Introduction
3.1 CANCER PROGRESSION

Cancer progression depends on the accumulation of certain metastasis supporting signaling molecules that modulate various signaling events resulting in dysregulated growth, overcoming of replicative senescence and metastasis formation (1). At the molecular level, acquisition of metastatic phenotype is generated by the expression of homing receptors, their ligands and extracellular matrix degrading proteases (2). Several growth factors and cytokines play pivotal roles in regulation of proliferation, survival, adhesion and migration of neoplastic cells. Tumor progression is a multistep process which depends on six essential characteristics identified as the "hallmarks of cancer," which includes self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (3-5).

The first targets of metastatic spread are typically regional lymph nodes that subsequently establish in other organs like lung, liver, brain, bone etc and hence accounts for 90% of human cancer deaths (6). However, the molecular mechanisms that define the role of various signaling molecules in tumor metastasis and the transcriptional and translational regulatory mechanisms that contribute to the induction of various downstream effector molecules leading to tumor growth and metastatic phenotype is not well defined.

Tumor growth, differentiation and metastasis depends on various elements like expression of homing receptors with associated signaling molecules, their ligands and alteration of gene expression and cell-to-cell and cell to substratum interaction.

Two sets of proteins are known to play a key role in host tumor interaction and metastasis.

a) Extracellular matrix (ECM) components which provide a substrate for tumor growth and migration as well as barrier for tumor invasion and metastasis and

b) Matrix degrading proteinases (uPA and MMPs) which degrades the extracellular matrix components
3.2 OSTEOPONTIN

Osteopontin (OPN), a secreted non-collagenous, sialic acid rich, chemokine like calcified ECM protein with pleiotropic properties plays a crucial role in determining the oncogenic potential of various cancers (7). It exerts its pro-metastatic effects by interacting with various integrins and CD44 receptors. Thus OPN and its receptors figure prominently in a wide spectrum of malignancies. Further studies in a variety of human cancers have correlated the high levels of OPN expression with the advanced stages of tumors. The pivotal role of OPN in tumor dissemination has been highlighted by gene transfer experiments. Overexpression of OPN results in an increase in malignant phenotype, whereas transfection with antisense oligonucleotides yields population with reduced malignant potential (8). Several groups have reported the role of OPN in atherosclerosis, bone remodeling, angiogenesis, wound healing and tissue injuries as well as certain diseases such as restenosis, arterial neointimal hyperplasia, myocardial necrosis, renal tubulointerstitial fibrosis, kidney stone formation and autoimmune diseases (9-12).

3.2.1 Structure of Osteopontin

OPN is an acidic glycoprotein with a protein backbone of about 32.5 kDa. It is rich in aspartic acid, glutamic acid and serine and contains about 30 monosaccharides, including 10 sialic acids. Carbohydrate is present as one N- glycosyl and 5-6 O-glycosyl side chains. Phosphorylation occurs to a variable extent, upto 28 sites (13), distributed throughout the molecule. Molecular weight of OPN varies between 45-75 kDa depending on the degree of post-translational modification.

OPN is divisible into multiple domains and its function is extensively regulated at the post-translational level. The molecule has a protease hypersensitive site that separates the integrin and CD44 binding domains. The thrombin cleavage motif in this region has a conserved sequence, RSK, present in most species (14). While binding of the C-terminal fragment of OPN to CD44v6 occurs through a protein-protein interaction (15, 16) it may also bind CD44v3 via a heparin bridge. In contrast, activation of the RGD containing N-terminal domain is a prerequisite for integrin ligation. Cleavage by thrombin and phosphorylation of the N-terminal fragment are required for the efficient engagement of OPN to αvβ3 integrin (16, 17) (Fig.1).
Fig. 1. Schematic representation of the domain structure of OPN. OPN structure can be divided into multiple domains. The thrombin cleavage motif has a conserved RSK sequence and cleavage at this site splits the protein into two functional domains. The GRGDS sequence at the N terminal region plays a crucial role in ligation of OPN to various integrins. The C-terminal fragment binds to CD44 and regulates various cellular events independently of post translational modification. Between the RGD motif and the CD44 binding domain is the recognition site for \( \beta_1 \) integrin (SVVYGLR) which thought to be revealed upon thrombin cleavage.

Thus the thrombin cleavage of secreted osteopontin releases the two receptor binding domains which carry out distinct functions in the cascade of events leading to tumor cell dissemination.

1) Chemotactic activity exerted by the C-terminal fragment is phosphorylation independent that leads to attraction of cells to the cleavage site and cellular attachment to the OPN N-terminal fragment.

2) Haptotactic activity ("cell crawling") exerted by the N terminal fragment or crosslinked OPN is phosphorylation dependent that leads to cancer cell spreading and degradation of extracellular matrix through activation of matrix metalloproteases.

OPN also serves as substrate for liver transglutaminase as well as plasma transglutaminase factor XIIIa (18, 19). The catalyzed reaction results in modification of OPN on glutamines 34 and 36, leading to the formation of \( \gamma \)-glutamyl-\( \varepsilon \)-lysine thereby
linking OPN to specific extracellular locations (20). This has been associated with extracellular matrix remodeling and may influence its haptotactic properties that are mediated by integrins. Multiple forms of OPN are generated by alternative splicing in the N-terminal portion (21) (Fig. 2)

As the biological function of OPN is regulated at the post-transcriptional and post-translational levels, OPN from various cellular sources have diverse structural characteristics that are reflected in distinct physiological roles (22, 23). Studies in OPN−/− gene targeted mice have suggested the existence of structural and functional differences between tumors derived OPN and the OPN forms that are relevant for host defenses (23). Evidence suggested that tumor derived OPN is unique (i.e structurally different from OPN derived from untransformed cells) and lacks important domains. Thus expression of structurally altered OPN by cancer cells represents a mechanism of immune evasion (24). Earlier reports indicated that OPN acts as a novel substrate for MMP-3 and MMP-7 and these cleaved fragments enhanced adhesion and migration in vitro through cell surface integrins indicating the reciprocal regulation of OPN on MMP activity (25).
3.2.2 Cellular distribution of osteopontin

OPN is expressed by cells in a variety of tissues including bone, dentin, cementum, hypertrophic cartilage, kidney, vascular tissues activated macrophages, lymphocytes and in specialized epithelia found in mammary glands (26, 27). Although the secreted protein is incorporated into the matrix in mineralized connective tissues, in many situations it appears in biological fluids including blood, milk (lactopontin), urine (uropontin) and seminal fluid. The concentration of serum OPN is markedly elevated with increased tumor burden in cancer patients (28). The elevated level of expression of OPN is characteristically observed in pathological and pathophysiological conditions.

3.2.3 Role of osteopontin in human pathology

Since its first identification in bone by biochemical means, OPN has been reported to play a multifaceted role in regulating bone resorption and matrix turnover (29). OPN acts as an important mediator of communication between osteoclasts and osteoblasts and plays a crucial role in the formation, migration and attachment of osteoclasts. The polyaspartic acid sequence and the phosphate groups facilitate the binding of OPN to mineral crystals in normal and pathological states (30). OPN expression is strongly stimulated by vitamin-D3 that promotes osteoclast differentiation and bone resorption. Recent knockout studies revealed that OPN-deficient (OPN$^{-/-}$) mice are resistant to ovariectomy-induced bone resorption compared with wild type mice (31). Thus OPN plays a pivotal role in calcification, homeostasis, development and bone remodeling.

Earlier reports have indicated that expression of OPN induces medial thickening without injury and neointimal formation after injury thus suggesting that OPN plays a crucial role in the development of atherosclerosis, vascular remodeling and restenosis after angioplasty (31, 32). Previous studies aimed at elucidating the role of OPN and its specific receptor αvβ3 integrin in the pathogenesis of coronary restenosis indicated that the levels of OPN at both the mRNA and protein level were significantly higher in the coronary atherosclerotic tissues of patients when compared to controls (10). The potential relevance of OPN expression in human cardiovascular diseases was further suggested by the focal expression of OPN mRNA and protein in the atherosclerotic plaques but not in normal human arteries. Studies on the molecular mechanism involved in arterial remodeling have
indicated that any injurious stimulus induces the release of various vasoactive molecules including growth factors which in turn stimulates the expression of OPN, integrins and proteases required for smooth muscle cell and endothelial cell migration.

OPN is also involved in a wide range of physiological processes including inflammatory and immune response mediated by Th1 cells (33). Neutrophil dependent macrophage responses are a prominent part of delayed type immunity and healing processes and depend on T-cell secreted cytokines. An important mediator of this process is OPN which is secreted by activated T cells that supports host resistance by inducing immune cells particularly macrophages to migrate to sites of inflammation. In cellular immune response, OPN is an early acting cytokine that induces the macrophages to mediate IL-10 suppression through interaction with CD44 and IL-12 secretion via binding to αvβ3 integrin. These components are essential for wound healing. It is also associated
with host resistance to infections by parasites, bacterial and viral pathogens. OPN functionally activates dendritic cells and induces their differentiation towards a Th1-polarizing phenotype (34). Previous reports have indicated that OPN deficient mice have severely impaired type I immunity to viral infection (Herpes Simplex Virus-type I) and bacterial infection (Listeria monocytogenes) and do not develope sarcoïd type granuloma. OPN also plays a crucial role in the proliferation of B lymphocytes and enhances the generation of immunoglobulins (35) (Fig. 3).

3.3 Role of osteopontin in tumor progression and metastasis

Abundant secretion of OPN acts as a marker for breast and prostatic cancer, osteosarcoma, glioblastoma, squamous cell carcinoma and melanoma (36). Recent DNA microarray analysis data have shown that OPN is one of the most abundantly expressed genes in metastatic melanoma and transformed cells are characterized by increased secretion of OPN (37). Overexpression of OPN negative breast cancer cells with OPN cDNA has been shown to increase the tumorigenicity and skeletal metastasis through a mechanism that enhances angiogenesis (38). OPN knockout mice lead to impaired colony formation in soft agar and slower tumor growth in vivo as compared to tumors in wild type mice (39). The level of serum OPN in patients with breast, lung and prostatic cancers is higher compared with normal serum (40). Clinical studies also revealed a correlation between plasma OPN, tumor burden and prognosis in patients with breast cancer metastasis (41). Furthermore, blocking of OPN expression by RNA interference reduced melanoma cell numbers in vitro suggesting that OPN may be acquired early in melanoma development and progression and may enhance tumor cell growth in invasive melanoma (42).
OPN is highly expressed in a wide spectrum of malignancies and ligates with either integrins or CD44 receptors and induces various cellular signaling events. The activation of various kinases (PI 3 kinase, NIK, PLC, PKC, MAPK) induces the transactivating potential of various transcription factors including NFκB and AP-1 leading to inhibition of apoptosis. This ultimately results in the activation of various matrix degrading proteases like uPA and MMPs leading to cancer cell motility, tumor growth and metastasis.

