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Drug development against metastasis-related genes and their pathways: A rationale for cancer therapy

Megumi Iizumi, **Wen Liu**, **Sudha K. Pai**, **Eiji Furuta**, and **Kounosuke Watabe***
Department of Medical Microbiology, Immunology and Cell Biology, Southern Illinois University, School of Medicine, Springfield, Illinois, USA

Abstract

It is well recognized that the majority of cancer related deaths is caused by metastatic disease. Therefore, there is an urgent need for the development of therapeutic intervention specifically targeted to the metastatic process. In the last decade, significant progress has been made in this research field, and many new concepts have emerged that shed light on the molecular mechanism of metastasis cascade which is often portrayed as a succession of six distinct steps; localized invasion, intravasation, translocation, extravasation, micrometastasis and colonization. Successful metastasis is dependent on the balance and complex interplay of both the metastasis promoters and suppressors in each step. Therefore, the basic strategy of our interventions is aimed at either blocking the promoters or potentiating the suppressors in this disease process. Toward this goal, various kinds of antibodies and small molecules have been designed. These include agents that block the ligand-recepter interaction of metastasis promoters (HGF/c-Met), antagonize the metastasis promoting enzymes (AMF,uPA and MMP) and inhibit the transcriptional activity of metastasis promoter (β-Catenin). On the other hand, the intriguing roles of metastasis suppressors and their signal pathways have been extensively studied and various attempts have been made to potentiate these factors. Small molecules have been developed to restore the expression or mimic the function of metastasis suppressor genes such as NM23, E-cadherin, Kiss-1, MKK4 and NDRG1, and some of them are under clinical trials. This review summarizes our current understanding of the molecular pathway of tumor metastasis and discusses strategies and recent development of anti-metastatic drugs.

1. Introduction

Cancer is the second leading cause of death in the USA, and more than half a million people succumb to the disease every year [1]. Despite significant improvements in screening methods and treatment options, the majority of cancer patients are still diagnosed at an advanced stage, and more than 90% of patients ultimately die from sequel of metastatic disease. Therefore, metastasis is a hallmark of malignancy, and no effective therapeutic option is currently available for those patients. Although the clinical importance of tumor metastasis is well recognized, advances in understanding the molecular mechanism involved in metastasis formation have lagged behind other developments in the field of cancer research. This is attributed to the fact that cancer cells are extremely heterologous in nature and that metastasis involves multiple steps with a high degree of complexity, and each step requires coordinated

*Corresponding author: Kounosuke Watabe, PhD., Department of Medical Microbiology, Immunology and Cell Biology, Southern Illinois University School of Medicine, 801 N. Rutledge St., P.O. Box 19626, Springfield, Illinois 62794-9626. Tel: (217) 545-3969, Fax: (217) 545-3227, E-mail: kwatabe@siumed.edu .

action of many promoters and suppressors. However, extensive efforts in the past decade have led to the discoveries of many previously unknown factors involved in metastasis and also unveiled several novel concepts in this research field [2,3]. These findings have shed new light on molecular pathways of metastasis, which also provided valuable information about potential targets for the treatment of metastatic disease. This review discusses our current understanding of molecular mechanism of metastatic process and summarizes recent information of drug development specifically targeted to the metastatic pathways.

2. Tumor metastasis involves multi-step process with high complexity

A primary tumor generally consists of heterogeneous cell types including a small number of cancer stem cells that are able to perpetually proliferate without responding to tumor suppressor function. The current theory predicts that these cancer stem cells originate from a normal stem cell or a cancer cell, which acquired a stem cell-like ability [4]. When a tumor grows more than $1mm^3$ in size at the primary site, it acquires active supply of oxygen and nutrients by promoting angiogenesis. Tumor cells accomplish this task by generating hypoxic environment followed by secretion of angiogenic growth factors (Fig 1). Tumor cells that gain growth advantage further proliferate and acquire metastatic phenotypes due to additional mutations. The first step in metastasis is the detachment of these tumor cells from the primary tumor mass by acquiring an invasive phenotype that results in the loss of cell-cell adhesion and cell-extracellular matrix adhesion followed by proteolytic degradation of the matrix (Fig 1) [5]. It is believed that autocrine motility factor(AMF) and hepatocyte growth factor (HGF) are critical components of motility and that degradative enzymes including serine-, thiol-proteinases, heparanases and metalloproteinases such as MMP2 and 9 play critical roles in the invasion [6–8]. When tumor cells intravasate surrounding tumor vasculature and neighboring lymphatic vessels, they must survive in this hostile environment that includes mechanical damage, lack of growth factor from the original environment and the host immune system (Fig 1) [9]. Tumor cells in the circulation often aggregate with platelets and fibrin, and they embolize in the capillaries or directly adhere to the endothelial cells by a mechanism similar to leukocyte adhesion at the inflammatory site [10–12]. In some cases, arrested tumor cells extravasate before proliferating themselves using the same hydrolytic enzymes that are used in the initial step of invasion (Fig 1) [13]. However, in many cases, cancer cells actually proliferate within the lumen of vessels to create a considerable tumor mass that can eventually obliterate the adjacent vessel wall by pushing aside the barrier composed of endothelial cells, pericytes and smooth muscle cells that previously separated the vessel lumen from the surrounding tissue [14,15]. After extravasation, cancer cells lodge at the secondary sites, where the cells must also proliferate and colonize for successful metastasis (Fig 1). These processes are controlled by various metastasis promoters and suppressors, and they must be well coordinated to establish successful distant metastasis (Table 1) [2]. Recent advancement of research in this field has revealed the complex interplay of metastatic factors and many novel concepts of signal pathways leading to metastasis (Fig. 2 a,b). Based on this information, the current research is gradually moving toward translational stage by aiming at development of targeted anti-metastatic drugs (Table 1). The following sections summarize up-to-date information of the promoters and suppressors of metastasis that are currently under active investigation for drug development.

