CHAPTER I

INTRODUCTION

1.1 Statement and significance of problem

Cancer is one of the major public health problems worldwide, and now has a very high mortality rate in Thailand. Cancer cells are typically classified by uncontrolled cell growth and cell division leading to a mass of unwanted cells called a tumor. Besides, many cancer cells have an ability to relocalize to distant tissues and these distant settlements of cancer cells consequently account for the major cause of death in cancer.

Carcinogenesis is a complicated multi-step process in which initiation and progression of cancer in cells involve genetic/epigenetic aberrations of human genome. A typical cancer cell does not result from a single mutation, but rather the multiple mutations of numerous genes. Such alterations in the genome lead to either over-activation (e.g. proto-oncogenes) or inactivation (e.g. tumor suppressor genes) of specific groups of genes. Altered regulation of such genes in cancer cells eventually causes uncontrolled-cell proliferation, invasion, and metastasis. Understanding regulation of the gene expression is thus necessary for development of novel efficient cancer therapy.

Maspin (Mammary Serine Protease Inhibitor) is belonging to a SERPIN superfamily of proteins. Several studies have revealed the tumor suppressor role of

Maspin as an effective inhibitor of cancer cell invasion, metastasis and angiogenesis (1). Maspin is originally found in normal mammary epithelial cells, but reduced or absent in carcinomas cells. Re-expression of Maspin in carcinoma cell lines leads to inhibition of cell invasion and metastasis both *in vitro* and *in vivo* (2, 3). Therefore, Maspin becomes a promising target for both prognosis/diagnosis and therapeutic intervention against cancer.

Promotion of carcinogenesis involves several factors including physical, chemical, or biological, which induce the growth of mutated cells leading to abnormal proliferation of the cells or tumor. Inflammation is one of the critical processes that take place in concurrent with carcinogenesis as the immune cells are recruited and release inflammatory cytokines such as interleukin-1β (IL-1β), tumor growth factor-β1 (TGF-β1) and tumor necrosis factor-α (TNF-α) etc. to the area of tumor. Depending upon different stages of cancer, the effects of these cytokines are varied on each signaling pathways. Many inflammatory cytokines possess an anti-cancer activity at the early step of tumorigenesis as a defense mechanism of immune response (4). In contrast, the presence of cytokines in later stage of cancer may cause the induction of adhesion molecules and metalloproteinases, both of which provide mechanisms for tumor invasion (4). Thus, blocking cytokines may be an effective approach for prevention of cancer metastasis if administered at the appropriate time and sufficient concentration.

In this study, effect of pro-inflammatory cytokines on Maspin expression will be explored to gain a further insight into a connection between inflammation and cancer invasiveness. The investigation of cytokine effect on modulation of Maspin

expression will further enhance our understanding of cancer biology. In addition, the results will provide an avenue to develop Maspin's potential as a diagnostic/prognosis marker for cancer progression, and a promise of cytokines as a potentially powerful therapeutic target in the fight against cancer.

1.2 Literature reviews

Carcinogenesis

Carcinogenesis that leads from a normal cell to cancer can be divided into three phases: initiation, promotion and progression. Initiation involves genomic alterations, promotion the proliferation of genetically altered cells, and progression an increase in the size of the tumor, the spreading of the tumor and the acquisition of additional genetic changes (5).

Initiation: Neoplasia initiation is crucial irreversible changes in appropriate target somatic cells. During the tumor initiation, the DNA of the cell is mutated by chemical or physical carcinogens, leading to the activation of oncogenes and/or the inactivation of tumor-suppressor genes (most commonly those that encode KRAS and p53, respectively). The cellular genome undergoes mutations, creating the potential for neoplastic development (6), which predisposes the affected cell and its progeny to subsequent neoplastic transformation. Such tumor gene mutations can have profound effects on cellular behavior and response. They can also lead to dysregulation of genes involved in biochemical signaling pathways concerned with control of cell proliferation and/or disruption of the natural processes of cellular communication, development and differentiation. As shown in Figure 1, DNA repair and cell death

pathways operate to repair or eliminate damaged cells, respectively. Failure to repair or get rid of damaging cells can result in fixation of DNA damage into mutation. Rapidly proliferating cells are more likely than slowly proliferating cells to fix DNA damage into mutations. A mutated cell has the potential to transform and can ultimately give rise to a cancer after clonal expansion The transformed cell undergoes continuous division with fidelity to the transformed karyotype and, possibly, with further mutations, before a malignant lesion is manifested.

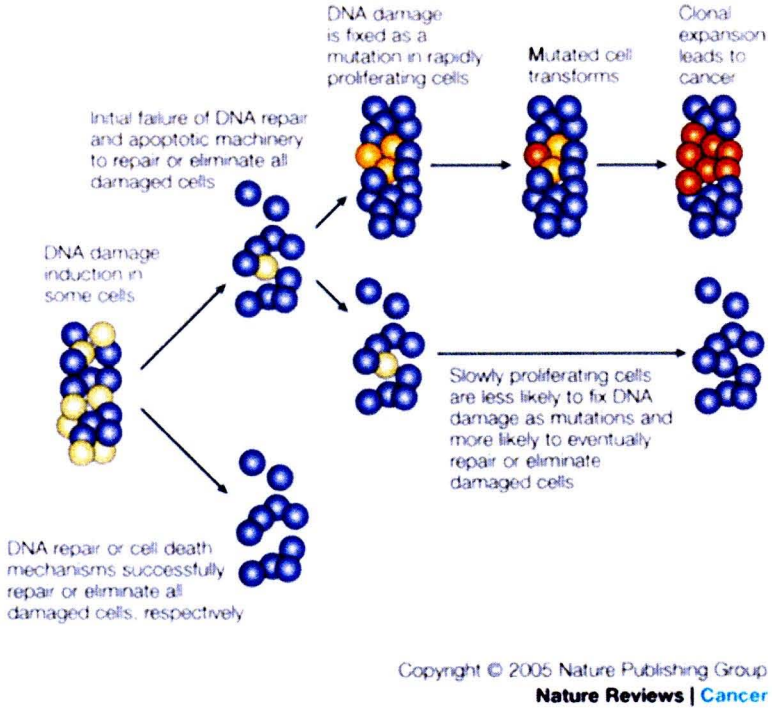

Figure 1 A diagram of the initiation process (7).

Promotion: The transformed (initiated) cell can remain harmless, unless and until it is stimulated to undergo further proliferation, upsetting the cellular balance. The second phase of tumorigenesis, tumor promotion, is characterized by the clonal expansion of initiated cells, owing to increased cell proliferation and/or reduced cell death. The subsequent changes of an initiated cell leading to neoplastic transformation

may involve more than one step and requires repeated and prolonged exposures to promoting stimuli (8). Neoplastic development is influenced by the intra- and extracellular environment. Expression of the initial mutation will depend not only on interaction with other oncogenic mutations but also on factors that may temporarily change the patterns of specific gene expression, i.e. lipid, metabolites cytokines, and certain phorbol esters. This may result in an enhancement of cellular growth potential and/or an uncoupling of the intercellular communication processes that restrict cellular autonomy and thereby coordinate tissue maintenance and development.