OPN also contributes to cancer progression by regulating the tumor surveillance mechanism and inhibiting apoptosis of neoplastic cells (Fig. 4). The expression of bone sialoprotein (BSP) and OPN confers survival advantage in tumor cells by interacting with αvβ3 or CD44 followed by sequestration of Factor H to the cell surface thereby inhibiting complement mediated cell death (43). Previous reports have indicated that soluble OPN inhibits apoptosis of HUVEC cells depleted of growth factors and cytokines (44). OPN inhibits DNA fragmentation and stimulates Bcl-xl mRNA levels in these cells. Furthermore, melanocyte growth factor receptor induces OPN secretion, which promotes antiapoptotic signaling thereby regulating melanoma progression (45). OPN deficient cardiac fibroblasts undergo increased cell death in response to H2O2 through a caspase-3 independent pathway (46). Previous studies have indicated that OPN reverses the phorbol-ester induced apoptosis in breast cancer cells through protein kinase C (PKC) mediated pathway (47). The anti-apoptotic effect of OPN depends on its phosphorylation status and can be reversed by treating with anti-integrin antibodies (47). This clearly indicates the potential role of OPN in regulating the tumor microenvironment and further suggested that it may act as a prognostic marker for various cancers.
3.4 Integrins: versatile integrators in cell signaling

Integrins are a family of noncovalently associated membrane glycoproteins consisting of two subunits $\alpha$ and $\beta$. The primary structure of integrin subunit was deduced from sequencing of complementary DNA (48, 49). The $\alpha$ subunits are homologous to each other but not to the $\beta$ subunits which form their own homologous group. The extent of similarity at the amino acid sequence level within the $\alpha$ and $\beta$ subunit groups is 40-50%. Both the integrin subunits have a large extracellular domain, a transmembrane segment, and a cytoplasmic tail (50). The cytoplasmic portion is short with 30 to 50 amino acids in most of the subunits except in the recently sequenced $\beta$4 subunit. Some of the $\alpha$ subunits [$\alpha$1, $\alpha$2, $\alpha$L, am and $\alpha$x] contain an additional 180 amino acid segment that has homologies with certain collagen binding properties. The extracellular domains of the $\alpha$ subunits contain several calmodulin type divalent cation-binding sites while the $\beta$ subunit has one such site. Thus the divalent cation dependency of integrin function is due to the presence of these structures (51) (Fig. 5)

**Fig. 5. Schematic representation of general structure of integrins.** Two types of integrins are shown, one with an $\alpha$ subunit which is proteolytically processed into two disulfide-linked fragments at the cleavage site. Eg: $\alpha$V$\beta$3 which interacts with the RGD sequence of matrix proteins. Other integrins have $\alpha$ subunits which are not processed. Eg: $\alpha$L$\beta$2 integrin binds to its counter receptors, ICAM-1 and ICAM-2.
### 3.4.1 Integrin diversity

There are 12 $\alpha$-subunits and 8 $\beta$-subunits known (52-55). The $\alpha$ and $\beta$ subunits, in various combinations form at least 20 integrins. A single $\alpha$ subunit can become paired with more than 1 $\beta$ subunit. The $\alpha_v$ subunit is more versatile. It forms more than four integrin receptors by combining with different $\beta$ subunits (56). The $\alpha$ and $\beta$ subunits are noncovalently bound to each other, and this association is promoted by divalent cations. Cross-linking studies and analysis of the ligand specificities of individual integrins suggest that ligand binding site is formed by sequences from both subunits (57, 58). This diversity in integrins provides cells with varied capabilities to recognize adhesive substrates and extracellular matrix (Fig. 6). The cytoplasmic domains of the integrins are also reported to interact with cytoskeleton. Such interactions are regulated by phosphorylation of the $\beta$ subunit cytoplasmic tail (59, 60). These properties enable integrins to serve as a link between the cytoskeleton and the extracellular matrix.

**Fig. 6. Integrin Diversity.** Schematic representation of the various integrin subunits, subunit combinations and their known ligands. FN, Fibronectin, Fn. Alt, fibronectin Alternatively spliced domain; LM, Laminin; VN, Vitronectin; vWF, von Willebrand Factor FB, Fibrinogen; OPN, Osteopontin; BSP 1, Bone sialoprotein 1, ICAM, Intercellular adhesion molecules
3.4.2 Modes of integrin mediated adhesion

Adhesion of cells to extracellular matrix

Many integrins bind to extracellular matrix proteins, and thereby mediate cell-extracellular matrix interactions. Among the various extracellular matrix ligands are fibronectin, laminin, various collagens, entactin, thrombospondin, von Willebrand factor and vtronectin (56, 61).

Cell-cell adhesion

Certain integrins bind to cell surface receptors present on another cell ("counter receptors") and thereby mediate cell-cell adhesion. The intercellular adhesion molecule ICAM-1 and ICAM-2 has been identified as counter receptors for the leukocyte integrin LFA-1 (61, 62).

Cell aggregation through soluble adhesion proteins

In this mode of interaction, the major integrin in platelets, αIIb/βIIIa, or gp IIb/IIIa, promotes the binding of platelets to one another through soluble, multivalent mediator molecules such as fibrinogen and von Willebrand factor. These molecules have multiple binding sites for αIIb/β3 and as a result can bridge the αIIb/β3 integrin of adjacent platelets, causing aggregation. Such aggregation plays a crucial role in blood clotting (63, 64).

3.5 Role of integrins in tumor progression

Integrins recognize a variety of ligands including extracellular matrix proteins, cell surface proteins and plasma proteins (65) (Fig. 6). Integrin dependent adhesion controls cell growth, differentiation, gene expression, apoptosis and motility (66). Integrin-mediated signaling includes Ca$^{2+}$ influx, cytoplasmic alkalinization, potassium channel activation, activation of lipid mediators, tyrosine phosphorylation of cytoplasmic proteins and activation of a number of kinases leading to downstream target gene activation. The interaction of OPN with integrin receptors depends on the RGD motif of OPN and a high activation state of the integrin receptor. Earlier reports have shown that OPN interacts with αvβ1, αvβ3 and αvβ5 integrins (67). Moreover, α1 integrin chain may serve as an OPN
receptor in conjunction with integrin β chains, specifically β<sub>v</sub>, β<sub>4</sub> and β<sub>9</sub> and can induce the downstream signaling events (68). Recent data revealed that OPN also interacts with α9β1 and α4β1 integrins expressed on epithelial cells, smooth muscle cells, skeletal muscle cells and neutrophils through the N-terminal sequence SVVYGLR adjacent to but distinct from, the RGD motif (69). This cryptic sequence is revealed upon putative thrombin cleavage. The β1 integrin chain may also associate with CD44 in the cell membrane and thus serve as an OPN receptor (70). Due to its prominent role in a wide range of malignancies, the interference of the ligand-receptor interaction by using blocking antibody may have great implication in curtailing the dissemination of various cancers.

3.6 The α<sub>v</sub>β<sub>3</sub> integrin as target for cancer therapy

The role of ανβ3 integrin in cancer includes invasion of tumor cells, osteoclast activation in lytic bone metastases and neovascularization. The ligand osteopontin substantially contributes to all functions and hence inhibition of OPN-ανβ3 integrin interaction may serve as valuable therapeutic target for the treatment of various cancers.

3.6.1 Modulation of ανβ3 integrin expression on tumor cells

Exposure to steroids may suppress the ανβ3 integrin expression. According to the experiments with osteoblasts, short-term exposure of cells to dexamethasone may increase the expression of ανβ3 integrin. However long term exposure to this glucocorticosteroid downregulates its expression. The expression of ανβ3 integrin has been suppressed experimentally by transfection of a construct encoding an intracellular single chain antibody directed to the αv chain. The expression of an intracellular antibody in Saos-2 osteosarcoma cells had no effect on the integrin αν mRNA levels and translation of the precursor αν integrin subunit was not affected. However the maturation of αν integrins as glycoproteins was slow suggesting that the transport from endoplasmic reticulum to golgi complex was partially prevented. Further the depletion of αν integrins from Saos-2 cells led to a decreased ability to spread on fibronectin and vitronectin (71)
3.6.2 Inhibition of receptor ligation by osteopontin

The two principal strategies used for inhibiting the receptor ligation are based on the use of antibodies (A) and synthetic peptides (B).

1. The monoclonal anti $\alpha\nu\beta$3 integrin antibody 17E6 is effective in preventing the growth of melanomas through direct action on the tumors (72). However indirect effects may also contribute to this anti-tumorigenic effect of the antibody. Tumor-induced angiogenesis promotes vascular cell entry into the cell cycle and expression of $\alpha\nu\beta$3 integrin. A single dose of monoclonal antibody, LM609 to this integrin also induces apoptosis of the proliferative angiogenic blood vessels and leads to tumor regression. Tumors treated with LM609 contain significantly fewer blood vessels and are considerably less invasive than controls. This is also the case for tumors that do not express this integrin, suggesting that the effect is due to the inhibition of the integrin receptors expressed on proliferating endothelial cells (73, 74). The intravenous administration of the chimeric antibody c7E3 Fab has been considered in coronary angioplasty to prevent smooth muscle cell adhesion on migration to, vitronectin and OPN. PDGF-induced smooth muscle cell migration was also inhibited by c7E3 Fab and LM609, but to a much lesser extent (75). This antibody in combination may also suppress angiogenesis in tumors.

2. A cyclic RGD peptide prevents the growth of melanomas through direct action on the tumors (72). Vascular cells stimulated to enter the cell cycle undergo apoptosis in the presence on $\alpha\nu\beta$3 integrin antagonists such as a cyclic peptide (RGDFV) but not with a control peptide (RADfV) (73).

3. Several snake venoms inhibit platelet aggregation by blocking integrin receptors and thereby also inhibit cancer cell adhesion and invasion. Contortrostatin is a unique dimeric disintegrin isolated from southern copper-head snake venom, which antagonizes integrins $\alpha$5$\beta$1, $\alpha$11$\beta$3, $\alpha$V$\beta$3, and $\alpha$V$\beta$5 and prevents the invasion of cancer cells through matrigel (76).

4. A synthetic chemical peptide mimetic, $\beta$-[2-[[5-[[aminoiminomethyl] amino]-1-oxopentyl] amino]-1-oxoethyl] amino-3-pyridine propanoic acid, bistrifluoroacetate (SC-56631) recognizes integrins $\alpha$V$\beta$3, $\alpha$V$\beta$5 and less effectively $\alpha$V$\beta$1 and hence have great implication in cancer therapeutics (77, 78).
3.7 Melanoma cell adhesion molecule (MelCAM)

The ability of tumor cells to detach from the primary site and produce metastases in a distant organ is due to the survival and growth of unique subpopulation of cells with metastatic properties (79, 80). One tumor cell property that is essential for metastasis is the expression of CAMs, which mediate cell-to-cell or cell-to-matrix interaction. MelCAM, also known as MUC18 or MCAM, is a newly recognized cell adhesion molecule (CAM) belonging to the immunoglobulin superfamily (81-84). These molecules play an important role in a variety of biological and pathological processes including regulation of organogenesis, maintenance of tissue architecture, inflammatory and immune responses, wound healing, tumor invasion and metastasis (84, 85).