3. Metastasis promoters

3.1. AMF

Autocrine motility factor (AMF) was originally isolated as a C-*X*-*X*-C cytokine that stimulates random or directed motility of AMF-producing tumor cells in an autocrine manner [16]. Elevated serum AMF was found in patients with malignant tumors such as colorectal, lung, kidney, breast and gastrointestinal carcinomas and is well correlated with the development of metastasis [16–19]. AMF is a multifunctional molecule, also known as phosphoglucose

isomerase, neuroleukin, and maturation factor [20]. AMF causes tumor cell detachment from the primary site by promoting cell motility in an autocrine fashion. However, recent research revealed that AMF also contributes to malignant progression by stimulating the migration and proliferation of endothelial cells via its receptor AMFR, a unique seven transmembrane receptor (gp78), followed by activation of small Rho-like GTPase [16,21]. Therefore, tumor cells appear to induce aggressive angiogenesis by promoting crosstalk of signals between VEGF-VEGFR and AMF-AMFR which also promotes cell survival via activation of Akt and MAPK-dependent anti-apoptotic pathways (Fig. 2) [22]. A recent report by Raz et al. demonstrated a more direct role of AMF in tumor progression and metastasis. They have shown that overexpression of AMF in normal fibroblasts lead to a gain of tumorigenicity, whereas down-regulation of AMF by siRNA in mesenchymal tumor cells resulted in mesenchymal-to-epithelial transition (MET), the reverse process of epithelial-to-mesenchymal transition, as reflected by a loss of cell polarity, reduced proliferation and invasion *in vitro* and loss of tumorigenic properties *in vivo* [23]. Interestingly, they later also showed that silencing AMF expression in human fibrosarcoma cells resulted in an increased sensitivity to oxidative stress-induced and p21-mediated cellular senescence, which brought a novel insight into the function of AMF in tumor progression [24]. Collectively, neutralizing AMF, disruption of AMFR and blocking their signal pathways are considered to be rational approaches for anti-metastatic drug development.

It has been shown that specific carbohydrate phosphate inhibitors including E4P, D-mannose-6-phosphate and 5-phospho-D-arabinonate (5PAA) are able to block both AMF enzymatic activity and AMF-induced cell motility [25,26]. Treatment of tumor cells with these inhibitors has been shown to decrease the growth, DNA synthesis, migration and invasiveness of several types of cancer cells [22,23,27]. Since these carbohydrate phosphate inhibitors are among the smallest compounds that have AMF inhibitory activity, information of the known crystal structure may help in designing a lead compound to develop more effective AMF inhibitors.

Because AMF is a secretory factor, antibody against AMF may also be a rational approach. In fact, Talukder *et al.* showed that neutralizing antibodies against AMF were able to partially block HRG-induced invasiveness of human breast cancer MCF-7 cells [28]. Raz et al. also demonstrated that a monoclonal anti-AMF antibody induced apoptosis in human fibrosarcoma cell lines *in vitro* and effectively promoted drug-induced apoptosis *in vivo* [22]. Therefore, humanized anti-AMF holds promise for future therapeutic application. Interestingly, antibody against EGFR2 (Herceptin) was also shown by Talukder et al. to block AMF expression and its promoter activity [27]. Because Herceptin has been used as an effective drug for breast cancer, it is interesting to know whether this antibody also blocks the invasiveness of the tumor.

Ectopic expression of AMF makes some tumor cells become resistant to apoptosis induced by serum deprivation, and this resistance appears to be mediated via PI3K and PKC/MAPK pathways (Fig. 2A). Yanagawa et al. recently indeed showed that PI3K inhibitors (Ly294002 and Wortmanin), PKC inhibitor (GF109203) and MAPK inhibitor (PD98059) were able to recover the expression of Apaf-1 (Apoptotic protease activating factor 1) in the AMF-transfected HT1080 cells followed by induction of apoptosis [22]. In addition, GF109203X and Wortmanin were shown to inhibit AMF-induced expression of fms-like tyrosine kinase (Flt-1) and hence impair the proliferative signals of VEGF in endothelial cells. Therefore, AMF may be a good target for anti-angiogenic therapy, although potential side effects of such drugs are unknown. Finally, it is recently found that the stability of AMF protein is regulated through ubiquitin-lysosome system, which is mediated by poly (ADP-ribose) polymerase-14 (PARP-14). This new discovery may offer a novel target to block the AMF/AMFR signaling and deserves further investigation [29].

3.2. HGF/SF

Hepatocyte growth factor (HGF), also known as scatter factor (SF), was identified as the natural ligand for the c-Met receptor tyrosine kinase [30]. HGF/SF interacts with c-Met receptor and transduces multiple biological signalings that control proliferation, disruption of intercellular junctions of EMC, migration and protection from apoptosis [31,32]. HGF/SF signaling has also been demonstrated to play an important role in a wide variety of human cancers of both epithelial and mesenchymal origins [31]. The results of several clinical studies indicate the prognostic value of HGF/SF and c-Met in various types of cancer and that the expression of HGF and/or c-Met is frequently associated with the aggressive nature of the tumors and the poor clinical outcome [31,33]. The exact mechanism of up-regulation of these genes in cancer is not well understood. However, a recent study suggested that the up-regulation of c-Met and HGF may be due to the stress of tumor microenvironment such as hypoxia [34]. Therefore, HGF/SF is considered to be widely involved in the tumor metastatic process. HGF is a potential promoter of cell invasion by directly stimulating the motility and migration of cancer cells as well as affecting the microenvironment [32]. HGF can disrupt cell-cell adhesion and promote cancer cell growth, partly by inducing phosphorylation of β-Catenin and relocation of E-cadherin, which may result in down-regulation of cell cycle regulatory factors such as p27 (Fig. 2A) [35–37]. On the other hand, HGF can increase the adhesion between cancer cells and matrix by activating the FAK and paxillin pathways, which cooperatively regulate the expression of integrins in cancer cells and eventually lead to adhesion as well as migration of cancer cells to matrix [38]. HGF is also able to increase the expression and secretion of proteolytic enzymes from cancer cells including MMP2, MMP7, MMP9 and uPA that are involved in matrix and basement membrane degradation (Fig. 2) [36,39,40]. In addition, HGF is considered as an angiogenesis-promoting factor through its direct morphogenic and adhesive effects and indirect regulation of other angiogenic factors such as IL-8, VEGF and TSP-1 [41,42]. Furthermore, Boccaccio et al. have recently demonstrated that the c-Met oncogene was responsible for the induction of thrombohemorrhagic syndrome, suggesting that c-Met may give survival advantage to tumor cells in the circulation by promoting the aggregation of tumor cells with platelets [43,44]. Therefore, the HGF/c-Met signaling plays a critical role in the metastatic process and this gene as well as the down-stream signal can be potential targets for cancer therapy.