Progression: It is the process through which successive changes in the neoplasm give rise to increasingly malignant sub-populations. The process may be accelerated by repeated exposures to carcinogenic stimuli or by selection pressures favoring the autonomous clonal derivatives. The initiated cells proliferate causing a rapid increase in the tumor size. As the tumor grows in size, the cells may undergo further mutations, leading to increasing heterogeneity of the cell population. In the first phase of progression, the pre-neoplastic cells are transformed to a state in which they are more committed to malignant development. This may involve further gene mutations accumulating within the expanding pre-neoplastic cell clone. The dynamic cellular heterogeneity, in many instances, may be a consequence of the early acquisition of gene specific mutations that destabilize the genome. Examples are mutations of the p53 gene (9) or DNA mismatch repair genes (10). Many tumor types develop transforming sequences in their DNA during their progression from the normal to the cancerous state. An accurate mutation rate established relatively early in tumor development may, therefore, provide for the high-frequency generation of variant

cells within a premalignant cell population. Such variant cells, having the capacity to evade the constraints that act to restrict proliferation of aberrant cells, will tend to be selected during tumorigenesis.

Tumor metastasis: Metastasis is a complex, multi-step process involving cell migration, invasion through the basement membrane, and growth in a foreign microenvironment. As the tumor progression advances, the cells lose their adherence property, detach from the tumor mass and invade the neighboring tissues. The detached cells also enter the circulating blood and lymph and are transported to other organs/tissues away from the site of the primary growth and develop into secondary tumors at the new sites. Many of the steps in tumor metastasis involve cell invasion and cell-cell-matrix interactions. Malignant cells have reduced ability to adhere to each other, so that they detach from the primary tumor and invade the surrounding tissues.

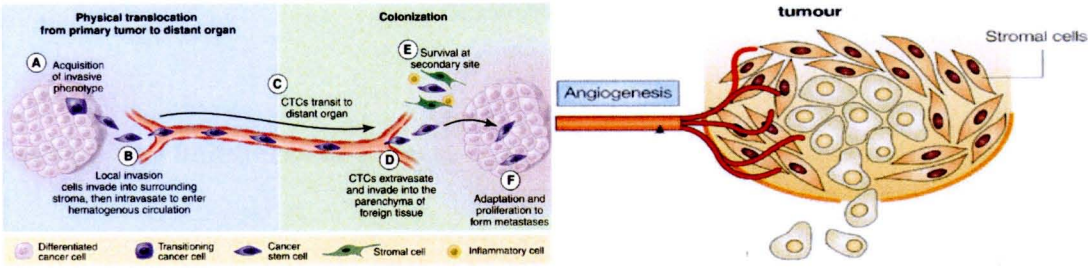

Figure 2 The processes of metastasis (11) and angiogenesis (12)

Tumor angiogenesis: Tumor growth depends on the supply of growth factors and efficient removal of toxic molecules, which comes through an adequate blood supply. Thereby, increase in tumor mass to more than 1-2 mm will depend on adequate blood supply through development of blood capillaries (angiogenesis).

In conclusion, six substantial alterations, some if not all, are acquired for characteristics of cancer cell a tumor cell: unlimited replicative potential, self-sufficiency in growth signals, insensitivity to growth inhibitors, evasion of programmed cell death, ability to develop blood vessels, and tissue invasion and metastasis (13). In addition, an inflammatory microenvironment is necessary for cancer progression(14).

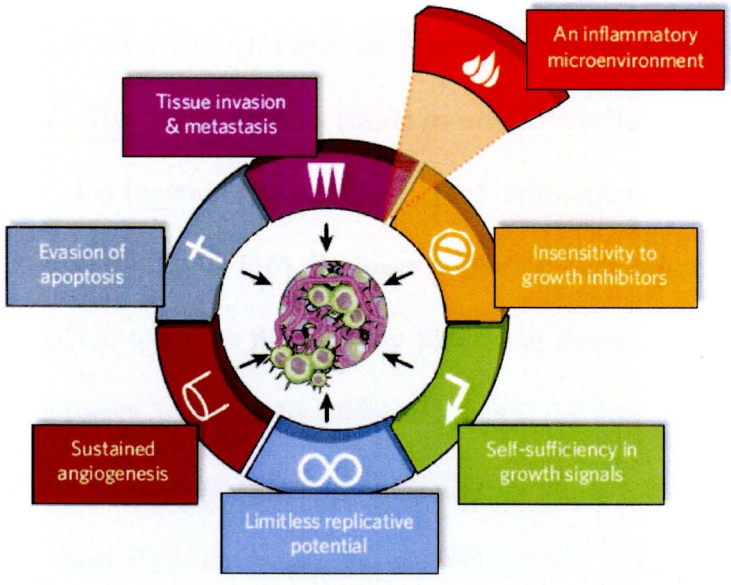

Figure 3 The hallmarks of cancer.

Maspin

Serine protease inhibitors (SERPINs) comprise a large protein family with diverse biological functions. As a member of the SERPIN family, Maspin (SERPIN B5 or Mammary Serpin) has been grouped with the ov-SERPIN subfamily (Clade B) because its primary structure exhibits a high sequence similarity to chicken ovalbumin (31%) (15). Currently, there are at least 13 known human ov-SERPINs, including plasminogen activator inhibitor 2 (PAI2) and the squamous cell carcinoma antigens 1 and 2 (SCCA1, SCCA2). Human ov-serpins reside mostly intracellularly, but several, including Maspin, may also function extracellularly (16). All human ov-SERPINs are known functional protease inhibitors (16). Protease inhibition occurs via the SERPIN reactive center loop (RCL), which is the primary functional domain of the SERPIN family. Despite its similarity to other SERPIN members, the Maspin RCL is not conserved and is shorter than most RCLs of the SERPIN superfamily. It has been hypothesized that the short RCL decreases the stability of the molecule, rendering Maspin extremely sensitive to limited proteolysis (17). Currently, no target proteases for Maspin have been known yet. Thus, as a non-inhibitory SERPIN, Maspin does not directly exert its biological functions as a protease inhibitor (17). Nonetheless, the Maspin RCL has proven necessary for some of Maspin's tumor suppressive functions, including adhesion to the cellular matrix and Maspin interactions at the cell surface (18-20).

Biological function of Maspin

Despite an unclear mechanism, Maspin's role in suppressing tumor growth and metastasis has been shown *in vitro* and *in vivo* in various types of cancer. Maspin has been consistently shown to suppress the aggressive tumor phenotypes, inhibiting cell invasion and mobility *in vitro* and inhibiting tumor growth and metastasis in experimental animal models (18-20). Extensive studies have been undertaken to determine the mechanisms of Maspin to produce its anti-metastatic effects.