**Structure of MelCAM**

MelCAM (MUC18 or CD146) is an integral membrane glycoprotein with an apparent molecular weight of 113 kDa. It was originally cloned and sequenced from a human melanoma cDNA library screened by a monoclonal antibody that specifically reacted with human melanoma cells (86, 87). It is a single copy gene located in the long arm of human chromosome 11 and is composed of 16 exons spanning over a chromosomal region of approximately 14 kilobases. However, currently no spliced isoforms of CD146 have been identified. The genomic sequence of CD146 is highly homologous to a number of cell adhesion molecules within the IgG gene superfamily like HEMCAM (88), BCAM (89), gicerin (90), ALCAM (91).

In the extracellular region, CD146 contains the characteristic V-V-C2-C2-C2-Ig like domain structure that consists of 43-70 amino acids forming β-pleated sheets. The amino acid stretch LKEEKN in the second immunoglobulin loop of CD146 is similar to the glycosaminoglycan recognition sequence (LKREKN) suggesting that CD146 is involved in the interactions between cells and the extracellular matrix. There is a single membrane spanning domain. The cytoplasmic domain of CD146 is relatively short and has potential recognition site for protein kinases (83). Previous studies indicated that activated CD146 is associated with complex formation between the cytoplasmic domain of CD146 and p59Fyn, a Src family kinase which phosphorylates p125FAK.
CD146 is highly glycosylated and approximately 35% of its molecular mass is composed of carbohydrates including sialic acid and other carbohydrate moieties (92). The composition of carbohydrate moieties in CD146 is cell-type specific. Previous reports indicate that the HNK-1 carbohydrate moiety in CD146 is expressed in melanoma cells, but is absent in CD146 that is expressed by the implantation site intermediate trophoblastic cells (92, 93).

**Regulation of MelCAM expression**

At the cellular level, MelCAM expression can be modulated by a variety of exogenous factors such as phorbol ester, cAMP, phytohaemagglutinin, T-cell receptor/CD3 stimulation etc. Analysis of the CD146 promoter sequence has revealed putative binding sites for several transcription factors including AP-1, AP-2, Sp1, CREB, c-myb as well as CArG-box motif. In human melanoma cells, phorbol ester and cyclic AMP have been shown to modulate MelCAM expression (94). The expression of MelCAM in T lymphocytes can be induced by phytohaemagglutinin treatment via super antigen and T cell receptor/CD3 stimulation in a restricted subset of T lymphocytes (95). The ectopic expression of carcinoembryonic antigen by a melanoma cell line enhances the expression of CD146 (96). Furthermore, the expression of CD146 in melanocytic cells is environmentally regulated by epidermal keratinocytes through direct cell-cell contacts. Normal melanocytes and naevus cells isolated in culture express CD146 and the expression is downregulated when the cells are cocultured with keratinocytes (97). This downregulation in the CD146 expression in melanocytic cells by keratinocytes is through direct cell-cell contacts mediated by E-cadherin. On the other hand, melanoma cells from advanced primary and metastatic lesions that lack E-cadherin expression constitutively express CD146 and keratinocytes have no modulatory effect. This contact dependent regulation of CD146 expression by keratinocytes is restored by overexpressing E-cadherin in melanoma cells. These findings support the view that the interactive cellular network between melanocytic cells and keratinocytes play a critical role in determining the MelCAM expression in melanoma system, which ultimately regulates the malignant phenotype.
Functions of MelCAM

MelCAM, also known as MUC18 or CD146 is a membrane glycoprotein which functions as a Ca\(^{2+}\)-independent cell adhesion molecule is involved in heterophilic cell-cell interactions. MCAM can mediate heterotypic adhesion between cells, but the counter receptor or ligand for MCAM is yet to be identified (98). Immunohistochemical studies have indicated that CD146 expression has been demonstrated in a relatively limited spectrum of normal human tissues and malignant neoplasms. The lineage specific expression pattern of MelCAM can be useful in the differential diagnosis of certain lesions including melanomas and various types of gestational trophoblastic lesions (99). Although the biological role of CD146 in normal tissues and malignant tumors remains unclear, CD146 plays an important role in tumor progression, implantation and placentation. CD146 promotes tumorigenesis and metastasis in human melanoma by enhancing the interaction between melanoma and endothelial cells. In contrast, CD146 acts as a tumor suppressor in breast carcinoma. It is not expressed in breast carcinomas and its overexpression in breast carcinoma cells results in a more cohesive cell growth and formation of smaller tumors in nude mice (100).

During implantation and placentation, CD146 expressed by the intermediate trophoblast in the placental site binds to its putative receptor in uterine smooth muscle cells and limits trophoblastic invasion in the myometrium (101). In early gestation, differentiation of implantation site intermediate trophoblast is correlated with the CD146 expression (102). CD146 immunoreactivity increases in intensity and distribution in villous intermediate trophoblastic cells from their proximal origin to the distal tip of the trophoblastic columns (103).

3.7.1 Role of MelCAM in tumor progression

Earlier reports have indicated a positive correlation between MCAM expression and the ability of human melanoma cells to produce metastases in nude mice (104). The malignant potential of cutaneous melanoma is directly related to the vertical thickness of the lesion (104, 105). Analysis of primary melanomas indicated that the expression of MelCAM on thin tumors (< 0.75 mm) that have a low probability of metastasizing, and on benign nevi is weaker and less frequent but the expression is very high in advanced and
metastatic tumors (83, 106). The studies in murine models have suggested that MelCAM contributes to tumor growth and metastases in vivo (101). The ectopic expression of MelCAM in radial growth phase melanoma cell lines (MCAM negative) resulted in an increase in their tumorigenicity and metastatic potential in nude mice (105). The transfected cells displayed increased homotypic adhesion, increased attachment to human endothelial cells, upregulation of the matrix metalloproteinase-2 (MMP-2) and increased invasion through the matrigel coated filters (106). Recent reports indicated that the production of tumorigenic variants from a non-tumorigenic melanoma cell line is accompanied by upregulation of MCAM expression (107).

MelCAM also plays a crucial role in angiogenesis and invasion, although the ligand for it is yet to be identified. Growth and metastasis of human melanoma cells depend on their ability to develop an adequate vasculature. The progression of neoplasms from the benign to malignant state is associated with a switch to an "angiogenic phenotype" representing an increase in proangiogenic molecules produced by the tumor cells and organ specific environments (108, 109). Melanoma cells secrete a variety of proangiogenic molecules like basic fibroblast growth factor, vascular endothelial growth factor and interleukin 8 (109). MelCAM indirectly affects angiogenesis and invasion of human melanoma cells through upregulation of MMP-2 and cell interaction with extracellular matrix and vascular endothelial cells.

However MelCAM acts differently in the progression of breast carcinoma. It is expressed in normal and benign proliferative breast epithelium and its expression is frequently lost in in situ and infiltrating breast carcinomas (95). Earlier reports indicate that transfection of CD146 cDNA into CD146 negative breast carcinoma cells induces a more cohesive cell growth pattern and results in the formation of smaller tumors than mock transfectants in nude mice. Thus loss of MelCAM expression in most breast carcinomas but not in normal or benign epithelial cells in breast may represent an intrinsic defect in CD146 expression and/or an epigenetic phenomenon unique to tumor progression in breast cancer. Thus CD146 (MelCAM) is differentially expressed in melanoma and breast cancers and functions as a tumor promoter in melanoma but it acts as tumor suppressor in breast carcinoma. Thus MelCAM acts as a candidate mediator of tumor growth, angiogenesis and
metastasis in human melanoma and lend credence to the rationale that blockade of MelCAM may act as a potential target for immunotherapy against melanoma.

3.8 Focal adhesion kinase: structure, functions and regulation

Focal adhesion kinase (FAK) is a protein tyrosine kinase with a molecular mass of 125 kDa. It is expressed in most tissues and cell types and is evolutionarily conserved in mammalian species and in lower eukaryotic organisms. It is involved in a wide range of physiological and pathological processes like cell attachment, spreading, proliferation, survival and cell cycle progression (110). The major role of FAK which acts downstream of integrins is that it regulates cell migration (111). FAK is activated upon cell binding to extracellular matrix proteins and activates multiple signaling cascades.

3.8.1 Domain structure of FAK

FAK comprises a central catalytic domain flanked by large N- and C- terminal domains. The N-terminal domain has a protein sequence similarity to a family of proteins that are referred to as FERM (erythrocyte band four.-ezrin-radixin-moesin) domains (112). Under in vitro conditions, the N-terminal domain of FAK binds to sequences in the cytoplasmic domain of integrin; however the direct interaction between FAK and integrin is not well documented. The FERM domain plays a crucial role in the interaction of FAK with various other cell surface receptors such as EGFR (113). Previous evidences also suggest the involvement of the FERM domain in regulating the catalytic activity and subcellular localization of FAK (114). Thus FERM domain may direct FAK to the sites of integrin or growth factor receptor clustering as well as regulating its interaction with other potential activating proteins (Fig. 7).
The C-terminal region of FAK contains a domain of 100 amino acid residues called FAT (focal adhesion targeting) which directs FAK to newly formed or pre-existing complexes (115). This domain contains a four helix bundle which resembles a number of other adhesion proteins like vinculin, α- catenin etc in its structure (116). The C-terminal domain of FAK is also referred to as FRNK (FAK related-non-kinase). This domain functions as a negative regulator of FAK kinase activity (117). Overexpression of FRNK inhibits cell spreading, cell motility and growth factor mediated signals to MAPK pathways in many cells (117-119).

The kinase domain of FAK shares sequence similarity with other receptor and non-receptor protein tyrosine kinases. The crystal structure of FAK kinase domain shows the presence of disulphide bond in the N-terminal lobe (120).

3.8.2 Regulation of FAK kinase activity

As the COOH domain of FAK interacts with various integrin associated proteins, FAK is activated upon cell binding to extracellular matrix proteins and forms a transient signaling complex with Src family protein tyrosine kinases (121, 122). Thus clustering of integrins results in rapid recruitment of FAK to the focal adhesion complex leading to its phosphorylation at Tyr 397 (Y397FAK). This phosphorylation status of FAK correlates with its kinase activity (123). Recent evidences suggested that transient dimerization of
FAK also leads to intermolecular phosphorylation of FAK at Tyr 397. Previous reports have strongly suggested the role of Src-family kinases in the activation of FAK (122). The other phosphorylation sites on FAK include Tyr 925 (Y925 FAK).