Recently, rapid progress has been made toward drug development against HGF/SF for the purpose of cancer therapy. These include HGF antagonists, anti-HGF and anti-cMet antibodies, small molecules targeting c-Met and its signaling pathways as well as compounds interfering with HGF-elicited biological activities [45]. Antagonizing ligand binding that block the activation of downstream signaling is a conventional therapeutic strategy for most carcinomas. NK4 is one of the antagonists that compete with HGF for the c-Met receptor, and it has been known to block HGF-induced cellular adhesion, invasion and metastasis in various types of cancer cells including breast, bladder, colorectal, lung, prostate, glioma, pancreatic and gastric cancers *in vitro* [46]. Moreover, NK4 also acts as angiogenesis inhibitor, and this activity is independent of its action as HGF-antagonist [47,48]. As expected, treatment of mice via intraperitoneal or intratumoral administration of NK4 protein or recombinant adenoviruses expression vector effectively blocked tumorigenesis, angiogenesis and metastasis in various mouse xenograft models including pancreatic and gastric cancers [46,49]. Another antagonist is an uncleavable HGF, which was engineered with a single amino-acid substitution at the proteolytic site of HGF [50]. The uncleavable HGF competes with endogenous pro-HGF for the catalytic domain and thus inhibits endogenous pro-HGF maturation. The peptide also binds to the c-Met receptor with high affinity and displaces the mature ligand. More strikingly, both local and systemic administration of uncleavable HGF in a xenograft mouse model significantly suppressed tumor growth and tumor angiogenesis, and notably inhibited the formation of spontaneous metastases without affecting vital physiological functions [50]. In a

separate study, neutralizing anti-HGF antibodies were first developed by Cao et al. who demonstrated that a minimum of three antibodies, each of which act on different HGF epitopes, were required to block c-Met tyrosine kinase activation and the biological outcomes [51]. Moreover, Burgess et al. have shown that fully humanized monoclonal anti-HGF antibodies effectively suppressed HGF-dependent tumor growth in tumor xenograft mouse model [52]. Another fully human HGF antibody, AMG102, was recently tested for its pharmacokinetics and safety in monkeys and further clinical investigation was warranted [53].

It is recently suggested that MET functions in certain human cancers as “oncogene addiction”, the concept formulated in the late 1990s, indicating a constant requirement of MET in these tumors [54]. Therefore, targeting the activated c-Met holds a great promise as an anti-cancer therapy at least for certain tumor types. Regarding c-Met tyrosine kinase receptor inhibitors, a set of low molecular weight compounds including PHA-665752, SU11274, and K252a, which are able to compete for the ATP binding and prevent receptor transactivation and recruitment of the downstream effectors, have recently been tested and shown to effectively inhibit the kinase activity and block the subsequent signaling pathways [55–58]. Particularly, PHA-665742 is capable of inhibiting the autophosphorylation of c-Met with a relatively high specificity compared to other tyrosine and serine-threonine kinases [55,59]. In addition, PHA-665752 was shown to induce massive apoptosis in human gastric cancer cell lines that had amplified MET genes, while it did not affect other cell lines without c-Met receptor amplification [59]. Furthermore, Salgia et al. has recently shown that PHA-665752 treatment inhibited tumorigenicity and angiogenesis in a mouse model of lung cancer xenografts [60]. These results strongly support a potential utility of these compounds for a therapeutic application in the future. Designing a drug that binds the extracellular domain of the c-Met receptor and thus impairing receptor dimerization has been considered as another c-Met blocking strategy. Recently, Petrelli et al. showed that a monoclonal antibody, DN30, prevented c-Met activation and abrogated its biological activity [61]. In addition, soluble recombinant Sema proteins or anti-Sema antibodies against the extracellular Sema domain that is involved in ligand binding and receptor dimerization of c-Met have been generated [62]. As expected, they suppressed the downstream signaling triggered by the c-Met receptor even in the presence of HGF. Another alternative strategy for specifically blocking the receptor is a gene silencing technology. Using adenovirus vectors carrying small-interfering RNA targeting c-MET, Shinomiya et al. demonstrated that the siRNA drastically reduced the c-MET gene expression followed by significant inhibition of proliferation and invasion of various tumor cells lines both *in vitro* and *in vivo* [63]. Collectively, recent information about the mechanistic insight of HGF/c-Met signaling in tumor progression has greatly facilitated the development of a variety of strategies for anti-HGF/cMet therapies, and some of these compounds hold great promises for future clinical application.

3.3. TGFβ

Transforming growth factor-β (TGFβ) is a secreted polypeptide cytokine that plays multiple roles in cell proliferation, differentiation, extracellular matrix production, migration and apoptosis [64,65,66]. Notably, in normal epithelial cells and at an early stage of tumorigenesis, TGFβ inhibits the proliferation of cells by inducing cell cycle arrest, promoting apoptosis, and enhancing genomic stability [65,66]. However, as the tumor develops, cancer cells become resistant to TGF-β-mediated growth inhibition because of the loss of TGFβ signaling, mutations of cell cycle regulators, or alteration of cross-talk signaling pathways such as activation of Ras [67].

TGFβ1 has been shown to be over-expressed in 74% and 60% of patients with breast and colon cancers, respectively. Interestingly, more intense staining patterns for TGFβ1 are observed in various types of metastatic cancer including breast, colon, liver, lung, prostate and stomach

compared to primary tumors, emphasizing the importance of TGFβ signaling for pro-metastatic activity [68]. Transplanting cell lines stably over-expressing TGFβ1 into athymic mice has been shown to cause increased tumor growth and metastases *in vivo* [69,70]. In another study, transgenic mice that co-express MMTV-Neu and MMTV-TGFβ1 developed mammary tumors with the same latency as the control MMTV-Neu transgenic mice; however the co-transgenics showed significantly more local invasion and elevated numbers of circulating tumor cells and lung metastases [71]. Thus, over-expression of TGFβ can enhance and stimulate tumor growth and malignant progression at least in particular subtypes of tumors. Therefore, TGFβ has been recognized as a tumor promoter at an advanced stage of some tumors, probably by stimulating tumor cell invasion, angiogenesis and immunological surveillance [65,66].

It has been shown that mouse and human carcinomas often over-express TGFβ, which promotes Epithelial-mesenchyma Transition (EMT) via the Smad pathway [66]. Furthermore, Shen et al. have shown that TGFβ was capable of inducing the expression of guanine exchange factor NET1 via Smad3 followed by activation of the Rho GTPase pathway, which results in local disassembly of the actin cytoskeleton and tight junction breakdown [72]. On the other hand, TGFβ can also activate various non-Smad signaling effectors including Ras, Rho GTPase, Erk1/2, PI3K and NF-κB that all play critical roles in EMT, which eventually promotes tumor metastasis [67,73,74]. It has been shown that the motility of metastatic breast carcinoma cells responding to autocrine TGFβ1 did not require Smad activation but rather the activity of the PI3K pathway [74]. In addition, Vogelmann et al. have shown that in polarized epithelial cells, TGFβ blocked cell-cell adhesion by inducing tyrosine phosphorylation of α- and β-Catenin which disrupts the E-cadherin/catenin complexes with actin, and by inducing the expression of transcriptional repressors of the E-cadherin gene such as Snail, Slug and LEF1 [75,76]. Wikstrom et al. showed that the ectopic expression of TGFβ in human prostate cancer correlated with increased angiogenesis around the tumor and eventually lead to a high rate of metastasis of prostate carcinoma cells [77]. The ability of TGFβ to promote angiogenesis is considered to be the action of either inducing expression of VEGF, which directly stimulates the proliferation and migration of endothelial cells, or its chemoattractant activity for monocytes that release angiogenic cytokines [78]. It should be also noted that, in breast cancer, TGFβ stimulates the expression of pTHrP (parathyroid hormone related protein) which promotes osteolytic metastasis and also suppresses late stages of osteoblast differentiation, which leads to net bone loss [79]. Furthermore, TGFβ plays a role in helping tumor cells to escape from the immunological surveillance through its ability to inhibit B and T lymphocyte proliferation and differentiation [80]. TGFβ is also able to deactivate macrophages and thus protect the tumor cells from the immune surveillance [81]. Collectively, because TGFβ often promotes tumor progression in particular subtypes, the components of the TGFβ signaling pathway are being considered as prognostic biomarkers for such tumors as well as potential therapeutic targets [68].