One line of evidence suggests that Maspin regulates cell invasion by altering integrin profiles of the cell. Specifically, treatment of MDA-MB-435 breast cancer cells with recombinant Maspin (rMaspin) resulted in increased levels of α5- and α3-containing integrins (21). These changes to the cellular integrin profile were accompanied by increased adherence to fibronectin and a reduction in invasion through a fibronectin/gelatin matrix. Importantly, the addition of a blocking antibody to the α5b1 integrin, a classical fibronectin-binding integrin, mitigated the response induced by rMaspin (21). In this study, it was unclear whether rMaspin was being internalized and functioning intracellularly or whether it was working at the cell surface. In support of a cell surface event, it has been reported that cell surface-associated Maspin is primarily responsible for Maspin's anti-invasive properties (3).

In conjunction with Maspin's ability to alter the cellular integrin profile, Maspin re-expression also alters signaling pathways involved in motility and invasion. Treatment with rMaspin resulted in decreased Rac1 activity concomitant with an increase in PI3K and ERK1/2 activities which resulted in a decrease in cell motility

and an increase in cell adhesion (22). These changes in cell signaling are accompanied by increased focal adhesions and stress fibers and a reversion of the aggressive breast cancer cell line MDA-MB-231 to a more epithelial-like phenotype. This study suggests that Maspin may regulate cell migration by regulating Rho GTPase signaling pathways. Another anti-invasive mechanism apparently utilized by Maspin targets the urokinase plasminogen activator (uPA)/urokinase plasminogen activator receptor (uPAR) complex at the cell surface. The uPA/uPAR complex is involved in the conversion of plasminogen to plasmin, an active protease with broad specificity, which is able to cleave a number of extracellular matrix proteins such as fibronectin, fibrin, and laminin. Although Maspin does not directly inhibit uPA activity, it was shown to reduce cell surface-associated uPA/uPAR by inducing their internalization (23, 24).

Maspin is localized both in cytoplasmic and nuclear compartments, but may also be secreted. Many biological functions of Maspin have been studied and proposed as shown in Figure 4. The role of cytoplasmic and nuclear Maspin is not well understood; however, some indication can be taken from the effect of Maspin interaction with proteins in these two parts of cells. In the nucleus, Maspin binds to and inhibits histone deacetylase (25) and binds to interferon regulatory protein-6, IRF-6 (26). The presence of Maspin prevents IRF-6 induced increases in N-cadhedrin 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 (27). The implications of Maspin in the nucleus vs the cytoplasm await more in depth researches.

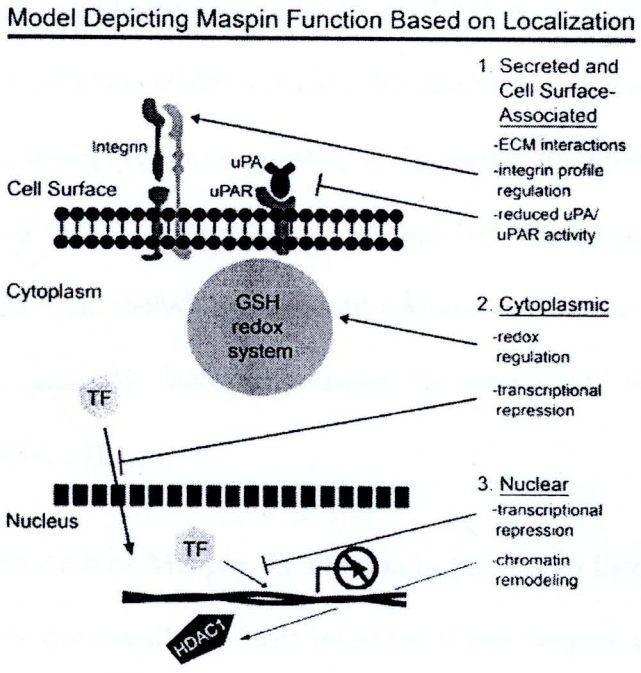

Figure 4 A model depiciting possible functions for Maspin based on it's localization within or without the cell. Abbreviation: TF, transcription factor; uPA, urokinase plasminogen activator; uPAR, uPA receptor; HDAC1, histone deacethylase-1; ECM, extracellular matrix; GSH, glutathione (18).

Expression of Maspin

Maspin expression has been demonstrated in multiple tissues including epithelium of the breast, prostate, epidermis, lung, and in stroma as well as endothelium of the cornea (28-30). Depending on the type of cell, Maspin also demonstrates a broad localization pattern. In mammary epithelial cells, Maspin is present primarily in the cytoplasm, but can also localize to the nucleus (in myoepithelial cells), secretory vesicles, and the cell surface (15, 31). The localization of Maspin appears important to its function(s) since in some tissues, aberrant Maspin localization can be indicative of a neoplastic lesion (Table 1). Sood et al.

demonstrated that the cytoplasmic localization of Maspin in ovarian carcinoma coincides with a poor prognosis, whereas nuclear Maspin localization in these cancers is indicative of a more benign lesion, suggesting an important tumor-suppressive role for nuclear Maspin (15, 31). This paradoxical Maspin localization pattern has been seen in other cancers as well, including non-small cell lung carcinoma and pancreatic cancer, where predominantly nuclear Maspin is associated with favorable morphologic features (32, 33).

Table 1 Tissue Distribution of Maspin. In addition to the tissues listed in this table, Maspin has been detected in small and large intestine, testes, tongue, thymus, vagina (42) and placenta (43), and in the absence of further information, they have not been included in the table. nd: not determined

Tissue	Cell type	Neoplasic alteration	Subcellular site	Ref
Breast	Epithelial	↓	Cytoplasmic	(15, 34)
Prostate	Epithelial	↓	Cytoplasmic	(15)
Skin	Keratinocytes	↓	Cytoplasmic	(35, 36)
Pancreas	Epithelial	↑	Nuclear	(37, 38)
Ovary	Epithelial	↑	Nuclear	(34)
Lung	Epithelial	↓	nd	(39)
Larynx	nd	↑	Cytoplasmic	(40)
Colon	Epithelial	↑	Cytoplasmic	(41)
Hair follicle	nd	↓	Cytoplasmic	(35)
Sebaceous gland	nd	↓	Cytoplasmic	(35)
Cornea	Endothelail	nd	nd	(28)
Cornea	Stromal	nd	Cytoplasmic/nuclear	(28)
Cornea	Epithelial	nd	nd	(28)

Regulation of Maspin expression

Cloning Maspin promoter led to identification of Ets, activator protein 1 (Ap1), hormone-responsive element (HRE), HIF, and p53 binding sites within the 1-kb promoter region (44). Activation of transcription in normal breast epithelial cells is due to the Ets and its synergy with the Ap1 site (Figure 5). The HRE is a negative regulator, acting through the androgen receptor in prostate (16). Nonadjuvant androgen ablation and anti-estrogen (tamoxifen) therapies resulted in the induction of Maspin in prostate cancer cell line (45) and breast cancer cell line MCF-7 (46). In an effort to define downstream targets of p53, Zou *et al.* (2000) demonstrated that adenoviral delivery of wild-type p53 to breast and prostate cancer cell lines could induce the re-expression of the Maspin tumor suppressor gene (47). The expression of wild-type p53 maintains regulatory checkpoints, including the regulation of Maspin expression. The regulation of Maspin by p53 could explain the role of p53 in cell invasion and metastasis, and hypothesize that cancer cells expressing mutant p53 would be more likely to metastasize, in part due to the inability to upregulate the Maspin gene.