### 3.8.3 Role of FAK in tumor progression

Cell migration and invasion are fundamental processes in tumor cell metastasis. FAK plays an important role in promoting growth factor- and integrin-stimulated cell motility in both normal and transformed cells (123). Many malignant human tumor samples exhibit increased FAK expression and tyrosine phosphorylation (124). These changes are correlated with the acquisition of an invasive cell phenotype and increased metastasis (117). FAK-null (FAK<sup>-/-</sup>) fibroblasts form an abundance of actin stress fibres and focal contact sites in cell culture that result in refractory motility responses (125). FAK<sup>-/-</sup> reconstitution studies have shown that c-Src recruitment to FAK is an initial event in promoting focal contact turnover and enhanced cell motility (126, 127). FAK is a v-Src substrate, and the formation of v-Src-FAK signaling complex is associated with increased cell invasion and the generation of invadopodia (128). Stable expression of a dominant negative inhibitor of FAK termed as FAK related non-kinase (FRNK) in v-Src transformed NIH3T3 fibroblasts resulted in the inhibition of matrigel cell invasion in vitro and experimental metastasis in vivo without effects on cell motility or v-Src enhanced cell growth (129, 130). Previous reports have indicated that FAK deficient cells spread more slowly on the extracellular matrix and hence migration is retarded in response to chemotactic and haptotactic signals (131-133). FAK deficient cells also exhibit increased directional persistence but FAK expressing cells respond to exerted forces by reorienting their movement and forming prominent focal adhesions (134). FAK acts as an important regulator of cytoskeletal rearrangement and lamellipodia formation (135). Expression of dominant negative forms of FAK in endothelial cells significantly inhibits cell migration, increases the number of stress fibers and size of the focal adhesions (136).

Integrin engagement or cell spreading on ECM substrate activates ERK signaling pathway (137). Various signaling pathways have been reported regarding the integrin signaling which converges at the level of MAPK. Phosphorylation of FAK on Tyr-925 has
been reported to recruit the SH2 domain of Grb2, resulting in the activation of the SOS-Ras-Raf-MEK-ERK pathway (138).

Cancer cells exhibit profound changes in the cytoskeletal organization, adhesion, motility, growth regulation and survival. Elevated expression of FAK has been observed in human tumor specimens and also in cell lines derived from tumors (139). In such cells, increased FAK expression has been correlated with increased cancer cell motility, invasiveness and proliferation (134, 139, 140). Inhibition of FAK expression and activity either by using antisense oligonucleotide or siRNA treatment or by overexpression of FRNK leads to reduction in the secretion of matrix degrading proteases and hence inhibition of cancer cell motility, invasion and proliferation (119, 140, 141). In some cancer cells, inhibition of FAK by overexpression of FRNK or in combination with other growth factor receptor inhibitor signaling leads to induction of apoptosis (141, 142).

### 3.9 Nuclear factor inducing kinase (NIK)

Nuclear factor inducing kinase (NIK) is a member of MAP kinase kinase kinase (MAPKKK) family which was originally identified as a protein interacting with the TRAF-2 component of the tumor necrosis factor-α receptor complex (143). This MAP3K physically interacts with IKKα and IKKβ and stimulates the catalytic activity of IKKs. NIK interacts with IKKs via its C-terminal region, and this interaction is required for the activation of NFκB following phosphorylation and degradation of IκBα. Previous reports have indicated that overexpression of NIK results in activation of NFκB and kinase-inactive mutants of NIK act as dominant negative inhibitors that suppress NFκB activation mediated by TNF, IL-1, TRAD, TRAF2, TRAF5, TRAF6 (144). Thus NIK is a common mediator of NFκB signaling cascades. Earlier reports have suggested that NIK may also be involved in the regulation of transcription factor, AP-1 as its activation leads to the induction of c-Fos that associates with c-Jun to form an AP-1 heterodimeric complex that can promote targeted gene expression. NIK also forms homodimers or oligomers when expressed in the mammalian cells (145).
3.9.1 Structure and domain organization of NIK

The domain structure of NIK consists of an N-terminal negative regulatory domain and a C-terminal region. The N-terminal non-catalytic region of NIK contains 400 amino acids (146). In contrast to the C-terminal region, the majority of the N-terminal sequences are dispensable for NIK function. Previous studies have indicated that deletion of up to 348 amino acids does not block the ability of NIK to activate IKKα and IKKβ. However, complete inactivation was observed when 377 or more amino acids were deleted. The C-terminal region of NIK undergoes phosphorylation at multiple sites and hence plays a crucial role in its signaling function (147).

Sequence analysis of the N-terminal negative regulatory domain (NRD) of NIK revealed two structural motifs. The first motif is similar to the basic region (BR) of basic leucine zipper (bZIP) motifs present in various transcription factors such as members of the Fos/Jun and CREB/ATF families (148-151). The bZIP motif is composed of a BR and a downstream leucine zipper. The NIK motif contains a perfect BR but lacks a leucine zipper. The second structural domain observed in the negative regulatory region of NIK is proline-rich repeat (PRR) sequence located between amino acid 250 and amino acid 317. This domain is composed of a number of short repeats which share a consensus sequence, PXPXPXP. Previous studies have indicated that deletion of the BR led to a marked increase in the NFκB inducing function of NIK. The deletion of PRR motif or PRR along with BR resulted in a more striking increase in NFκB inducing activity of NIK indicating that both BR and PRR motifs play a negative regulatory role in controlling the signaling function of NIK (Fig. 8).
3.9.2 Negative regulation of NIK

In response to various external stimuli, the MAP3K gets phosphorylated and activate the downstream kinases, mitogen activated protein kinase kinases (152). Activation of MAP3K is mediated through its phosphorylation by upstream kinase (153) or by caspase mediated proteolytic cleavage involving the removal of its N-terminal regulatory domain (154). The negative regulatory domain (NRD) of NIK functions by interacting with C-terminal region. This intramolecular interaction induces conformational changes in NIK which affects the function of its kinase domain thereby inhibiting the binding of NIK to its substrate IκB kinase. Thus NIK is an important member of the MAP3K family which plays a crucial role in the regulation of various transcription factors thereby controlling downstream target gene expression ultimately controlling various physiological and pathophysiological processes.

3.10 Role of MAPK in cell signaling and cancer progression

A diverse array of growth factors, cytokines and proto-oncogenes transduce various extracellular signals through mitogen activated protein kinases (MAPK) and hence play a crucial role in various cellular processes including cell proliferation, differentiation and death.

There are three main groups of MAP kinases in mammalian cells, the extracellular signal regulated protein kinase (ERK) (155), the p38 MAP kinases (156), and the c-Jun
NH2- terminal kinases (JNK) (157). These MAP kinases are activated by dual phosphorylation on the tripeptide motif Thr-Xaa-Tyr. The sequence of this tripeptide motif is different in each group of MAP kinases:

- ERK (Thr- Glu- Tyr)
- p38 (Thr- Gly- Tyr)
- JNK (Thr- Pro- Tyr)

The dual phosphorylation of Thr and Tyr is mediated by a conserved protein kinase cascade. The ERK MAP kinases are activated by the MAP kinase kinases MKK1 and MKK2; the p38 MAP kinases are activated by MKK3, MKK4 and MKK6; and the JNK pathway is activated by MKK4 and MKK7. These MAK kinase kinases are activated by different MAP kinase kinase kinases (MAPKKK). Different upstream signals can lead to the activation of MAPKKK (Fig. 9). However small G proteins have been reported to play a significant role in the activation of these signaling cascades.
The ERK pathway can be activated by Ras via the Raf group of MAP3K. In contrast, the p38 and JNK MAP kinases are activated by Rho family GTPases, including Rac and Cdc42. The candidate MAPKKK that are activated by Rho proteins include members of the MEKK and mixed lineage protein kinase groups.

Anchorage independent cell cycle progression is one of the hallmark of neoplastic phenotype (158, 159). The various growth factors and cell adhesion receptors that are involved in regulating anchorage dependent cell cycle entry stimulate a myriad of signaling pathways including the PI 3-kinase and MEK-ERK cascades. Cell proliferation is mainly facilitated by the activation of various growth factor receptors leading to stimulation of various downstream signaling events that ultimately result in induction of cyclin D1 and downregulation of endogenous cyclin dependent kinase (CDK) inhibitors such as p21 (also called as WAF1 or CIP 1) and p27 (also called as KIP 1) (160, 161). Previous studies have indicated that suppression of ERK activation resulted in abrogation of thrombin induced proliferation of Chinese hamster lung fibroblast (162). Similarly dominant negative forms of ERK as well as ERK antisense oligonucleotides inhibited proliferation of NIH3T3 fibroblasts (163). In addition, studies using pharmacological inhibitors of MEK (164, 165) or activated MAPK phosphatase 1 (MKP 1), a negative regulator of MEK (166), significantly altered the ability of these cells to proliferate in response to growth factor stimulation.

The phosphorylation status and expression levels of a range of early gene products of the MAPK signaling pathway such as transcription factors Fos, Myc and Jun have also been reported to dictate the biological outcome (167). Fos has been shown to function as a sensor for ERK signal duration. When ERK activation is transient, its activity declines before the Fos protein accumulates. In contrast, sustained ERK signaling results in phosphorylation of Fos leading to its stabilization thereby positively impacting cell cycle entry. Most tumors have sustained activation of the Raf-MEK-ERK pathway. The MAPK signaling pathway not only contributes to tumor progression by promoting cell cycle and proliferation but also by controlling the induction of apoptosis. Due to its multifaceted role in cancer progression, MAPK are regarded as valuable therapeutic target.
Table: Human tumors exhibiting activated MAPK signaling

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Pathway Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>KRAS (55%)</td>
</tr>
<tr>
<td>Colon</td>
<td>KRAS (45%), BRAF (12%)</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>KRAS (90%)</td>
</tr>
<tr>
<td>Ovarian</td>
<td>BRAF (30%)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>NRAS (15%), BRAF (66%)</td>
</tr>
<tr>
<td>Non-small-cell-lung</td>
<td>KRAS (35%)</td>
</tr>
<tr>
<td>Papillary thyroid</td>
<td>HRAS, KRAS, NRAS (60%);</td>
</tr>
<tr>
<td></td>
<td>BRAF (35-70%)</td>
</tr>
<tr>
<td>ALL, AML</td>
<td>NRAS (30%)</td>
</tr>
</tbody>
</table>

Knockout studies have indicated that ERK⁻/⁻ mice are fertile and have no apparent abnormalities. However they have a two fold reduction in the number of mature thymocytes (168). Subsequent demonstration that ERK⁻/⁻ fibroblasts were defective in fibronectin mediated cell migration despite the fact that MEK2 and ERK activation were normal indicated that MEK1 might have additional roles apart from activation of ERK (169). Thus there exists a strong rationale for exploring the potential of MAPK pathway inhibitors as anti-cancer agents as majority of the tumors exhibit activated signaling through MAPK pathways (Table).