On the contrary, to the tumor promoting activity of TGFβ, this molecule also has tumor suppressive function at an early stage in some types of cancer. Therefore, TGFβ is considered as a target for chemoprevention for the population with high-risk cancer incidence. To this end, several compounds have been examined and these include FTI-277, Dietary ω-3 fatty acids, Captopril, Suberoylanilide hydroxamic acid (SAHA) and triterpenoids. They are capable of enhancing the expression of TGF receptor (TβRII and TβRI) at mRNA and protein levels, thus increasing the responsiveness of tumor cells to TGFβ with respect to growth arrest and cytostatic effect [82–86]. However, considering the pro-tumorigenic actions of TGFβ, such drugs may have dreadful effects by promoting tumor invasiveness and metastasis. Therefore, current effort is more focused on drugs that block the tumor progression at a later stage. These strategies include developing small molecule inhibitors, affinity- or antibody-based drugs and anti-sense RNA.

Intense high-throughput screenings have led to the development of selective small molecule inhibitors against the enzymatic activity of the TβRII and TβRI kinases. These inhibitors including SD-208, SD-093, SB-431542, A-83-01 and LY2109761 act as ATP-binding analogues and thus competitively block the catalytic pocket of the receptor kinase [68]. SD-208, an orally active specific TβRI kinase inhibitor, was previously tested in a glioma model, which depends primarily on the pro-tumorigenic action of TGFβ. In this study, SD-208 was found to effectively inhibit the TGFβ-induced glioma cell migration and invasiveness and also to enhance the immunological surveillance [87]. Recently, Reiss et al. also showed that SD-208 treatment resulted in decreased angiogenesis in a mouse model of mammary carcinoma [88]. In addition, Wong et al. showed that SD-208 reduced primary tumor growth and decreased the incidence of metastasis in an orthotopic xenograft mouse model of pancreatic adenocarcinoma [89]. Thus, this inhibitor holds a great promise for future clinical application. Another small molecule for TβRI kinase inhibitor, SD-093, has been shown to strongly decrease the *in vitro* motility and invasiveness of pancreatic carcinoma cells without affecting their growth [90]. Another set of TβRI inhibitors, SB-431542, A-83-01 and LY2109761, all potently affect TGFβ-dependent transcriptional activation and inhibit TGFβ-induced EMT [73]. Interestingly, SB-431542 was demonstrated to reduce colony formation of human lung adenocarcinoma cells, which are growth-dependent on TGFβ; however, it also induced anchorage independent growth of human colon adenocarcinoma cells whose proliferation is promoted by TGFβ [91]. Furthermore, SB-431542 showed no effect on a cell line that failed to respond to TGFβ, which further strengthens the rationale in using this compound as a therapeutic agent of human cancer responsive to tumor-promoting effects of TGFβ. A-83-01 is structurally similar to SB-431542 while it has shown even more potent effect of suppressing TβRI [73]. LY2109761 is a specific pharmacologic inhibitor of TβRI and TβRII kinases. It was demonstrated that this drug was capable of inducing the expression of the Coxsackie and adenovirus receptor (CAR), a tight junction component whose expression is required to be downregulated for EMT [92]. Currently, some of the above-mentioned specific inhibitors of TβRI have already entered the phase I clinical trials for various human cancers (Table I).

Neutralizing anti-TGFβ antibodies and the soluble extracellular domain of TβRII with receptor-binding activity have also been pursued as anti-TGFβ approaches. Interestingly, the results of pre-clinical studies have shown that these drugs had a weak and transiently negative effect on primary tumor growth but strongly suppressed metastasis [73]. Pietenpol et al. have demonstrated that the neutralizing antibody 2G7 which has high affinity to three mammalian isoforms of TGFβ showed moderate inhibitory effect on the growth of the primary tumor in an animal model of MDA-MB-231 xenograft, while it almost completely blocked the abdominal and lung metastasis [69]. In addition, enforced expression of the extracellular domain of TβRII has been demonstrated to enhance tumor immune surveillance and strongly inhibit metastasis in animal models of human pancreatic carcinoma [93]. These observations led to a development of a fusion protein of immunoglobulin Fc fragment with the soluble extracellular domain of TβRII (Fc: TβRII) as a therapeutic approach [94]. When tested in vitro, this fusion protein indeed effectively induced apoptosis and inhibited migration of breast cancer cells. Furthermore, Wakefield et al. found that when Fc:TβRII was expressed in the mammary gland of MMTV-based transgenic mouse model followed by a challenge of melanoma cells or by crossing it to the MMTV-Neu mouse, it completely blocked lung metastasis without any adverse side effect [95]. The clinical potential of this experiment is significant especially because the chronic presence of Fc:TβRII did not show obvious adverse effects. Similarly, Sun et al. have shown that over-expression of soluble extracellular domain of β-glycan (sRIII) antagonized TGFβ in the breast carcinoma cells, which resulted in significant inhibition of metastasis of the tumor cells to the lung, while it moderately blocked the tumorigenic ability [96].

Finally, the anti-sense DNA or RNAi technology have recently brought a promising development in anti-TGFβ therapy. The oligonucleotide AP12009, which is directed against human TGFβ2, has been tested by administering into brain tumors with continuous infusion and showed better survival time after recurrence than other current chemotherapy against gliomas [97]. Also, RNAi for both TGFβ1 and TGFβ2 in human glioblastoma has been reported to be effective in restoring the proper immune response, which significantly decreased the glioma cell motility and invasiveness [98]. Further investigations in this research field are expected to provide valuable information to improve the efficacy of these compounds and to develop a better delivery system for eventual clinical use of anti-TGFβ therapy.

