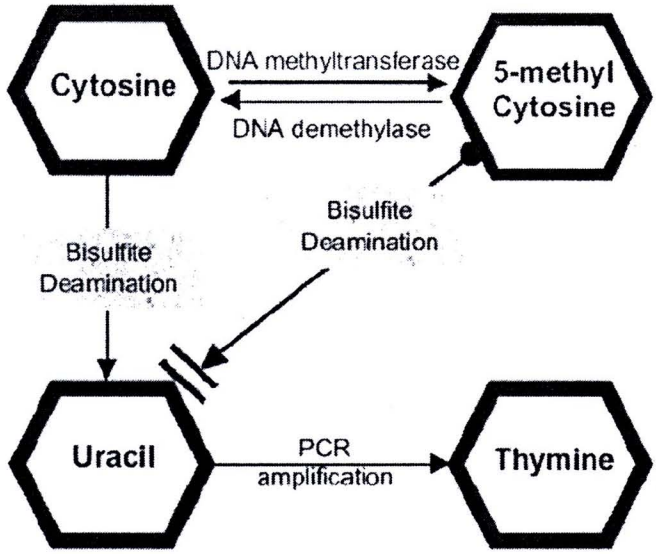

Figure 20 The biochemical reaction pathways of cytosine *in vitro*. Sodium bisulfate can convert cytosine into uracil through steps of sulfonation, hydrolytic deamination, and subsequent desulfonation with alkali. 5-Methylcytosine is, however, protected from this bisulfate reaction owing to the presence of the methyl group, which blocks the sulfonation by bisulfate.(Trygve O, 2004)

2.5.1 DNA extraction

Total genomic DNA was extracted using Trizol reagent (Invitrogen, USA), according to the manufacturer's instruction. In brief, the cell monolayers were directly lysed in a culture dish by adding 1 mL of Trizol reagent to a 3.5 cm diameter dish, and passed several times through a pipette. The cleared homogenate solutions were transferred to a fresh tube. The homogenized cells were incubated for 5 min at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes and then 0.2 mL of chloroform per 1 mL of Trizol reagent was added into each tube. The tubes will be

vigorously shaken for 15 sec and incubated at 15 to 30°C for 2 to 3 min. After centrifugation at 12,000xg for 15 min at 4 °C, the mixture is separated into a lower red, phenol-chloroform phase, an inter-phase, and a colorless upper aqueous phase. The remaining aqueous phase overlaying the interphase was removed, and the DNA precipitated from the interphase and organic phase using .15 mL of absolute ethanol. Next, the samples were stored at 15 to 30°C for 2-3 min and the DNA was sedimented by centrifugation at no more than 2,000 xg for 5 min at 4°C. The phenol-ethanol supernatant was rremoved, and the DNA pellet was washed twice in a solution containing 0.1M sodium citrate in 10% ethanol 0.5 mL. At each wash, the DNA pellet was stored in the washing solution for 30 min at 15 to 30 °C and centrifuged at 2,000xg for 5 min at 4°C. Following these two washes, the DNA pellet was suspended in 75% ethanol 1 mL, stored for 10-20 min at 15 to 30 °C and centrifuged at 2,000xg for 5 min at 4 °C. the DNA was air-dried for 5 to 15 min in an open tube, and dissolved in 8 mM NaOH. The pH of NaOH should be easily adjusted with TE or HEPES once the DNA is in solution. The DNA was stored at -20°C.

The quantity and quality of total DNA were assessed by the ratio of A_{260} / A_{280}. The concentration of DNA was determined by GeneQuant pro (Amersham Biosciences, Germany). An OD of 1.0 at the wavelength of 260 nm corresponds to approximately 50 µg/mL for DNA. The obtained DNA is diluted 50 times in water before measuring. The concentration of DNA is calculated by using the formula below:

Concentration of DNA (µg/mL) = OD_{260} x 50 x dilution factor

The quality of the DNA is essential to overall success of the analysis. OD_{260} is frequently used to measure DNA concentration, and OD_{280} protein concentration. For further analysis, it is imperative that the extracted DNA should have high purity, displaying ratio of OD_{260}/OD_{280} values between 1.7 and 1.9. Smaller ratio usually indicates contamination of protein or organic chemicals.