Loss of Maspin expression during tumor progression was demonstrated to result, in part, from the absence of Ets, Ap1 and p53 transactivation in combination with epigenetic silencing by methylation (48). 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 (49). 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 (37, 50, 51). As

Maspin's potential is a possible therapeutic agent, many laboratories have sought a better understanding of the regulation of Maspin expression.

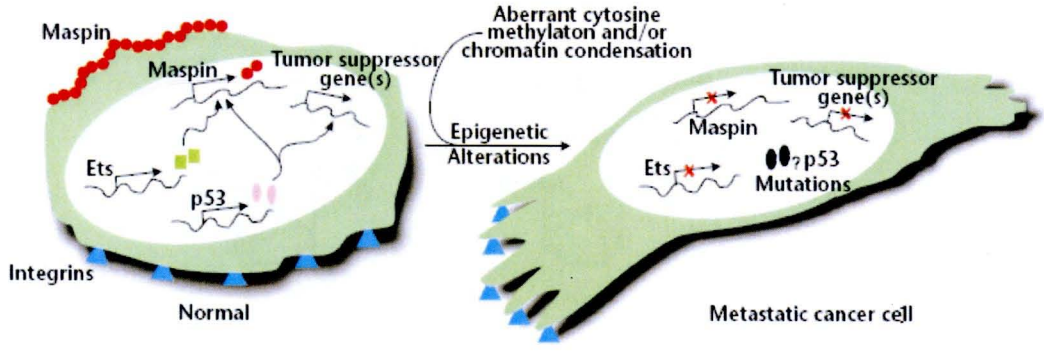

Figure 5 Hypothetical model for the expression of Maspin (52). The tumor suppressive function of Maspin is intact along with transactivation through the (Ets) element. Maspin is commonly found at the cell surface along with specific integrins involved in maintaining the epithelial phenotype of stationary cells. Loss of Maspin expression or silencing of this tumor suppressor gene resulting from epigenetic alterations, aberrant cytosine methylation and/or chromatin condensation and/or p53 mutations leads to an aggressive, metastatic phenotype.

Epigenetic regulation of gene expression

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. Two of the principle epigenetic events that contribute to diversity are DNA methylation and post-translational modifications of histones (Figure 6). The pattern of methylation can change during differentiation from one cell type into another or during carcinogenesis. In mammalian cells, between 2% and 7% (depending on the species and tissue) of all cytosine residues present in the genome are methylated on

position 5 in the pyrimidine ring. DNA methylase or DNA methyltransferases (DNMTs) catalyze the DNA methylation by transferring a methyl group to the cytosine residues in CpG dinucleotide sequences (Figure 6A).

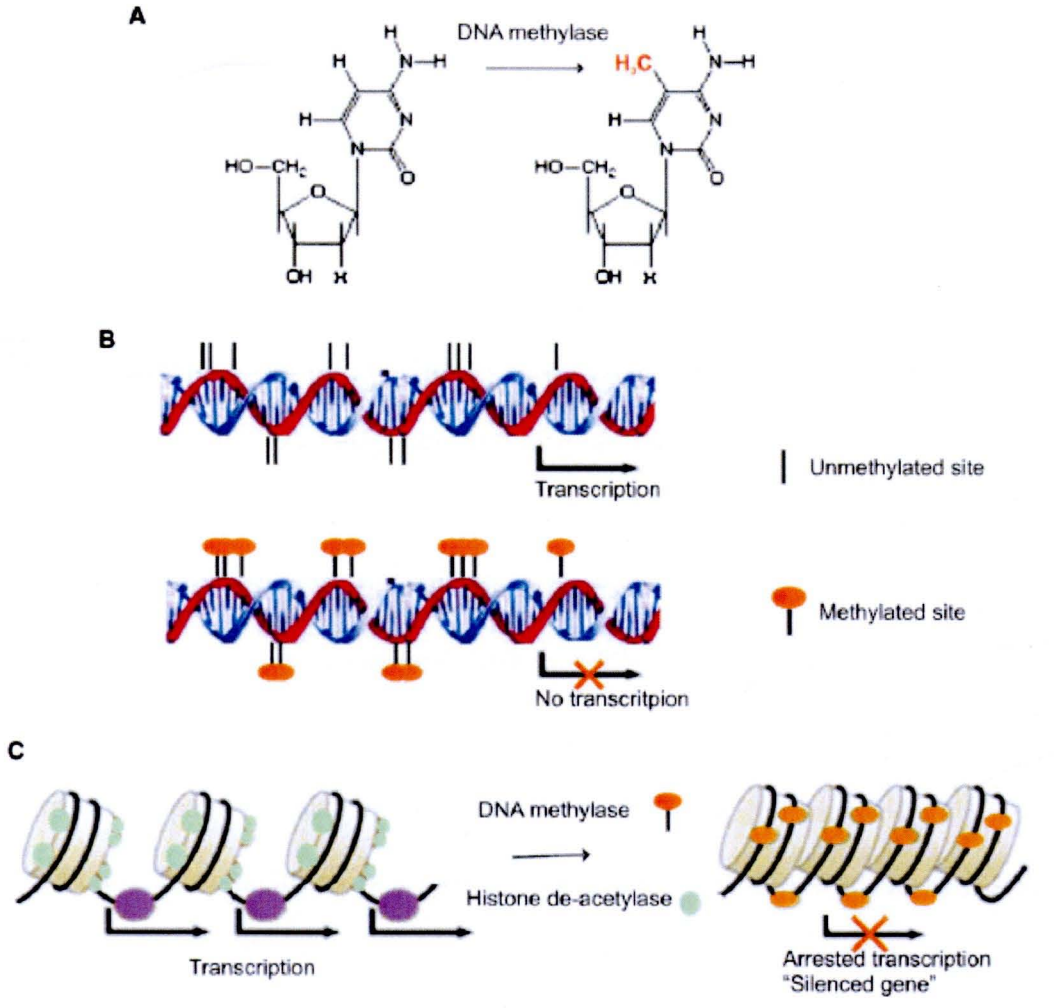

Figure 6 Picture of epigenetic regulation mechanism (53).