3.4. MMP

Matrix metalloproteinases (MMPs), a group of zinc-dependent endopeptidases, was originally identified to have roles in ECM disruption and thus associated with invasion and metastasis in late stages of cancer progression (Fig. 2A). Years of intense investigations of MMPs have highlighted the significance of these molecules in cancer. MMPs contribute to the formation of a complex microenvironment that promotes malignant transformation in early stages of cancer, suppresses tumor cell apoptosis, and enhances angiogenesis as well as impairs the host immunological surveillance [99]. Several studies have indicated that cleavage of particular substrates such as insulin-like growth factor binding proteins (IGFBPs) and TGFβ by MMPs can have direct effects on tumor growth [100,101]. In transgenic animals, over-expression of certain MMPs such as MMP1 and MMP3 was sufficient to generate fully malignant tumors in the absence of specific carcinogens [102,103]. In the normal cells or at an early stage of tumor, MMPs can target substrates that influence the apoptotic process of the cells, which is also linked to the chemotherapeutic resistance. Particularly, MMP7 is able to release a soluble form of the death protein Fas Ligand (FasL), which has lower death-promoting potency than the membrane anchored form but has more flexibility to interact with its cognate receptor Fas [104,105]. Thus, the weak but constant apoptotic signal acts as a selective pressure for tumor cells that have elevated anti-apoptotic signals and those that have propensity to acquire additional mutations, which further promote tumor progression. This mechanism is also considered to be the basis of induction of chemoresistance to certain types of tumors [106].

MMPs also play critical roles in angiogenesis. Angiogenic factors such as basic fibroblast growth factor (bFGF) and VEGF are usually localized in the matrix and cannot interact with their receptors until freed by MMPs, particularly by MMP9 through ECM proteolysis [107, 108]. In addition, MMP9, when recruited to the tumor cell surface and interact with the docking receptor CD44, can proteolytically cleave latent TGFβ and thus promote tumor invasion and angiogenesis [100]. Furthermore, an elegant work of Hanahan and Coussens has shown that MMP9 is predominantly expressed in the tumor-associated stromal cells as well as in macrophages, neutrophils, mast cells and endothelial cells rather than in tumor cells themselves in many cases, which regulates the vascular formation and architecture [109–111]. Intriguingly, Hiratsuka and colleagues have recently shown that MMP9 plays a role in priming premetastatic sites for primary tumor. They demonstrated that tumor associated macrophages (TAM) induced MMP9 in endothelial cells and in TAMs, which facilitated tumor cell invasion and also prepared the lung as premetastatic niche for the growth of tumor cells in a manner dependent on VEGFR-1 [112].

Escaping from host immune response is a significant problem associated with many cancers. Some MMPs alter the behavior of chemokines and cytokines by specific proteolytic cleavage. For example, MMP9 can suppress the development and propagation of T lymphocytes by disrupting IL-2Rα signaling, resulting in attenuation of a T cell-mediated anti-tumor response [113]. Likewise, CXCL12, also known as SDF1 has been identified as a substrate of MMP2.

MMP2 – mediated cleavage renders CXCL12 unable to bind its receptor CXCR4, which consequently influence the metastatic dissemination of tumor cells [114].

The strong correlations between altered expression of MMPs at mRNA and protein levels in different human cancers with poor disease prognosis have been well established [99,115]. The over-expression of many MMPs, including MMP-1,-2,-7,-9,-13,-14, is positively associated with tumor progression and metastasis [115]. On the other hand, human breast tumor cells with reduced expression of MMP-8 were found to acquire the metastatic ability compared to their non-metastatic counterpart [116]. Interestingly, Balbín et al. has revealed that MMP8-null mice exhibit an increased tumor susceptibility compared to the wild type because of the attenuation of adaptive immune responses due to the loss of MMP8 [117]. Similarly, MMP-3 knockout mice exhibited increased rate of initial skin tumor growth [118]. However, altered expression pattern or levels of individual MMPs in tumor or stromal cells do not always correlate in the primary tumors and secondary metastatic sites [115]. Interestingly, over-expression of MMPs is frequently accompanied with a corresponding increased expression of natural inhibitors (TIMPs) of MMPs, which result in reduced tumorigenesis in some model systems but does not necessarily inhibit metastasis [119,120]. These discrepancies point out the complexity of MMP functions *in vivo*.

The link between MMPs activity and malignant progression has stimulated serious effort in developing pharmacological inhibitors of MMPs (known as MMPIs) as a potential therapeutic modality since the 1980s [121]. A variety of MMP inhibitors including Marimastat (BB-2516), Prinomastat (AG3340), Tanomastat (BAY12-9566) and BMS-275291 Neovastat were found to be orally active and achieved effective blood levels and displayed high specificity to MMPs while sparing most other types of proteases [122]. These MMPIs have been shown to be effective in controlling cancer progression in animals. However, most clinical trials have come to a crashing halt with the repeated failure in multiple large-scale phase III stage [122]. Even worse, some compounds caused severe side effects such as inflammation, musculoskeletal pain and joint stricture [122]. Considering the ability of MMPs to cleave not only ECM but also a variety of other factors, cytokine precursors and chemokines, it may not be surprising to see unwanted chaotic immune responses. Therefore, this area of research requires newer strategies.

A recent work of Taketo and colleagues has provided valuable insights regarding a possibility of targeting the MMP-producing cell instead of inhibiting MMPs themselves [123]. They found that immature myeloid cells expressing CC chemokine receptor (CCR1), MMP2 and MMP9 infiltrated the tumor invasion front and migrated toward the CCR1 ligand CCL9, whereas blocking CCR1 expression resulted in the accumulation of MMP-expressing cells at the invasion front and suppressed tumor invasion in an animal model. Although an application of this “cellular target” concept is still premature and is waiting to be confirmed by multiple studies, it is expected to cause fewer side effects than the systemic “molecular target” therapy using MMP inhibitors. One important lesson we learned from the past clinical trials of MMPs inhibitors is the need for attention to the stage and type of cancer and the critical selectivity of MMPs inhibitors since the expression pattern of MMPs varies in various cancer types and stages [122]. For example, small cell lung cancer is known to over-express MMP11 and MMP14 rather than MMP2, thus the MMP2 specific inhibitors like Tanomastat and Prinomastat would lead to a poor outcome [124]. One possible strategy is to take advantage of both the frequent over-expression of MMPs in malignant tumors and the catalytic functions of these enzymes, and this strategy led to the development of protease-activatable retroviral vectors, which contain engineered MMP-cleavable linkers [125,126]. Another approach is to employ macromolecular carriers that are linked to anticancer drugs or immune response-stimulating drugs that can be released from its carrier when encountered with MMPs in the tumor environment [127,128]. Alternatively, designing an inhibitor which targets substrate-specific binding sites of MMPs resulting in reduced binding and cleavage of specific substrates

of the corresponding MMP opened a possibility of blocking the unwanted catalytic activity of MMPs during tumor progression [99]. Finally, re-screening for MMPs inhibitors from the current anti-cancer drug pool may be worth a consideration. Notably, Bisphosphonates (BP), a class of pyrophosphate analogues widely used in the treatment of breast cancer patients with osteolytic tumors for the past 20 years, was found to significantly inhibit proteolytic activity of MMPs without reducing the expression of MMPs [129]. Although past efforts in developing anti-MMP drugs have been less fruitful than expected, there are still strong rationales and hopes to continue this line of research using more innovative approaches.