3.11 NFκB Transcription Factor

Melanoma progression is regulated by a complex network of autocrine and paracrine positive and negative growth factors. The understanding of the functional role of various transcription factors as important control elements of cell growth, differentiation and apoptosis can lead to a new understanding of tumor development. Changes in transcriptional regulation of various genes, which are either controlled by the tumor microenvironment or by mutations or by transformation of the cell itself, represent an early event in tumorigenesis.
NFκB is a family of homo/heterodimeric transcription factors. It is activated by proinflammatory cytokines, ECM proteins, UV irradiation, oxidative stress, growth factors, and microbial, fungal and viral infections and by other physiological and pathophysiological stimuli. The target genes of NFκB fall into four broad functional categories: immunoregulatory and inflammatory genes, anti-apoptotic genes, genes that positively regulate cell proliferation and genes that encode negative regulators of NFκB. Genes of all four categories contribute to tumorigenesis (170). The activation of NFκB not only enables malignant transformation and tumor progression, but also provides a mechanism by which tumor cells escape immune surveillance and resist therapy.

**Structure and Regulation of NFκB**

NFκB is a collective name for the complexes formed by the multigene NFκB-Rel family. The Rel proteins can be divided into two groups depending on their mode of synthesis and transactivation properties. One class includes p65 (also called as Rel A), Rel B and c-Rel which are all directly synthesized in their mature forms. All share a ~300 amino acid region of extensive homology in their N terminus referred to as Rel Homology Domain (RHD). It plays a crucial role in dimerization, DNA binding and further transcription by modulating various domains at their carboxyl termini (Fig. 10).

The second class consists of p105 (also known as NFκB 1) and p100 (also known as NFκB 2) that are synthesized as large precursors with an N terminal RHD and a C terminal series of ankyrin repeats. This C-terminal domain is removed via a ubiquitin-dependent proteolysis upon phosphorylation of p100 and p105 respectively. The mature proteins p52 and p50 respectively contain only the RHD but lack the transcription modulating domains (171). The most commonly detected dimers are p65/p50, p65/p65 and p50/p50. The DNA consensus sequence for the binding of members of the NFκB family corresponds to GGGRNNYYCC (172). Due to the presence of a strong transcriptional activation domain, p65 (Rel A) is responsible for most of the NFκB transcriptional activity.

NFκB represents a paradigm for inducible transcription factors that are regulated mainly by their subcellular compartmentalization (173). The multiple cellular activities of NFκB are controlled through their interactions with the IκB family inhibitor proteins. In
unstimulated cells, NFκB is retained in the cytoplasm in complex with the IκB proteins, which function by masking the nuclear localization signals (NLS) of NFκB (174). Upon cellular stimulation, the IκB proteins become phosphorylated on the two serine residues located within the N-terminal region (175). Phosphorylation occurs on Ser 32 and Ser 36 of IκBα and on Ser 19 and Ser 23 of IκBβ. Phosphorylation of the IκB proteins results in rapid ubiquitination and subsequent proteolysis by the 26S proteosome. Proteasome dependent degradation of the IκB proteins results in the liberation of NFκB leading to its nuclear translocation and initiation of the downstream target gene expression (171, 173).

Fig. 10. Schematic structure of NFκB and IκB proteins
Structure and Regulation of I\(\kappa\)B family proteins

I\(\kappa\)B is a diverse family of transcription factor inhibitors which includes I\(\kappa\)B\(\alpha\), I\(\kappa\)B\(\beta\), I\(\kappa\)B\(\epsilon\), I\(\kappa\)B\(\gamma\) and Bcl-3 (176-178). Each of the I\(\kappa\)B family proteins contains six or seven imperfect copies of the 33 amino acid structural motif known as ankyrin repeats. Ankyrin repeat containing domains which are present in many proteins of diverse functions are primarily involved in the regulation of protein-protein interaction modules (179). The two principal members of the I\(\kappa\)B family of proteins are the I\(\kappa\)B\(\alpha\) and I\(\kappa\)B\(\beta\). Both the proteins possess an amino terminal signal response domain (SRD) which contains a pair of conserved serine residues for inducible phosphorylation. I\(\kappa\)B\(\alpha\) and I\(\kappa\)B\(\beta\) also bear carboxyl terminal segments rich in the amino acids proline, glutamic acid, serine and threonine (PEST). This highly acidic PEST region represents a site for constitutive phosphorylation by protein kinase caesin kinase II (CK II) (180).

I\(\kappa\)B\(\alpha\) and I\(\kappa\)B\(\beta\) both bind preferentially to p65 and c-Rel- containing NF\(\kappa\)B homo- and heterodimers (181). Despite extensive primary structure similarities, I\(\kappa\)B\(\alpha\) and I\(\kappa\)B\(\beta\) exhibit significant functional differences in vivo. The most striking of these differences is that I\(\kappa\)B\(\beta\) activates NF\(\kappa\)B persistently in a cell type and stimulus specific manner; where as regulation of NF\(\kappa\)B by I\(\kappa\)B\(\alpha\) is rapid but transient (182). The distinct functional roles arise due to the fact that active NF\(\kappa\)B upregulates expression of I\(\kappa\)B\(\alpha\), but not I\(\kappa\)B\(\beta\). Newly synthesized I\(\kappa\)B\(\alpha\) can enter the nucleus and dissociate transcriptionally competent NF\(\kappa\)B/DNA complexes (183). I\(\kappa\)B\(\beta\) has been shown to be a weaker inhibitor of NF\(\kappa\)B-DNA binding as compared to I\(\kappa\)B\(\alpha\) (184). Recent studies have also indicated that both free I\(\kappa\)B\(\alpha\) and NF\(\kappa\)B p50/p65 heterodimer/I\(\kappa\)B\(\alpha\) complexes shuttle between the nucleus and cytoplasm by virtue of a free p50 nuclear localization signal (NLS) (185-186). However inactive NF\(\kappa\)B/I\(\kappa\)B\(\beta\) complexes reside permanently in the cell cytoplasm as both NLSs of the NF\(\kappa\)B dimer are masked.

The classical pathway of NF\(\kappa\)B activation which involves phosphorylation induced ubiquitination and degradation of the I\(\kappa\)B proteins mainly depends on another protein complexes, the I\(\kappa\)B kinase (IKK) complex and E\(^3\)I\(\kappa\)B ubiquitin ligase complex (187). The first two subunits of the IKK complex to be identified were IKK \(\alpha\) and IKK \(\beta\) (also referred to as IKK 1 and IKK 2). These are components of a high molecular weight
complex migrating between 600 and 900 kDa that phosphorylates the IκB proteins (187-192). These two proteins contain a Ser/Thr kinase domain in the N-terminal portion of the protein, a leucine zipper, which is responsible for heterodimer formation, and a helix-loop-helix domain in the carboxy-terminal region. Although these proteins can form stable homodimers in vitro, majority of the kinase activity is associated with the formation of IKKα/IKKβ heterodimers. Although these kinases have a lot of similarities, IKKβ has at least 20 fold higher level of kinase activity for IκB than IKKα (187, 192, 193-197). The mitogen activated protein kinase kinase kinase (MAP3K) family members such as NIK (197) and MEKK1 (189, 193, 194, 197) have been reported to stimulate IKK activity via phosphorylation of serine residues in their activation loop. Mutation of serine residues to alanine in their activation loop at positions 176 and 180 in IKKα and at positions 177 and 181 in IKKβ inactivates IKK kinase activity, whereas replacement of the serine residues with glutamates results in the generation of constitutively active kinases (198).

In addition to IKKα and IKKβ, there are additional components of the IKK complex. One of the recently identified components of the IKK complex is the IKKγ/NEMO also called as NFκB essential modifier (199, 200). This 48-kDa glutamine rich protein contains a leucine zipper domain and two coiled-coil motifs but has no known enzymatic activity (Fig. 11). It was first identified in a genetic complementation assay as a cellular factor that was able to restore NFκB activation but did not respond to a variety of activators of this pathway (201, 202). Cells lacking IKKγ/NEMO are unable to neither form the high molecular weight IKK complex nor respond to cytokines in this pathway (203, 204). Although IKKβ preferentially binds to IKKγ/NEMO as compared to IKKα, IKKα has also been shown to bind to IKKγ/NEMO using extracts prepared from IKKβ knock-out cells (204). Gene disruption studies of the IKKγ/NEMO demonstrate that although male mice die in utero, heterozygous female mice develop granulocytic infiltration and both hyperproliferation and increased apoptosis of keratinocytes (205). Homozygous deletion of the IKKγ/NEMO leads to embryonic lethality due to massive hepatic apoptosis (206).
In addition to IκB-dependent regulation, NFκB transcriptional activity is also positively modulated by signaling events that result in direct phosphorylation of NFκB subunits. It has been reported that phosphorylation on Ser-276 by the catalytic subunit of protein kinase-A (PKAc) can contribute to the intrinsic transcriptional capacity of the p65 subunit of NFκB (207). The phosphorylation of p65 on Ser-276 enhances its intrinsic transcriptional activity by facilitating its interaction with the transcriptional co-activators, CBP (cAMP-responsive element (CRE) binding protein) and the closely related factor p300 (208). Similar to phosphorylation on Ser-276, TNF-induced phosphorylation of p65 at Ser-529 does not affect the nuclear translocation signal or the DNA-binding activity but increases the transcriptional potential of p65. However a site directed mutant of p65 (Ser-276 to Ala-276) is still phosphorylated at Ser-529 in response to TNF, suggesting that multiple physiological stimuli modulates p65 through distinct phosphorylation sites thereby controlling its transcriptional activity (209).
Another less explored pathway of NFκB activation is the tyrosine phosphorylation of IκB protein in response to oxidative stress (non-degradative pathway) leading to an increase in the transcriptional activity of NFκB. Tyrosine phosphorylated IκB activates NFκB in the absence of proteosome mediated pathway. In this pathway, a member of the protein tyrosine kinase family (PI 3-kinase and Src family kinase) interacts and modulates the IκB activity (210). Most of the signaling pathways converge at the level of activation of IKK, phosphorylation of IκB and subsequent degradation leading to activation and translocation of NFκB (Fig. 12).

3.11.1 Role of NFκB in oncogenic transformation and tumorigenesis

Accumulating evidence indicates that NFκB plays a crucial role in the development of cancer progression. Retroviruses that encode v-Rel, a viral homologue of c-Rel, are
highly oncogenic and cause aggressive tumors in chicken. The genes that encode c-Rel, NFκB2 (p100/p52), p65 (Rel A) and Bcl-3 proteins are all located within regions of the genome that are involved in rearrangements or amplifications. Constitutive activation of NFκB has been detected in some lymphomas, melanomas and breast cancers (211, 212). Mutations in the IκBα gene have been detected in Hodgkin’s lymphoma and are suggested to contribute to the constitutive activation of NFκB in Hodgkin’s cells. NFκB is also linked to cellular transformation independent of chromosomal translocation events. For example, it has been shown that NFκB is activated by a number of viral transforming proteins and is required for virus-induced transformation. Thus the Tax protein from the human T cell leukemia virus-1 (HTLV-1) transcriptionally activates NFκB through direct interaction with the IKK complex (213). Activation of NFκB has been shown to be required for the transformation of rat fibroblasts by the HTLV-1 Tax protein (214). Studies with ER -ve human breast cancers have revealed an enhanced level of NFκB when compared to ER +ve breast cancers (215, 216). This also correlated with the enhanced level of EGFR in ER-ve cells and further suggested that NFκB can act as a potential therapeutic target for estrogen receptor negative breast cancers (216, 217).