Methylation of the CpG islands in the gene promoter region inhibits gene expression (Figure 6B). Levine *et. al.* (54) found that inhibition of promoter activity correlated with the density of methyl CpG sites at the preinitiation domain of the promoter (TATA) box but was not affected by methyl CpG sequences distant from this domain. Their evidence also suggested that a methyl CpG binding protein was

involved in this inhibition. Interestingly, some DNA templates were able to establish functional preinitiation complexes even in their methylated. Furthurmore, histone acetylation, another epigenetic control, plays an essential role in the regulation of gene expression. Hyperacetylated chromatin is transcriptionally active whereas hypoacetylated chromatin is silent. Methyl-CpG-binding proteins interact with histone deacetylase causing gene silencing (Figure 6C).

Tissue specific control of Maspin expression by DNA methylation

The methylation status of the Maspin gene is evidently tissue specific; thus, epigenetic regulation of this tumor suppressor gene can lead to either activation or silencing of Maspin. It is expressed in a cell type– restricted manner and is expressed in epithelial cells of the airway, breast, skin and prostate, but not in skin fibroblasts, lymphocytes, bone marrow, heart and kidney (50). At the transcriptional level, Maspin is regulated by AP-1 and p53- binding sites, and can be negatively regulated by a hormone-responsive element recognized by the androgen receptor (52). As shown in Figure 7, methylation is a primary impediment to Maspin expression and thus determines the cell type–specificity. Acetylation of histones limits histone–histone interactions and thus provides an open chromatin structure that is required for Maspin expression. In contrast, the promoter in skin fibroblasts is completely methylated, associated with hypoacetylated histones and adopts an inaccessible transcriptionally inactive state. Methylation of DNA allows the binding of methyl CpG–binding proteins (MeCP), which can recruit histone deactylases (HDAC) and chromatin remodelling complexes to direct a local change in chromatin organization.

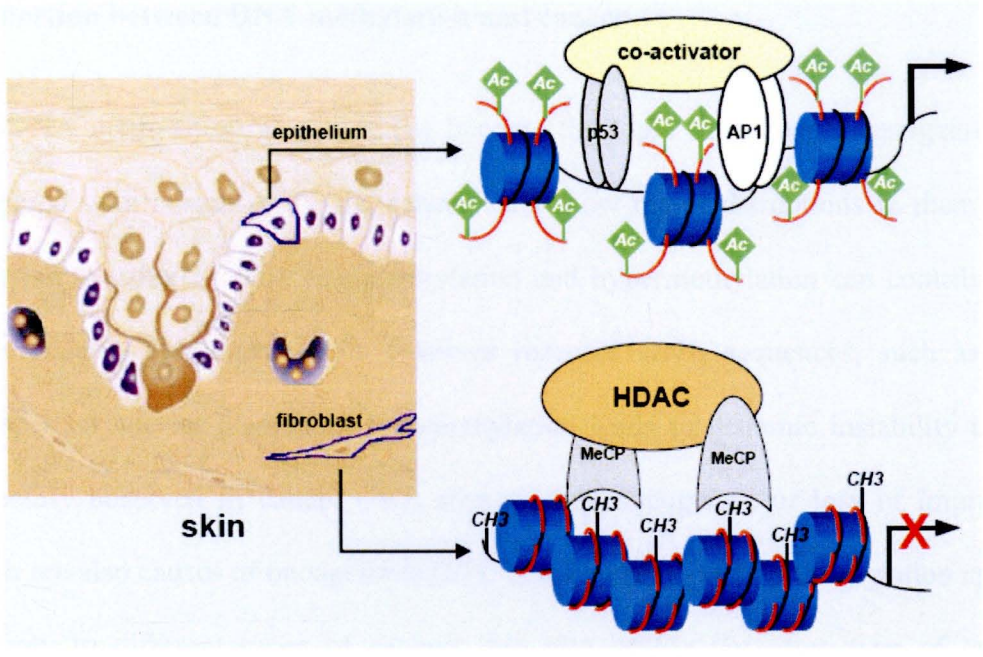

Figure 7 Model of the cell-type–specific control of Maspin expression by methylation (55). Abbreviations; Ac: acetylated, CH3: methylated, HDAC: histone deacetylase; MeCP: methyl CpG–binding proteins

In normal human cornea, Maspin is mainly expressed in corneal stromal keratinocytes but upon phenotypic differentiation, maspin mRNA and protein were dramatically down-regulated. Using sodium bisulfite sequencing method, Horswill *et al.* (2008) demonstrated that the Maspin promoter in the freshly isolated stromal keratocytes is hypomethylated while both the primary stromal cells and the first passage cells which are cultured in the presence of serum-free defined medium with fibroblast growth factor-2 and transforming growth factor -β1 are hypermethylated. Down-regulation of Maspin synthesis is also concerned with histone H3 dimethylation at lysine 9. Therefore, both Maspin promoter hypermethylation and histone methylation occur with down-regulation of Maspin synthesis in corneal stromal cells.

Connection between DNA methylation and cancer

DNA methylation in cancer has become the topic of intense investigation. As compared with normal cells, the cancer cells show major disruptions in their DNA methylation patterns. Both hypomethylation and hypermethylation can contribute to tumorigenesis. Hypomethylation involves repeated DNA sequences, such as long interspersed nuclear elements. Hypomethylation leads to genomic instability that is frequently observed in cancer (56), activation of oncogenes, or loss of imprinting which are also causes of oncogenesis (57). The variability of hypomethylation applies not only to different types of cancers, but also within the same type of cancer. Genomic hypomethylation increases the probability of genomic rearrangements through chromosomal instability and also contributes to oncogene activation that can lead to the development of cancer. Clinical advances are currently being made toward the classification of tumors based on genomic hypomethylation as well as the diagnosis and prognosis of cancer.

In many different types of cancerous cells, a profound loss of global 5-methylcytosine genomic content occurs in cancer cells with discrete areas of dense hypermethylation (56, 58, 59) occurring in the CpG islands located in the promoters of certain tumor-suppressor genes (56, 58, 59) leading to gene silencing. Such silencing of genes can contribute to many of the hallmarks of cancer including evading apoptosis, insensitivity to antigrowth signals, sustained angiogenesis, limitless replicative potential, and tissue evasion and metastasis. The progression of cancer is also greatly impacted by DNA methylation which is involved in gene silencing, chromosomal instability, and differentiation of cancer stem cells. Therefore,

hypermethylation may provide one of the most promising targets of cancer therapy and major advances are currently underway to further develop drugs that target hypermethylation abnormalities in cancer.

Expression of Maspin is down-regulated upon conversion of many epithelial cells to carcinoma cells such as mammary and prostate cancer (15, 21, 60). Nonetheless, Maspin is not expressed in some epithelial cells but is up-regulated in pancreatic, ovarian, and gastic carcinoma (31, 60, 61). Epigenetic changes of Maspin expression occur in the 5' regulatory region of the Maspin gene and involve cytosine methylation, histone deacetylation, and chromatin accessibility (62). Hypermethylation of the promoter of the Maspin gene is often found and plays a role on gene silencing in several cancers e.g. breast, thyroid, lung, skin, and colon (30, 41, 63-65). Studies have indicated that overexpression of Maspin in gastric, pancreatic, and ovarian cancers results from the promoter CpG demethylation (66, 67). This clearly indicates that both methylation and demethylation of Maspin promoter could regulate Maspin gene expression in cancer.