3.5. uPA

The urinary-type plasminogen activator (uPA) is a serine protease and able to proteolytically degrade various ECM components and the basement membrane around the primary tumors. It also activates multiple growth factors and MMPs that further contribute to the degradation of the ECM, and thus facilitates tumor cell invasion and intravasation (Fig. 2) [130,131]. Interestingly, a newly identified metastasis suppressor, p75 neurotrophin receptor ($p75^{NTR}$), has recently been demonstrated to suppress metastasis in part by down-regulating specific proteases such as uPA [132]. uPA is produced and secreted as a zymogen (pro-uPA) which binds to the cell surface uPA receptor, uPAR. The pro-uPA is then cleaved by plasmin to become an active form of uPA, which has plasminogen-activating property to convert plasminogen to the active matrix-degrading serine protease plasmin [131]. The proteolytic activity of uPA is regulated by the serine protease inhibitors, plasminogen activator inhibitor-1 (PAI-1) and PAI-2. PAI-1 is able to react with uPA/uPAR-complex and induces internalization of the complex, which results in the intracellular degradation of uPA and PAI-1. On the other hand, PAI-2 forms a complex with uPA and uPAR without internalization, and it is degraded once bound to uPA/uPAR [133]. Because the activity of uPA is dependent on its binding to uPAR, this receptor is also considered to play a crucial role in metastasis [130]. Besides the role in proteolysis, uPAR can interact with and regulate other cell surface proteins such as integrins, growth factor receptors and G-protein coupled receptors to exert its biological functions including chemotaxis, cell migration and invasion, adhesion, proliferation and angiogenesis [134].

Several recent studies have shown that uPAR is also involved in activation of the signaling of other metastasis-promoting factors such as basic fibroblast growth factor (bFGF), VEGF, TGFβ and HGF (Fig. 2) [130,135,136]. Most normal tissues have little or no detectable uPAR, while uPAR is over-expressed across a variety of carcinomas including colon, breast, ovary, lung, kidney, liver, stomach, bladder, endometrium and bone [131,137,138]. uPAR expression has also been shown to be strongly correlated with advanced metastatic cancer, and it is typically found to be abundant at the invasive boundary between tumor cells and normal tissue [139,140]. This localization of uPAR expression in the invasion front may be due to the fact that uPAR is a hypoxia-inducible gene [141,142]. Importantly, the uPAR expression has been found to correlate with a poor prognosis and mortality of patients with various types of solid tumors [141–143]. Currently, the PAI-1 is considered as one of the most informative prognostic markers in several cancer types and a high PAI-1 level is significantly associated with a poor prognosis in these cancers [144–147]. The precise role of PAI-1 in tumor growth and metastasis is yet to be elucidated, but PAI-1 shows diverse functions depending on the cell context and the expression level [148]. Interestingly, several reports indicated that unlike PAI-1, PAI-2 functions as a tumor suppressor and blocks metastasis, and therefore, is associated with a favorable outcome in patients [143,149]. In addition, uPA and PAI-1 have also been reported to be associated with resistance to hormone therapy in advanced breast cancer [150]. Therefore, uPA/PAI-1 can also be used to predict resistance to specific therapies for breast cancer patients. These studies of uPA/uPAR and PAI-1 so far indicate the critical roles of these molecules in

tumor progression, suggesting that these proteins serve as excellent therapeutic targets for cancer patients.

In the past, various approaches have been developed to inhibit uPA and its signals. WX-UK1 and WX-671, synthetic serine protease inhibitors developed by WILEX, are the first inhibitors of uPA in world wide clinical trials. Both of them have shown to effectively block metastasis formation and to reduce primary tumor growth in preclinical studies, and they have already entered the phase-I/II clinical trials as a single agent and/or in combination with other chemotherapeutics for the treatment of patients with metastatic tumors [148]. Bikunin, a Kunitz-type protease inhibitor, is discovered as a potent and selective inhibitor for trypsin and plasmin, while it is moderately effective in inhibiting the catalytic activity of uPA [151]. Kobayashi et al. have also shown that Bikunin was able to down-regulate the expression of uPA and uPAR [152]. Furthermore, Bikunin has been shown to inhibit MAPK and PI3K/Akt signaling, and to effectively inhibit growth and invasiveness of several types of tumor cells [153–155]. Recently, the possibility of using Bikunin as oral therapy was examined in an ovarian cancer model in animal. Results of these experiments have shown that once-daily oral administration of Bikunin had no significant side effects and strongly suppressed the expression of uPA and uPAR, suggesting a utility of Bikunin for an anti-metastatic therapy in humans [156].

DX-1000, another Kunitz domain-based inhibitor of plasmin with specificity, has been previously shown to block tumor growth and metastases *in vivo* with few side effects [157] However, DX-1000 has a quick clearance and short half-life in circulation that challenges the practical utility of this compound in patients. To circumvent these problems, Henderikx et al. conjugated the DX-1000 with polyethyleneglycol (PEG) to prolong *in vivo* half-life. The PEG-conjugated DX-1000 was indeed shown to be effective *in vitro* and significantly blocked tumor proliferation, vascularization and metastasis *in vivo* [158]. More recently, Fishe et al. have shown that 1- Isoquinolinylguanidines (UK-356,202) and its derivatives were able to reversibly inhibit uPA enzymatic activity with selectivity over tPA and plasmin, and it has been selected as a candidate for clinical evaluation [159]. There are also several other strategies currently under active investigation and these include receptor ligand analogues to interfere with the cellular uPA/uPAR interaction, antibodies for PAI-1 and recombinant PAI-2 (231Bi-PAI2) [160–162].