More recent evidence confirms the involvement of NFκB in oncogenesis. It has been reported that blockade of the constitutive NFκB activity in metastatic human prostate cancer cells by expression of the super repressor IκBα resulted in suppression of angiogenesis, invasion and metastasis (218, 219). Recently, it has been reported that inhibition of NFκB in head and neck squamous cell carcinoma inhibits cell survival and tumor growth (219). Consistent with the role of NFκB in transformation and tumorigenesis, many human derived solid tumor cell lines, display increased nuclear levels and/or increased NFκB dependent reporter activity in comparison to non-transformed control cell lines (220). The ability of tumors to invade surrounding tissues, enter the blood stream and metastasize to distant sites is one of the most significant factors contributing to mortality of cancer patients. NFκB plays an important role in this process through its ability to regulate the expression of various cell adhesion molecules such as ICAM-1, VCAM-1, matrix metalloproteinases such as MMP-9, chemokine receptors such as CXCR4, urokinase type plasminogen activator (uPA) and cyclooxygenase-2 (COX-2) (221). Solid tumors also
induce vasculogenesis and thereby overcome the effects of hypoxia and nutrient starvation induced by rapid increases in proliferation (222). NFκB contributes to this process by inducing vascular endothelial growth factor (VEGF) thereby stimulating angiogenesis and metastasis. Thus NFκB plays a pivotal role in regulating cell proliferation, migration and cell survival leading to neoplastic transformation (223).

3.12 AP-1 transcription factors: structure, functions and regulation

The transcription factor AP-1, activator protein-1 is involved in a wide range of cellular processes such as cell proliferation, survival, differentiation, oncogenic transformation, apoptosis etc by regulating the target gene expression in response to a plethora of physiological and pathological stimuli (224). AP-1 is a group of basic leucine zipper (bZIP) transcription factors consisting of the Fos (c-Fos, Fos B, Fra-1 and Fra-2), Jun (c-Jun, Jun B, JunD) families (225, 226). The Fos members heterodimerizes with the members of the Jun family. However the Jun proteins either homodimerize with the other members of the Jun family or heterodimerize with Fos members to form transcriptionally active AP-1 complexes (227). This homodimerization of Jun proteins or heterodimerization of Fos and Jun proteins results in the formation of transcriptionally active complex. This active AP-1 complex recognizes specific DNA sequences known as tetradecanoyl phorbol acetate-responsive elements (TRE) or AP-1 binding sites which are found in the regulatory regions of a variety of cell cycle related genes and AP-1 genes themselves. AP-1 converts extracellular signals into changes in the expression of specific target genes that harbour AP-1 binding site(s) in their promoter or enhancer regions. AP-1 activity is modulated by interactions with other transcriptional regulators and is further controlled by upstream kinases that link AP-1 to various signal transduction pathways (228).

Regulation of AP-1 by physiological and pathophysiological stimuli

AP-1 was identified as a transcription factor that contributes to both basal gene expression as well as TPA-inducible gene expressions (229, 230). AP-1 activity is regulated by a wide range of physiological and pathophysiological stimuli such as serum, growth factors, proinflammatory cytokines (IL-1 and TNF-α), neurotransmitters,
polypeptide hormones, cell-matrix interactions, bacterial and viral infections and a variety of physical (UV exposure), chemical and genotoxic stress (231-235).

Several mechanisms are involved in the stimulation of AP-1 activity by growth factors, proinflammatory cytokines and UV radiation. The most important mediator of the growth factor response is the ERK/MAP kinase (MAPK) cascade. It induces the phosphorylation of the ternary complex factors (TCF) resulting in the induction of 'fos' genes. These proteins then heterodimerizes with Jun proteins to form more stable AP-1 dimers. As AP-1 sites are present in the c-jun promoter, the newly formed Jun:Fos heterodimers lead to increased c-Jun transcription (236). The induction of c-Jun by ERK activation also occurs through another group of transcription factors known as MEF-2 proteins that bind to c-Jun promoter (237).

The induction of AP-1 in response to proinflammatory cytokines and UV radiation are mainly dependent on the other two MAPK cascades, JNK and p38 (238). In addition to phosphorylation of TCFs and Fos gene induction, the p38 MAPK also contribute to c-Jun gene induction through phosphorylation of MEF2C (239). The induction of c-Jun by UV occurs through JNKs. This MAPK family member induces the phosphorylation and enhances the transcriptional activity of two major players in c-Jun expression, c-Jun itself and ATF-2 (240). Thus these phosphorylation events result in potentiation of transactivation leading to downstream gene expression but do not affect the DNA-binding.

Role of AP-1 in wound healing

The physiology of skin can be dissected into two major compartments, the epidermis representing the outermost skin layer, and the dermis, both of which are separated by the basal lamina. Homeostasis of the epidermis relies on a tightly regulated balance between proliferation and differentiation. Expression of c-Jun, Jun-D and Fra-1 are coordinately expressed in the basal and spinous layers of the epidermis. Basal cells also express c-Fos, Jun-B and Fra-1, but not Fos-B. Jun-B and Fra-2 are the only AP-1 members which are also found in the granular layer, suggesting the existence of subgroups of AP-1 target genes involved in keratinocyte proliferation and differentiation.

AP-1 plays a dominant role in the transcriptional activation of a majority of matrix metalloproteinases (MMPs), which are involved in cell migration, degradation of
extracellular matrix and tissue remodeling during the wound healing process. Basal transcription as well as activation of MMP promoters in response to phorbol esters, cytokines, growth factors, cell-matrix interactions and altered cell-cell contacts requires the specific interaction of AP-1 and ETS proteins (241-243). In acute murine excisional skin wounds, interstitial collagenase (MMP-1; MMP-13), MMP-9, MMP-3 and MMP-10 and their physiological inhibitor TIMP-1 are strongly induced showing a unique spatial and temporal transcription pattern (244). Tissue culture as well as animal studies have provided evidence for the importance of the AP-1 target genes MMP-1 and MMP-3 during wound healing in keratinocyte migration and wound contraction respectively (245, 246).

3.12.1 Role of AP-1 in tumorigenesis

Tumorigenesis is a multistep process, which involves cell transformation, invasive growth and metastatic spread to distant sites. Components of the AP-1 transcription factor have been implicated in many of these processes (247) (Fig. 13).

c-Fos is the cellular homologue of v-Fos, the transforming oncogene of the murine osteosarcoma viruses, FBJ-MuSV and FRB-MuSV (248). Like v-Fos, c-Fos also has oncogenic activity which is dependent on its ability to heterodimerize with c-Jun protein and bind to the promoter sequence of the target gene (249-251). The oncogenic activity of c-Fos depends on the integrity of several structural motifs present in the N- and C-terminal transactivation domains, constitutive c-Fos expression and DNA 5-methylcytosine transferase activity (252-253). When overexpressed in vivo, c-Fos transforms chondroblast and osteoblast and thus indicating that these two cell types as cellular targets of c-Fos induced tumorigenesis (254, 255). However Fos-B transgenic mice do not form tumors (256). The transforming activity of Fra-1 and Fra-2 are weak in comparison to the other Fos proteins. Though Fra-1 overexpression in fibroblasts does not lead to morphological transformation or focus formation, it results in anchorage independent growth in vitro and tumor formation in nude mice (257, 258). Fra-2 has transforming activity in chicken embryo fibroblasts, but not in rat fibroblast (259). Ectopic expression of c-Fos in mammary epithelial cells causes loss of epithelial polarity, epithelial fibroblastoid cell conversion and invasive growth in collagen gels suggesting the ability of c-Fos to modulate the potential of tumor cells for invasive growth.
c-Jun is a cellular homologue of v-Jun, the transforming oncogene of the avian sarcoma virus 17 (260). Transgenic mice expressing oncogenic v-Jun develop no spontaneous tumors, but form fibrosarcomas at the sites of wounding (261). c-Jun can lead to oncogenic transformation in mammalian cells only when coexpressed with an activated oncogene such as Ras or Src (262). Transformation by c-Jun also requires an intact transactivation domain and N-terminal phosphorylation of serines 63 and 73 (263, 264). However c-Jun cooperates with c-Fos in the formation of skeletal osteosarcomas and efficient c-Fos-induced transformation of osteoblast depends on c-Jun phosphorylation (262). Unlike c-Jun, both Jun-B and Jun-D lack transforming activity (265, 266).

Ectopic expression of Jun-B and Jun-D inhibits oncogenic transformation by activated Ras in fibroblasts indicating that both these proteins act as anti-oncogenes (267). Jun-B deficient myeloid progenitor display increased levels of GM-CSF a receptor, antiapoptotic proteins Bcl-2 and Bcl-xL, whereas the expression of the cell cycle inhibitor
p16 is reduced. These results suggest that Jun-B inhibits proliferation and promotes apoptosis in myeloid progenitors thereby suppressing leukaemogenesis. However the skin tumor development caused by constitutive activation of the Ras pathway by expressing an activated form of SOS-K5-SOS-F is impaired in jun AA/AA mice further supporting the in vivo significance of c-Jun N-terminal phosphorylation in Ras induced tumorigenesis (263). Reducing AP-1 activity by targeted expression of a dominant negative c-Jun (TAM67) in the basal keratinocytes of transgenic mice inhibits the development of chemically induced pappilomas (264). Further, AP-1 response element is present in the promoter region of matrix degrading proteases like uPA, MMP-9 etc and hence this transcription factor plays a crucial role in regulating cancer progression (265).

3.13 Urokinase plasminogen activator

The process of extracellular proteolysis plays a crucial role in vascular remodeling, angiogenesis, wound healing, tumor growth and metastasis. These ECM degrading proteases are located on the cell surface and hence degrade the pericellular matrix components leading to the destruction of cell-cell contacts. Urokinase type plasminogen activator (uPA) is a member of serine protease family that interacts with a specific cell surface receptor (uPAR/CD87) located at the leading edge of migratory cells and facilitates the conversion of inert zymogen plasminogen into widely acting serine protease plasmin. This results in the activation of matrix metalloproteinases (MMPs) leading to proteolysis of the extracellular matrix components. In vascular system, the plasminogen activators are key components of the fibrinolysis system. Moreover, uPA-uPAR complex also interacts with other integrins and plays a crucial role in intracellular signaling thereby controlling cell adhesion, migration and proliferation (268).