Table 2 The promoter methylation status of Maspin in cancer.

Tissue	Promoter status in Neoplastic alteration	Ref
Breast	Methylation	(15, 34)
Prostate	Methylation	(15)
Gall Bladder	Hypomethylation	(68)
Biliary tract	Hypomethylation	(51)
Pancreas	Hypomethylation	(37, 38)
Ovary	Hypomethylation	(34)
Lung	Hypomethylation	(39)
Bladder	Hypomethylation	(69)
Colon	Hypermethylation	(41)
Hair follicle	Methylation	(35)
Sebaceous gland	Methylation	(35)

Relationship between cancer and inflammation

The hallmarks of cancer-related inflammation include the presence of inflammatory cells and inflammatory mediators (for example, chemokines, cytokines and prostaglandins) in tumor tissues, tissue remodeling and angiogenesis similar to that seen in chronic inflammatory responses, and tissue repair (70). Indeed, inflammatory cells and mediators are present in the microenvironment of most, if not all, tumors, irrespective of the trigger for development (Figure 8). Whereas many of pro-inflammatory cytokines promote tumor development, the immune system also restrict cancer development and progression through immunosurveillance and

immunoediting mechanisms (71). The inflammatory cells and cytokines found in tumors are more likely to contribute to tumor growth, progression, and immunosuppression than they are to mount an effective host anti-tumor response. Moreover, cancer susceptibility and severity may be associated with functional polymorphisms of inflammatory cytokine genes, and deletion or inhibition of inflammatory cytokines inhibits development of experimental cancer.

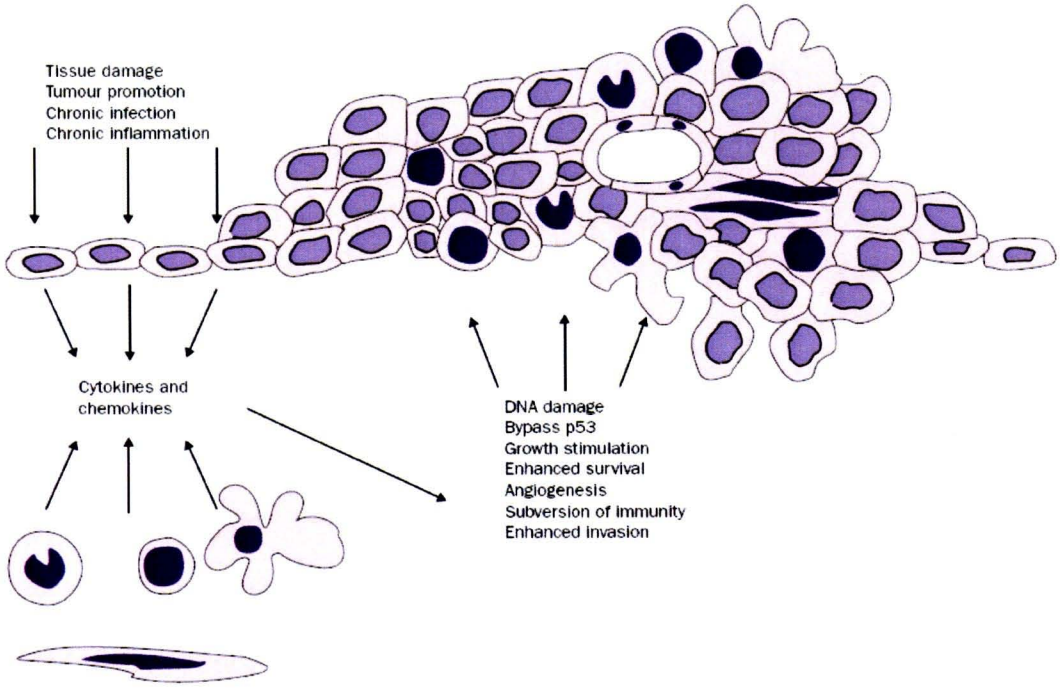

Figure 8 Chronic inflammation, tissue damage, and chronic infection may stimulate cytokines and chemokines that contribute to development of malignant disease (72).

The cytokine network of several common tumors is rich in inflammatory cytokines, growth factors, and chemokines but generally lacks cytokines involved in specific and sustained immune responses (73). There is now evidence that inflammatory cytokines and chemokines, which can be produced by the tumor cells

and/or tumor-associated leucocytes and platelets, may contribute directly to malignant progression. Recent results have indicated that inflammatory cells can provide growth signals that promote the proliferation of malignant cells. Thus, inflammation seems to be an important tumor promoter, and several cytokines such as interleukin-1β (IL-1β), IL-6 and tumor-necrosis factor (TNF-α) can promote tumor growth. In addition, there is strong evidence that IL-10 and transforming growth factor-β (TGF-β) are tumor suppressing rather than tumor promoting, most probably as a result of their anti-inflammatory properties (74-76). Many cytokines and chemokines are inducible by hypoxia, which is a major physiological difference between tumor and normal tissue. Examples are tumor necrosis factor (TNF-α), IL 1 and 6, and chemokines.

Role of cytokines in cancer development

Cytokines are released in response to a diverse range of cellular stresses, including carcinogen-induced injury, infection and inflammation. In these settings, cytokines function to stimulate a host response that is aimed at controlling the cellular stress and minimizing cellular damage. Whereas effective containment of the insult promotes tissue repair, the failure to resolve the injury can lead to persistent cytokine production and to an exacerbation of tissue destruction. As such, host reactions to cellular stress can impact on several stages of cancer formation and progression.

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and/or tumor-associated leucocytes and platelets, may contribute directly to malignant progression (Figure 9). Cytokines secreted by tumor and inflammatory/immune cells can either promote tumor development and tumor cell survival or exert antitumor effects. Chronic inflammation develops through the action of various inflammatory mediators, including TNF-a, IL-6, and IL-17, leading to eradication of antitumor immunity and accelerated tumor progression. However, TRAIL, through direct induction of tumor cell apoptosis, IL-10, through anti inflammatory effects, and IL-12, through activation of CTLs and NK cells and expression of cytotoxic mediators, can lead to tumor suppression. The multiple actions of TGF-ß (cytotoxic in colon cancer cells, and having both positive and negative effects on the tumor microenvironment) and IL-23 have dual roles in tumor development.