3.6.β-Catenin

β-Catenin is an essential component of the cadherin-catenin complex and plays a critical role in the Wnt signaling pathway [163]. The product of the tumor suppressor gene APC (adenomatous polyposis coli) forms a complex with axin/axil, protein phosphatase 2A (PP2A) and glycogen synthase kinase3β (GSK3β) which leads to phosphorylation of β-Catenin thereby inducing degradation of this protein by ubiquitination-mediated proteasomes [164]. The abnormally activated Wnt signaling due to the mutations of APC results in accumulation of β-Catenin followed by promotion of tumorigenesis. Phosphorylation of β-Catenin also releases E-cadherin, which initiates tumor cell migration and tumor metastasis [165,166]. On the other hand, β-Catenin together with other proteins such as TCF/LEF complex, Reptin and p50, acts as a transcription factor to regulate metastasis-related gene including MMP-9 and KAI1 [167]. More recently, it has been reported that accumulated β-Catenin binds specifically to androgen receptor (AR) and augments the ligand-independent activity of AR in hormone-refractory prostate cancer [168]. Indeed, aberrant expression of β-Catenin has been reported in many types of cancer including colon, bladder, breast, prostate, lung cancer and adrenocortical adenomas [169]. Furthermore, the Wnt/β-Catenin signaling pathway has been shown to be involved in the self-renewal of embryonic stem cells and perhaps in progression of tumor stem cells [170]. Several agents targeting the Wnt/β-Catenin pathway including

Exisulind and Imatinib have been shown to inhibit self-renewal of cancer stem cells with varying levels of success [171]. Therefore, targeting β-Catenin and blocking APC/β-Catenin/TCF signals is considered to be a rational approach for developing new anti-cancer drugs.

Exisulind (Aptosyn) and two analogs CP461, CP248 belong to a new class of compounds of SAANDs (Selective Apoptotic Antineoplastic Drugs), which are oxidative metabolites of the non-steroidal anti-inflammatory drug (NSAID) sulindac. These drugs reduce β-Catenin activity and block Cyclin D1 followed by an induction of apoptosis and inhibition of tumor cell growth [164,172,173]. Currently, Exisulind is in Phase III clinical trials in combination with several chemotherapeutic agents [174,175]. Imatinib (Gleevec), originally identified as an inhibitor of platelet-derived growth factor (PDGF) receptor, has been used in treating chronic myelogenous leukemia (CML), gastrointestinal stromal tumors (GISTs) and a number of other malignancies. Interestingly, Imatinib has been shown to inhibit tyrosine-phosphorylation of β-Catenin, which otherwise releases E-cadherin and promotes cell migration and tumor metastasis [176]. Other strategies including RNAi, antisenseDNA and small molecule inhibitors for blocking β-Catenin have been developed [171,177]. The antisense approach has been used in colon and esophageal cancers as well as leukemia and lymphoma *in vitro*, which lead to reduction of β-Catenin expression and subsequent decrease in the expression of its downstream targets such as Cyclin D1 [177–179].

NSAIDS are also found to be effective in inhibiting the Wnt/β-Catenin signaling pathway. Among them, aspirin and indomethacin were shown to block the transcriptional activity of β-Catenin/TCF [180]. Celecoxib (a COX-2-inhibitor) blocked β-Catenin activity by inducing its degradation via GSK3 β and APC, leading to diminished tumor cell proliferation and survival [181]. R-Etodolac (an enantiomer of Etodolac) and its analog (SDX-308) have been shown to be able to decrease total and activated forms of β-Catenin via GSK3 β activation [182]. These drugs also increased β-Catenin and E-cadherin complex at the membrane site and inhibited β-Catenin-dependent TCF activity followed by decreasing the level of downstream target gene products, Cyclin-D1 and glutamine synthetase [183,184]. In addition to these efforts of directly blocking the β-Catenin activity, selective disruption of β-Catenin-TCF complex and reversing the localization of β-Catenin from cytoplasmic membrane to the nucleus are also considered to be effective approaches for anti-cancer therapy. Thiazolidinedione (TZD), a peroxisome proliferator-activated receptor-gamma ligand, has been demonstrated to completely inhibit lymph node and lung metastases in a xenograft animal model by promoting localization shift of β-Catenin from the nucleus to plasma membrane [185]. TZD also reduced tyrosine phosphorylation of β-Catenin and promoted enhanced expression of E-cadherin [185]. Recently, a crystal structure of β-Catenin-TCF complex has been clarified which shed new light on the molecular mechanism by which this stable and potent transcription factor complex forms [186–188]. Therefore, developing a drug which can disrupt the β-Catenin-TCF complex holds great promise, although how to effectively and selectively disrupt the complex without affecting β-Catenin-E-cadherin or APC complex is still a challenge.

4. Metastasis suppressors

4.1. NM23

NM23 is the first identified metastasis suppressor gene in this group. It is located on chromosome 17q21 and codes for an 18.5-kDa protein containing 166 amino acids which functions as nucleoside diphosphate kinase and protein-histidine kinase [189,190]. Clinically, NM23 has been shown to be down-regulated in a variety of tumors including breast and prostate cancers [191,192]. Ectopic expression of NM23 has also been shown to significantly reduce the *in vitro* and *in vivo* metastatic potential of highly metastatic carcinoma cell lines including breast, melanoma, colon, and oral squamous cells [190,193–195]. Recently, Hartsough et al. reported that NM23 formed a complex with Kinase suppressor of Ras1 (KSR1) and

phosphorylated this protein at Ser-392 and Ser-434, which resulted in blockade of Ras/ MAPK pathway (Fig 2b) [196]. More recently, Salerno et al. have shown that the NM23 expression level influenced the binding properties, stability and function of the KSR1 in breast carcinoma cells [197]. Hence, NM23 was hypothesized to inhibit MAPK/ERK activation via altering the scaffold function of KSR1 (Fig. 2b). Consistent with this hypothesis, MDA-MB-435 breast cancer cells that over-express NM23 showed reduced MAP kinase activity and cell motility *in vitro* as well as diminished incidence of metastasis *in vivo* [196,198,199]. Therefore, NM23 acts as a metastasis suppressor by inhibiting the MAP kinase pathway through the interaction with the KSR1 scaffold protein.

In an attempt to restore the expression of NM23 in tumor cells, several drugs have been found in the past. Among them, medroxyprogesterone acetate (MPA) and estradiol were reported to suppress metastasis through up-regulation of the NM23 gene (Table 1). Medroxyprogesterone is a progestin and commonly used as a component of hormonal contraceptives. Progesterone binds to the progesterone receptor which is then transferred to the nucleus and acts as a transcription factor by binding to the progesterone response elements (PRE) in the promoter region of target genes. Progesterone receptor is known to directly regulate the expression of Cyclin D1, beta-casein and $p21^{WAF1}$ as well as MAPK [200–205]. MPA has a long history of clinical use at a low dose as the contraceptive Depo-Provera and has also been used for hormone replacement therapy in combination with estrogen [206]. At a high concentration, it has been used for the treatment of advanced breast and endometrial cancers [207]. MPA can competitively bind to several steroid hormones including progesterone (PR), androgen (AR) and glucocorticoids (GR), and thus it is able to up-regulate NM23 by antagonizing the effect of glucocorticoid response element (GRE) on the NM23 promoter [208]. Ouatas et al. previously found that MPA inhibited the soft agar colonization of breast carcinoma cells by up-regulating the NM23 expression [209]. In *in vivo*, Palmieri et al. treated mice xenografted with breast carcinoma cells with MPA and found 27–36% reduction of metastasis incidence in the treated animals.