2.5.2 Sodium bisulfate treatment and polymerase chain reaction

Briefly, first the genomic DNA was extracted using Trizol reagent (Invitrogen, USA) from treated cell. Then, genomic DNA (200 ng) was treated with NaOH and sodium bisulfite using EZ DNA Methylation-Gold™ Kit (ZYMO RESEARCH, USA). The genomic DNAs were denatured to single-stranded DNA and all unmethylated cytosines were chemically converted to uracil. After bisulfite conversion, the upper and lower strands of the DNA were no longer complementary. In the next step, the DNA sequence under investigation was amplified by PCR with primers U2 and D2 which are specific to the bisulfite-modified sequences of the Maspin promoter as shown in Figure 21. The sequence of primer U2 is located at nt -284 to nt -256 (5'-AAAAGAATGGAGATTAGAGTATTTTTTGTG-3') and primer D2 nt +180 to nt +153(5'-CCTAAAATCACAATTATCCTAAAAAATA-3').

To ascertain the bisulfate conversion, primers specific to the original sequences of Maspin protomer were used as MP sense, 5'-TGGAGATCAGAGTA CTTTTTGTGCC-3', and MP anti, 5'-ACCTGGAGTCACAGTTATCCTGGAAA-3'. The PCR amplification was performed using genomic DNA or bisulfate-modified DNA in 50 μL of reaction mixture using 1 μM primer 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 (126).

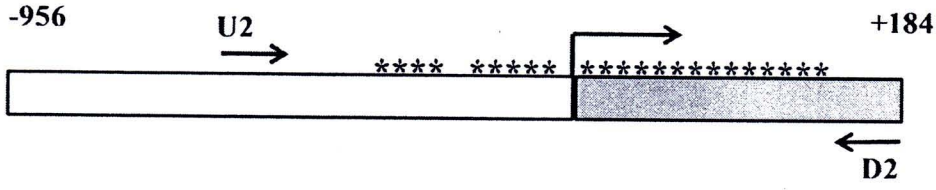

Figure 21 Diagram of the Maspin gene promoter region. The number refer to the sequence of the maspin promoter as defined by Sager and colors white and gray refer to the promoter and 5'UTR, respectively. The asterisks were denoted location of CpG positions at -247, -194, -178, -170, -163, -151, -147, -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 were indicated by U2 and D2 primers at positions -284 to -256 and +184 to +157, respectively(126).

During this process, uracil was amplified as thymine. In contrast, if the 5-methylcytosines remain unchanged, they will be amplified as cytosine in the PCR. The PCR products were run on a 1.5% agarose gel, then extracted and purified from the gel using the Qiagen II gel Extraction kit (Qiagen, USA). Next, the PCR fragment was ligated into pTG19-T PCR cloning vectors according to the manufacturer's instruction (Vivantis, USA). The ligated vector was transformed into competent *E.coli* cells (DH5α). The transformed cells were plated on selective agar containing 100 ug/mL ampicillin and Xgal/IPTG to allow screening of positive transformants by the appearance of white colonies. The positive clones were selected and grown in LB medium. Plasmids containing the DNA insert of interest were extracted and randomly selected clones were sequenced by a commercial service (1st Base, Singapore). According to the sequencing results, it can be distinguished if the cytosines in the CpG sites were methylated. The methylation percentage of each CpG site can be calculated according to the number of methylated and unmethylated cytosines in different clones (127).

2.6 Statistic analysis

All values are given as mean ± standard derivation (m ± SD) from triplicate samples of three independent experiments. The analysis of variance (ANOVA) with SPSS 15.0 software package was used to compare treated and control cells. Differences were considered statistically significant when $p < 0.05$.