Structure of uPA

uPA is secreted by monocytes, macrophages, smooth muscle, epithelial, fibroblast, vascular endothelial cells and also by transformed cells of different origin (269-271). uPA is secreted as an inactive single chain proenzyme (pro-uPA) of 52 kDa, consisting of 411 amino acid residues (272).

This pro-uPA consists of three domains:
1) the N-terminal domain homologous to the epidermal growth factor
2) the kringle domain
3) the C-terminal catalytic domain

The growth factor like domain is responsible for the binding of uPA to its receptor uPAR/CD87 receptor (273). The kringle domain contains a sequence that interacts with the specific uPA inhibitor called PAI-1 (274). The active site of the catalytic domain has serine protease amino acid triad His-204, Asp-255 and Ser-356. Each domain has internal disulfide bonds maintaining its rigid structure. This pro-uPA has only 1/250 the activity of the mature enzyme and can convert plasminogen to plasmin (275). This plasmin in turn activates uPA by cleaving the Lys158-Ile159 peptide bond and converts single chain uPA into two-chain forms. In the two-chain urokinase, the polypeptide chains A (light chain) and B (heavy chain) are connected by the Cys148-Cys279 disulfide bond (268). A subsequent plasmin action on this form leads to the cleavage of the growth factor like domain and the kringle domain resulting in the generation of the 32 kDa uPA (268).

![Fig. 14. uPA domain structure and fragments generated upon proteolytic processing of uPA on the cell surface. G, growth factor like domain; K, Kringle domain; P, protease domain](image-url)
The full length single and two chain forms of uPA can bind to the uPAR on the cell surface. However, uPA lacking the growth factor like domain cannot interact with uPAR/CD87 (268). uPA can also bind the surface of smooth muscle cells either through kringle domain or through protease domain suggesting the presence of receptors other than uPAR/CD87 (268). Active uPA has high specificity for the Arg 560-Val561 bond in plasminogen and cleavage between these residues results in the formation of active plasmin (276) (Fig. 14).

**uPA-uPAR functions**

uPA receptor/CD87 which binds to uPA has a molecular weight of about 55-60 kDa and it contains three homologous sequence repeats (277). The urokinase receptor lacks a transmembrane sequence and is anchored on the plasma membrane by a glycosyl phosphotidyl inositol (GPI) moiety (278). The location of uPAR on the cell surface depends on the functional state of the cells. In resting cells, it is uniformly distributed on the cell surface, however in migratory cells, it is clustered on the leading edge (279). The receptor bound single chain urokinase is activated by plasmin more efficiently than free urokinase (280). uPAR has the capacity to prevent urokinase from undergoing further degradation by plasmin. Thus, plasmin mediated elimination of growth factor like domain of uPA takes place more slowly when it is bound to uPAR (278). Since uPAR lacks both transmembrane and cytoplasmic domain, it associates with the integrin family of adhesion receptors to initiate intracellular signaling. In certain cell types, uPA is reported to physically interact with β1 and β3 integrin family members and transduces signals similar to integrin-mediated signaling (281, 282). Binding of uPA to uPAR results in the activation of FAK, paxillin, MAP kinases and also enhancement in STAT-1 and STAT-3 DNA binding activity.

uPA also plays a crucial role in the activation of matrix metalloproteinases (MMP-3, MMP-9, MMP-12 and MMP-13) leading to the degradation of extracellular matrix. The combined effects of plasmin, uPA and metalloproteinases on the plasma membrane promote cell migration due to destruction of the cell-cell contacts and matrix. Moreover uPA either directly or via plasmin also plays a crucial role in the release of a number of
growth factors like HGF, VEGF165, FGF-2 by the extracellular matrix which in turn binds to its specific receptors to initiate the various cell signaling events.

**Negative regulation of uPA activity**

The activation of plasminogen by uPA is regulated by two important inhibitors, plasminogen activator inhibitor-1 (PAI-1) and PAI-2. PAI-1 is a 52 kDa single chain protein which is secreted in an active but conformationally unstable form. The inhibitory activity of PAI-1 is stabilized by its binding to vitronectin (278). PAI-1 exerts its inhibitory activity by inhibiting the binding of uPA to uPAR. PAI-1 or PAI-2 forms a complex with uPA on the cell surface. Following which the uPA/uPAR/PAI-1 complex is internalized and the uPAR is recycled back to the cell surface through the endosomal compartment. PAI-1 also modulates cell migration and invasion by competing with binding to uPAR.

**Regulation of uPA expression**

The expression of uPA is regulated by a variety of extracellular signals such as phorbol ester, cytokines, growth factors, oncogenes such as v-Src and v-Ras etc. The transcription of the uPA gene is regulated by two upstream regions: the minimal promoter (MP) and the enhancer element. The minimal promoter (MP) of the uPA gene extends approximately 86 bp upstream of the transcription start site and the enhancer element is located about 2 kb upstream of the transcription start site which contains potential binding sites for various transcription factors. The various transcription factors which play a key role in the regulation of uPA expression are NFκB, AP-1, Sp-1 and PEA-3. The enhancer region contains an NFκB binding site at -1592/-1582 (283). An octameric AP-1 binding site combined with a PEA-3 site is located at the 5' region of the enhancer element while a heptameric AP-1 binding site is located at the 3' region. Both these binding sites are essential for basal and induced promoter activity (284). Transfection of cells with IκBα super-repressor construct results in a drastic reduction of uPA promoter activity. However mutation in the 5'-NFκB site leads to a 20% reduction in uPA promoter activity. The minimal promoter region of the uPA gene also contains 5 (high, 3 x GGGCGG, low, 2 x GGGAGG) affinity-binding sites for the Sp1 family transcription factors immediately upstream of the TATA box. This cis element binds Sp1 *in vivo* in PC-3 cells that
constitutively express uPA gene. Thus the uPA minimal promoter that contains multiple transcription factor binding sites play a very important role in uPA gene expression, invasion and metastasis formation in cancer cells.

Role of uPA in tumor progression

The key steps which are involved in the process of tumorigenesis and metastasis are: degradation of the basement membrane and interstitial matrix, endothelial and cancer cell migration, proliferation and formation of tubular structures with a lumen and a new basement membrane (285). Three of these steps critically depend on the proteolytic activity generated by the matrix metalloproteinases and the plasminogen activator/plasmin system. In particular, the role of uPA/uPAR system in tumor cell migration, invasion and metastasis has been under intense investigation. uPA interacts with uPA receptor (uPAR) and facilitates the conversion of inert zymogen plasminogen into widely acting serine protease plasmin which regulates cell invasion by degrading matrix proteins such as type IV collagen, gelatin, fibronectin and laminin or indirectly by activating matrix metalloproteinases (MMPs) (286, 287). The level of expression of uPA correlates with metastatic potential in a number of cancers (288).

uPA, its receptor uPAR and inhibitors PAI-1 and PAI-2 which constitute the urokinase plasminogen activator system play a vital role in not only cancer progression but also in a number of normal physiological processes like wound healing, liver regeneration and homeostasis. Its high levels are associated with cancers of lung, skin, breast, bladder, uterine cervix and soft tissue sarcoma (289). Earlier studies have indicated that blocking of uPA activity or uPA-uPAR interaction drastically downregulates tumor growth and metastasis (290-292). uPA enhances tumorigenesis by increasing the bio-availability of various growth factors like FGF, TGF-β etc in the tumor micro-environment which subsequently initiates various signaling events thereby promoting cell migration, invasion, tumor growth and metastasis. Further reports have indicated that uPAR also interacts with integrins leading to persistent activation of MEK-ERK pathways in response to fibronectin. The downregulation of uPA-uPAR results in the cell cycle arrest at G0/G1 phase in vivo leading to tumor dormancy (293).
uPA knock-out mice show minor phenotypic defect such as occasional fibrin deposition in the intestine and in the sinusoids of the liver (294). In another study, uPA deficient mice having electric and mechanical injury of arteries showed impaired neointima formation. This may be due to impaired migration of smooth muscle cells (295). Another study indicated that both wild type and uPA gene deficient mice developed blue nevi with same frequency upon chemical induction of melanocytic neoplasm. However, only wild type mice had the ability to develop melanomas suggesting the critical role of uPA in the progression of malignant phenotype (296).

It has been reported that the chemokine like ECM protein, OPN which is associated with the malignancy of various cancers induces cancer cell migration and invasion by inducing uPA secretion. Therefore, studies on the molecular mechanism by which OPN regulates uPA secretion and uPA dependent MMPs activation will be useful in identifying novel targets for cancer therapy.

3.14 MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMPs) belong to the matrixin subfamily of the metzincin superfamily of zinc dependent endopeptidases. MMPs also called matrixins plays a central role in regulating matrix turnover and contribute to matrix equilibrium and structural integrity (Fig. 15). Thus these ECM degrading enzymes play a crucial role in several biological processes such as embryogenesis, wound healing, and connective tissue remodeling, inflammatory responses and in pathologies such as arthritis and cancer (297).

This multigene family of proteases is characterized by several common features.

- They require Zn\(^{2+}\) ions or molecules to be active
- They are all active at physiological pH
- They are secreted as prepro-enzymes and is activated extra-cellularly
- They are inhibited by metal chelators and tissue inhibitors of metalloproteinases (TIMPs)

To date, 23 different MMPs have been cloned and additional members continue to be identified
Classification of MMPs

Mammalian MMPs are classified into two types based on the presence of transmembrane domain

1) Soluble type MMP and 2) Membrane anchored MMP

The soluble type MMP is secreted as inactive pro-MMP. They show broader accessibility to ECM and hence regulate tissue remodelling events. On the other hand, membrane anchored MMP are activated intra-cellularly and mostly expressed on the cell surface as an active enzyme. They are predominantly involved in the regulation of pericellular environments closely associated with cellular functions including migration and invasion.
Soluble type MMP

On the basis of substrate specificity, soluble MMP can be further classified into four different groups.
1) Collagenases, 2) Stromelysins, 3) Gelatinases and 4) Matrilysins

Membrane anchored MMP

The membrane anchored MMP are made of six membrane type (MT)-MMP and cysteine array (CA)-MMP (MMP 23), all of which are expressed as integral membrane zymogens. Based on the membrane anchoring mechanisms, these membrane anchored MMP can be further divided into three groups 1) Type I transmembrane MMP (MT 1-3 and MT 5-MMP), 2) the glycosphosphotidylinositol (GPI) - anchored MMP (MT4-MMP and MT6-MMP) and 3) Type II transmembrane MMP (MMP-23)

Although the classification system was developed based on the substrate specificity, it is now identified that there is some overlap between members of some families. Eg. MMP-2 which was classified under gelatinases as gelatinase-A has been reported to have the ability to cleave fibrillar collagen similar to the collagenases (298).