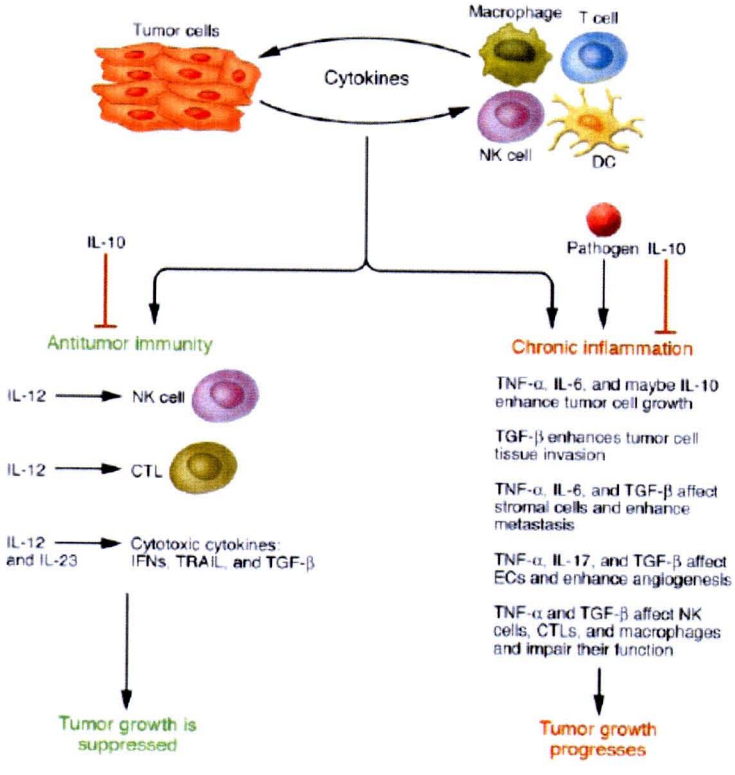

Figure 9 Two outcomes of interactions between tumor cells and infiltrating inflammatory and/or immune cells in the tumor microenvironment (77).

Transforming growth factor beta (TGF-β)

The TGF-β signaling pathway has been the focus of several recent reviews (78-80). Three TGF-β isoforms are expressed in mammals (TGF-β1, TGF-β2, and TGF-β3) and each is encoded by a unique gene and expressed in both a tissue-specific and developmentally regulated fashion. TGF-β1 is the most abundant and universally expressed isoform; most studies have either examined or been performed with exogenous TGF-β1. TGF-β superfamily ligands mediate and regulate cellular homeostasis, including embryonic development, differentiation, proliferation, immune surveillance, angiogenesis, motility, and apoptosis, in both a cell type and context specific manner (81). TGF-β superfamily signaling pathways also have diverse roles in human disease, with both increases and decreases in these signaling pathways resulting in human disease (82). In human cancers, TGF-β superfamily ligands and their pathways function as both tumor suppressors and tumor promoters (82-85).

TGF-β superfamily ligands function as tumor suppressors through the ability to inhibit cell proliferation, maintain tissue architecture, inhibit genomic instability, and induce senescence and apoptosis (86-88). Alterations in many components of the TGF-β signaling pathways, such as mutation or deletion of receptors and other signaling components, are frequent events in human cancers and lead to a loss of the tumor suppressor function of these pathways (87). Conversely, many late stage human tumors dysregulate or over-express TGF-β ligands, which can have tumor promoting effects on both the stromal compartment (suppressing immune surveillance, enhancing angiogenesis) and on the cancer cells themselves (inducing epithelial-tomesenchymal transition (EMT), enhancing migration and invasion) to promote

tumor invasiveness and metastasis (86, 87, 89-91) In addition, TGF-β–induced changes in the microenvironment, to favor angiogenesis, and inhibition of tumor-specific CD8+ T cells promote tumor development (91, 92). TGF-β signals mainly through activation of SMAD transcription factors, but it also leads to MAPK activation (80, 91). SMAD3 is a key mediator of the anti inflammatory and immunosuppressive activities of TGF-β in the colon (93). Defective TGF-β signaling due to mutational inactivation of the type 2 TGF-β receptor (TβRII) has been found to occur frequently in human colon cancer (94, 95). Such mutations occur at the adenoma-to-carcinoma transition or at a later stage, indicating that the tumor suppressor effect of TGF-β is mainly critical at late stages of colon carcinogenesis (96). Interestingly, TGF-β signaling prevents the release of IL-6 from Th1 cells during the late stages of colon cancer and therefore functions to control tumor growth (66). Conversely, IL-6–activated STAT3 signaling counteracts the TGF-β–mediated cytostatic effect through induction of inhibitory SMAD7 (97). In summary, the complex role of TGF-β in tumor suppression and progression might be stage and cancer cell type dependent. Precise mechanisms for this dual tumor suppressor/tumor promoter role for TGF-β ligands remain a fundamental problem in the TGF-β signaling field and major obstacle to targeting these pathways for the treatment of human cancers.

Tumor necrosis factor alpha (TNF-α)

Macrophages/ monocytes are the principal cells producing TNF-α. Moreover, TNF-α is also made by other cells including, B cells, T cells, NK cells, Kupffer cells, glial cells, and adipoyctes. TNF-α is a major mediator of inflammation, with actions

directed towards both tissue destruction and recovery. While inducing death of diseased cells at the site of inflammation, TNF-α stimulates fibroblast growth. It also can destroy blood vessels, but induce angiogenic factors (98). Likewise, in malignant disease, high-dose local TNF-α selectively destroys tumor blood vessels (99). This cytokine, when chronically produced, may act as an endogenous tumor promoter, contributing to the tissue remodelling and stromal development necessary for tumor growth and spread. TNF-α can be detected in malignant and/or stromal cells in human ovarian, breast, prostate, bladder, and colorectal cancer, lymphomas, and leukaemias, often in association with ILs 1 and 6 and macrophage colony stimulating factor (73, 100). In epithelial ovarian cancer, TNF-α mRNA is found in epithelial tumor islands, where there is a positive correlation with tumor grade (100). TNF-α is also implicated in the induction of a chemokine called monocyte chemotactic protein-1, which can regulate the macrophage and lymphocyte infiltrate, and of matrix metalloprotease-9, in the ovarian tumor microenvironment. In breast cancer, infiltrating macrophages are a major source of TNF-α, which may regulate thymidine phosphorylase, a key angiogenic enzyme in the tumor epithelium (101). In prostate cancer, tumor cell TNF-α production correlates with loss of androgen responsiveness. In non-Hodgkin lymphoma, myelogenous leukaemia, and chronic lymphocytic leukaemia, high circulating levels of TNF-α and its soluble receptors are associated with poor prognosis (102). There is also evidence for pro-cancer actions of TNF-α in animal models (103-105). For example, treatment of ascetic ovarian cancer xenografts with TNF-α promotes adhesion of free-floating tumor cells to the peritoneum and solid tumor formation (103), and overexpression of TNF-α confers invasive properties on

some tumor cell lines (104). Direct evidence for the involvement of TNF-α in malignancy comes from the observation that mice lacking the gene for TNF-α are resistant to skin carcinogenesis (106). TNF-α may be involved in the early stages of skin tumor promotion in normal mice, being transiently but extensively induced in keratinocytes after application of tumor promoter (106). Pentoxifylline (an inhibitor of inflammatory cytokine production) inhibits papilloma development in skin carcinogenesis models (107), and intraperitoneal injection of TNF-α enhances papilloma development and vascularisation of tumors.

Interleukin 1 (IL-1)

Three proteins comprise the IL-1 family, two of which are agonists, IL-1α and IL-1β; the third is IL-1 receptor antagonist (IL-1ra). IL-1α and IL-1β are derived from different genes but are functionally similar, and both bind to the same receptor (108-110). Although they exhibit similar biological activities, IL-1α and IL-1β differ in the manner in which they are processed and secreted. IL-1α is localized in the cytosol or cell membrane and is believed to regulate the intracellular environment (109, 110). In contrast, IL-1β is first cleaved by interleukin-1β-converting enzyme (ICE) to its mature active form and then secreted extracellularly. Patients with infectious or inflammatory conditions exhibited elevated plasma concentrations of IL-1β but not IL-1α, suggesting the systemic role of IL-1β (110, 111).

In mouse model of metastasis, a treatment with an IL-1 receptor antagonist (which inhibits the action of IL-1) had significantly decreased tumor development, suggesting that local production of this cytokine aids development of metastases.

Intraperitoneal injection of mineral oil in mice induces chronic inflammation followed by invasion and metastasis. Moreover, mice deficient in IL-1 were resistant to the development of experimental metastases (112). In human multiple myeloma, the malignant cells come to the bone marrow where they stimulate stromal cells to secrete the inflammatory cytokines IL-1, IL-6, and TNF. The cytokines stimulate myeloma cell growth and promote resistance to therapy (113).

Most studies of the mechanisms of cancer have focused on the early stages of cancer, but inflammatory mediators and cells are also involved in the migration, invasion and metastasis of malignant cells. chemokine receptors and their ligands direct the movement of cells during inflammation, cancer and the maintenance of tissue homeostasis, by affecting cell motility, invasiveness and survival (114). On transformation, many cells start to express chemokine receptors and thereby use chemokines to aid in their migration to, and survival at, sites that are distant from the original tumor (114-116).

Maspin and inflammation

Inflammation enhances tumor promotion through NF-kB dependent mechanisms (117). NF-kB has been proposed to promote metastatogenesis through epithelial–mesenchymal transition (118). Recently, it has become clear that inflammation and a pro-inflammatory microenvironment make important and critical contributions to tumor development (70). Many studies have exhibited an important tumor-promoting role for the inflammation-responsive IKK complex and its target NF-kB, acting both within cancer (or pre-malignant) cells and inflammatory cells (117). Even though

inflammation is expected to enhance metastatic progression, distinct genetically established mechanisms linking inflammation and metastasis are scarce. One study has described a novel mechanism, in which IKKa activation through binding of the pro-inflammatory cytokine RANKL to its receptor RANK, promotes prostate cancer metastasis (119). Interestingly, gene analysis revealed that IKKa exerts its pro-metastatic effect by repressing transcription of the Maspin gene (120). Suppression of Maspin expression requires nuclear translocation of catalytically active IKKa. Inactivation of IKKa increased Maspin expression and inhibited metastasis, whereas short interfering RNA (siRNA)-mediated Maspin knockdown elevated the metastatic potential. An excellent correlation between active nuclear IKKa, the level of Maspin expression and tumor progression was observed both in mouse and human prostate carcinoma.

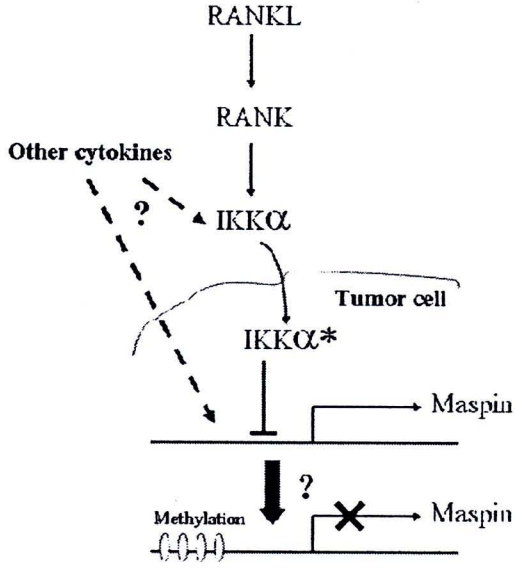

Figure 10 Inflammation suppress Maspin expression leading to Metastasis.

Hypothesis

Two hypotheses are herein proposed. Firstly, pro-inflammatory cytokines act as anti-cancer agent that up-regulates Maspin expression leading to anti-invasiveness of cancer. Alternatively, the cytokines down-regulate the expression of Maspin linking the processes of inflammation and cancer progression.

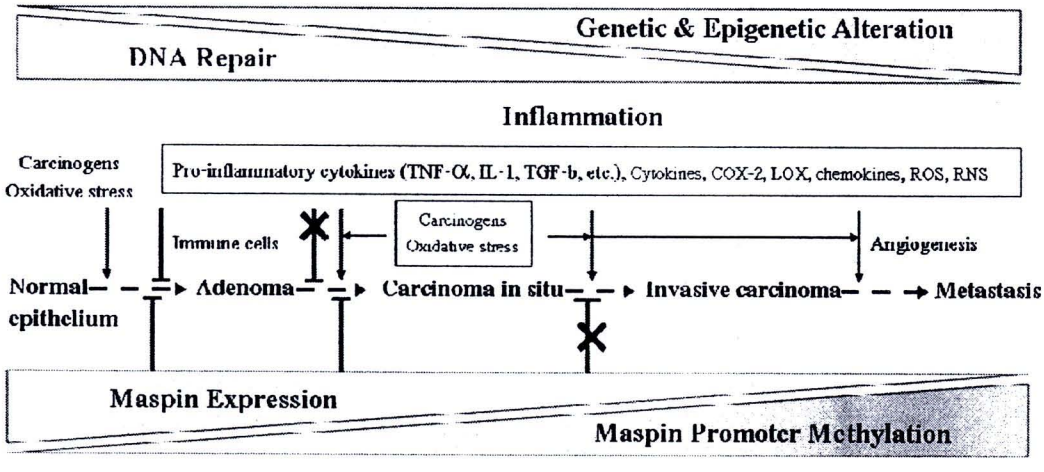

Figure 11 Hypothetical model of inflammation-altered Maspin expression.

1.3 Objectives

1. To study the effect of inflammatory cytokines; TNFα, TGF-β1, and IL-1β on Maspin expression (both mRNA and protein) in human cancer cell lines
2. To investigate the effect of cytokines-altered Maspin expression on cancer cell proliferation and invasion *in vitro*
3. To characterize the methylation status of Maspin promoter upon exposure to inflammatory cytokines that affect Maspin expression