3.14.1 Structure of MMPs

The protein and the cDNA sequences of the various cloned MMPs reveal a number of conserved regions in the family. The primary structure comprises of several domain motifs which generally varies for different MMPs. All pro-MMPs contain three domains: (a) a hydrophobic signal domain that is necessary for signal secretion; (b) an amino terminal propeptide domain that is cleaved upon activation and (c) the Zn$^{2+}$ containing catalytic domain (299-301).

The propeptide domain consist of about 80 amino acids and has a conserved PRCG (V/N) PD sequence (“cysteine switch”) that ligates the catalytic Zn$^{2+}$ to maintain the latency of pro-MMPs (302).

The catalytic domain consists of about 170 amino acid residues. It also contains a zinc binding motif HEXXXHXXGXXH and a conserved methionine, which forms a unique “Met- turn” (303). This domain consists of a five stranded $\beta$ sheet, three $\alpha$ helices and bridging loops which chelates the catalytically essential zinc atom. This domain also
contains an additional structural zinc ion and 2-3 calcium ions, which are required for the stability and the expression of the enzymatic activity. Except MMP-7 which is the smallest in size and has only the three basic domains, all MMPs have a hemopexin domain connected to the catalytic domain by the hinge region.

The C terminal hemopexin like domain consist of about 210 amino acid residues. It has an ellipsoidal disc shape with a four bladed \(\beta\)-propeller structure, each blade consist of four anti-parallel \(\beta\)-strands and a \(\alpha\) helix (302-303). This domain contains the TIMP binding site, which is believed to be involved in receptor binding and inhibiting the interaction between the cysteine in pro-domain and zinc in catalytic domain (304) (Fig. 16).

The basic three domain structure is present in all MMPs, however further subdivision exist based on the distinct structural variations. The membrane type metalloproteinases (MT-MMPs) are a unique subdivision characterized by the presence of a transmembrane domain at the carboxyl terminal anchoring the molecule to the cell surface (305). The gelatinases (MMP-2 and MMP-9) contains a fibronectin like domain within the catalytic domain responsible for collagen binding (306).
3.14.2 Regulation of MMP

Due to its multifaceted role in both normal and pathological processes, MMPs are very stringently regulated. The regulation of MMPs occurs at three levels,

- Alteration of gene expression
- Activation of latent zymogens
- Inhibition by tissue inhibitor of Metalloproteinases (TIMPs)

Under normal physiological conditions, there is controlled regulation of MMPs by these three parameters but any alteration results in a number of pathological conditions.

Regulation of gene expression

One of the characteristic features of MMP is that many of these genes are “inducible”. The effectors include growth factors (eg. Basic fibroblast growth factor, epidermal growth factor, and vascular endothelial growth factor), cytokines (interleukin family, tumor necrosis factor), chemical agents (eg. Phorbol esters) and oncogenic cellular transformation etc. The various factors that contribute to MMP regulation vary in different tissues resulting in variable expression pattern of the different MMP family members, thus complicating the understanding of gene regulation in both normal and pathological states.

Recent studies indicate that not only soluble factors but also cell matrix and cell-cell interaction are involved in regulation of MMP gene expression. Eg. The laminin peptide (AG-73) has been shown to enhance gelatinase production and increase lung and liver metastasis in melanoma cells (307). Vitronectin induces MMP-2 expression in melanoma cells (308). Collagen and elastin have also been reported to induce the expression of collagenases and gelatinases by either tumor cells or surrounding fibroblasts (309, 310). EMMPRIN (M6 antigen or basigin), a member of the immunoglobulin family expressed on tumor cell surface is involved in induction of MMP-1, MMP-2 and MMP-3 in fibroblast; induction of MMP-9 through leukocyte function associated antigen-1 (LFA-1), intercellular adhesion molecule (ICAM-1) mediated cell adhesion in T lymphoma cell (311).

The MMP-9 expression during macrophage differentiation is induced by α5β1 integrin and fibronectin interaction. Earlier reports also indicate that ultra-violet irradiation upregulates MMP-1, MMP-3 and MMP-9 expression in human dermal fibroblast (310,
Recent data indicated that the MMP expression can also be enhanced by factors like transforming growth factor β, retinoic acids and glucocorticoids (313, 314). Thus there exist a critical balance between the various inducers and suppressors that modulate MMP genes and also suggest that cell environment plays a key role in regulating MMPs.

**Activation of pro-MMPs**

An important aspect in the regulation of MMP activity is that they are secreted as inactive zymogens. This inactive proform contains a prodomain in which a Cys residue...
prevents the Zn\(^{2+}\) binding domain from becoming catalytically active (315). Elimination of this domain is an important prerequisite for MMPs to become active. The most widely accepted model for pro-MMP activation is the cysteine switch model which involves the destruction of Cys-Zn interaction (316). This process consists of a two-step reaction. An initial cleavage at the propeptide bait region leads to the removal of N-terminal polypeptide, which is followed by a second step, an autoproteolytic reaction that generates the stable active enzyme (by both intra and inter molecular mechanisms). The activation of pro-MMPs \textit{in vitro} occurs in the presence of destabilizing agents, such as the organomercurial APMA which initiate an autocatalytic cleavage of the prodomain. However the mechanism of pro-MMP activation \textit{in vivo} is very complex and less understood. This includes the participation of other serine proteases such as plasmin or furins or other MMPs. For eg. The activation of pro-MMP-2 occurs in a two step process. This process involves an initial cleavage of a \(M_r\) 72,000 precursor form at the Asn 37-Leu 38 bond by MT1-MMP that is followed by an autocatalytic conversion of the \(M_r\) 64,000 Leu-38 intermediate into a \(M_r\) 62,000 active enzyme with an amino terminal Tyr 81 residue (317) (Fig. 17). In the case of activation of MMP-9, the activation of Mr 92,000 precursor can be achieved by MMP-3 and MMP-2. In addition, the urokinase- plasmin system has been implicated in the activation of both MMP-2 and MMP-9 (318-320). This activation of pro-MMPs, including collagenases, stromelysins and matrilysins, can occur at distant sites in the cells, in the extracellular matrix and on the cell surface.

**Inhibition by Tissue Inhibitor of Metalloproteinases**

The third level of control of MMPs occurs through inhibition of its enzymatic activity (Fig. 17). Various physiological agents have an inhibitory effect on MMP activity including \(\alpha2\) macroglobulin and tissue inhibitors of metalloproteases, TIMPs (321). In the extracellular matrix, the activity of MMPs is controlled by a family of specific inhibitors known as TIMPs (TIMP-1, TIMP-2, TIMP-3 and TIMP- 4) (322). The amino terminal domain in all TIMP molecules is responsible for the MMP inhibitory activity. These inhibitors exert a double control on MMP activity by forming stable complexes with the active domain of MMPs and also by controlling the activation process (323).The TIMPs form high affinity, non-covalent complexes with active MMPs in a 1:1 stoichiometric ratio.
In addition, TIMP-1 and TIMP-2 can block the pro-forms of MMP-9 and MMP-2 respectively. TIMP-1 is a 28.5 kDa glycoprotein. Its functions include growth factor activity, stimulating cell morphology changes and inhibiting angiogenesis (324). TIMP-2, a non-glycosylated 21-kDa protein plays crucial role in suppression of tumor growth and metastasis (324). In contrast to TIMP-1 and TIMP-2, TIMP-3 is associated with the extracellular matrix. It is reported that TIMP-3 reduces invasion and induces cell death in vitro (325). TIMP-2 forms a complex with pro-MMP-2 that is responsible for controlling the activation of MMP-2 (326). TIMP-1 forms a proenzyme: inhibitor complex with pro-MMP-9 (327). However, much less is known about the exact mechanism by which it regulates MMP-9 activation.

**MMPs are involved in normal and pathological processes**

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**3.15 MMP-9: Structure, regulation and functions**

MMPs have been implicated in tumor progression in large part because of the repeated finding of the overexpression of this class of enzymes in tumors compared with their expression in normal tissues (328-330). MMP-9, which is also called as 92 kDa gelatinase/collagenase or gelatinase-B, is one of the members of the gelatinase family and it shares all the common features of MMPs. It is secreted as a 92 kDa molecule and is processed to 86-kDa form. Association with tissue inhibitor of metalloproteinases-1 (TIMP-1), a naturally occurring inhibitor of MMP-9 alters its processing in vitro. MMP-9
can be activated *in vitro* through exposure to 4-aminophenyl mercuric acetate, stromelysin, MMP-2 or plasminogen activator, but its physiological activators are currently unknown (331-333). Since MMP-9 can be activated by electrophoresis procedures, its gelatinase activity will be revealed on zymography or gelatin substrate electrophoresis even in its 92-kDa form. Once active, it has a wide range of proteolytic activity with the capacity to degrade matrix components, including denatured collagen or gelatin, type IV and V collagens and elastin. It cleaves galectin on cell surfaces and can also process tumor necrosis factor *in vitro*. MMP-9 bears considerable homology with another member of the gelatinase family, gelatinase A or MMP-2. These enzymes have similar substrate specificities when tested against synthetic peptides but show one major difference, MMP-9 is highly active against casein, whereas MMP-2 is not (334).

### 3.15.1 MMP-9: Role in tumor invasion and metastasis

Several reports have indicated the direct involvement of MMPs in tumor invasion and metastasis (335). This evidence has been supported by the studies that documented the abundant expression of many MMPs in human tumor specimens and the direct correlation between their expression and clinical outcome (336). Among the various MMPs, MMP-2, MMP-9 and MT1-MMP have been associated with invasion and metastasis of a large variety of cancers (337), including lung (338), prostate (339), breast (335) and colon (340). The release of MMP-9 has been correlated with metastasis in several systems. Metastasis by ras\(^H\)-transformed NIH3T3 cells has been correlated with MMP-9 release (341). Previous studies have indicated that U937 cells treated with 12-O-tetradecanoylphorbol-13 acetate show increased production of MMP-9 in culture associated with increased lung colonization and invasion *in vivo* (342). Transfection of transformed rat embryo cells with the oncogene E1A resulted in virtual shutoff of MMP-9 mRNA and released 92-kDa gelatinase. This correlated with inhibition of metastasis in lung colonization assay, although decreased stromelysin expression was also observed in this case (343). Thus the presence of MMP-9 in many potentially invasive and metastatic tumors and the experimental evidence demonstrating the involvement of MMP-9 expression in metastasis leads to the conjecture that MMP-9 plays a pivotal role in metastasis in many tumors (Fig. 18). Immunohistochemistry and *in situ* hybridization studies used to localize the production
of MMPs in the tumor tissues have indicated that host derived stromal cells and not tumor cells are the source for MMP production (344). Thus the matrix degrading proteolytic activity of a tumor is a result of a complex balance between several factors including pro-MMPs, active MMPs, MMP activators and TIMPs and this balance is controlled by interactions between tumor cells and host derived stromal cells.

**Fig. 18. Model depicting the role of MMPs in malignant tumor progression**