Estradiol works as an estrogen to modulate gene expression via binding to its intracellular receptor ERs [210]. Interestingly, Estradiol was found to be able to decrease the number of experimental lung metastases in nude mice when they were injected with breast cancer cell line MDA-MB231 with forced expression of ER (Table 1) [211]. Lin et al. reported that the level of NM23 mRNA and protein was induced by Estradiol in breast cancer cell lines with the extent that these effects correlated with the level of ERα expression [212]. In addition, Estradiol was shown to be able to decrease the invasive ability of ERα positive carcinoma cell lines MCF7 and BT-474, while it did not have any effect on BCM-1 cell which had virtually no ERα expression [212]. Therefore, it is suggested that Estradiol was able to suppress tumor metastasis by activating the expression of the NM23 gene in an ERα–dependent manner (Fig. 2b) [212].

Many of the therapeutic effects of nonsteroidal anti-inflammatory agent (NSAIDs) are clearly due to the inhibition of prostaglandin synthesis by inactivation of cyclooxygenase 1 and 2 (COX-1 and COX-2) [213]. The anti-tumor effect of NSAID has been recognized when Aspirin was found to reduce the risk of colorectal adenoma and carcinoma in animal models [214–217]. Interestingly, Yu et al. reported that Aspirin decreased the invasive potential of COX2 negative colon cancer cells via up-regulation of NM23 expression (Table 1) [217].

Another NSAID, Indomethacin, was also found to up-regulate the expression of NM23 in breast cancer cells and to alter the malignant choline phospholipid phenotype toward a less malignant tumor [218]. Reich et al. reported that indomethacin reduced the invasive ability of human fibrosarcoma and murine melanoma cell lines and that murine melanoma cells exposed to indomethacin prior to i.v. injection produced significantly fewer lung metastases (Table 1)

[219]. Kundu et al. also reported the anti-metastasis effect of indomethacin by oral administration in a murine model [220]. They transplanted a murine mammary adenocarcinoma cell line 410.4 and found that the metastatic ability of this cell line was reduced by almost 50% with the treatment of indomethacin (Table 1) [220]. Therefore, indomethacin has potential utility as an anti-metastatic drug and it is currently under clinical trial.

All-*trans* Retinoic Acid (ATRA) is known as the first successful targeted drug for cancer therapy. ATRA causes the differentiation of leukemic myeloid cells from mature myeloid cells by attaching to one of several retinoid receptors in the cell nucleus and then directly modulating gene expression [221–223]. The downregulation of several oncogenes including Ras and c-fms by ATRA has been reported [224,225]. Interestingly, the expression of NM23 was also shown to be up-regulated by ATRA in human hepatocarcinoma cell line and gastric cancer cell lines [226,227]. Liu et al. demonstrated that treatment with either ATRA or transfected NM23 cDNA reduced metastasis-associated phenotypes including chemotaxic cell migration and invasion of human hepatocarcinoma cell line [226]. Furthermore, Wu et al. examined the effect of ATRA treatment in xenografted nude mice and found that ATRA treatment significantly decreased the metastasis in liver and increased NM23 protein levels in experimental groups compared with a control group [227]. Since ATRA was also able to reduce cell growth *in vitro* and *in vivo* [227], the specificity of ATRA treatment on tumor metastasis is still unclear. However, a combination treatment of ATRA and IFN-alpha in a clinical trial was well tolerated, and patients who have metastatic osteosarcoma were found to be in stable complete remission 14 months after the end of therapy [228]. Therefore, further investigation of ATRA as an anti-metastatic drug is warranted.

4.2. KiSS-1

KiSS-1 was originally identified as a metastasis suppressor gene using a combined strategy of MMCT and differential display [229]. The introduction of an intact copy of whole human chromosome 6 into the C8161 human melanoma cell resulted in significant reduction of metastasis ability of this cell line without affecting tumorigenicity or local invasiveness in animals [229]. Later Lee et al. reported that the KiSS-1 gene was actually mapped on chromosome 1q region which is frequently deleted in late-stage human breast carcinomas [230]. They then transfected the KiSS-1 gene into human breast ductal carcinoma cell line MDA-MB-435 and found that KiSS-1 almost completely suppressed metastatic activity of MDA-MB-435 [230]. Therefore, although the KiSS-1 gene is located on chromosome 1, it is believed that chromosome 6 is responsible at least in part for its metastasis suppressive effects by harboring a gene that positively regulates KiSS-1 expression [231]. Clinically, the expression of mRNA of the KiSS-1 gene was found to be significantly down-regulated in metastatic tumors, which is in accordance with the idea that KiSS-1 is a metastasis suppressor [232].

Ectopic expression of the KiSS-1 gene was shown to significantly reduce the rate of three-dimensional growth in soft agar, but it did not affect invasion or motility [230]. These results suggest that KiSS-1 affects downstream of cell-matrix adhesion and perhaps involves cytoskeletal reorganization. On the other hand, Yan et al. reported that KiSS-1 transfected HT1080 cells showed substantially reduced enzyme activity of MMP9 with specific down-regulation of mRNA level of MMP9 and invasiveness of tumor cells *in vitro* [233]. They have further shown that this effect was partly attributable to the ability of KiSS-1 to reduce NF-kB binding to the promoter of MMP9 by enhancing I-kB activity (Fig. 2b) [233].

Metastin is a 54 amino acid peptide whose sequence is identical to a part of the KiSS-1 gene, and this peptide was found to act as a ligand for orphan G-protein coupled receptor (hOT7T175, AXOR12, GPR54) (Table 1) [234,235]. Interestingly, Ohtaki et al. have shown that Metastin significantly attenuated pulmonary metastasis in a mouse xenograft model using the B16-

BL6MR melanoma cell, while Metastin had no direct effect on the primary tumor growth [234]. Importantly, Metastin was found to be able to suppress the degree of pulmonary metastasis even when the peptide was administered to the mice that already had metastasis in the lung [234]. Therefore, Metastin is considered to be a promising agent for the treatment of metastatic cancer patients. In this regard, it is encouraging that the expression of the Metastin receptor genes was found to be normal even when KiSS-1 was significantly down-regulated in various types of cancers [236]. These results suggest that Metastin may be effective even in advanced cancer that has lost Kiss-1 expression.

4.3. MKK4

Chekmareva et al. has previously demonstrated a prostate cancer metastasis-suppressor activity encoded by a discontinuous ~70cM region of human chromosome 17, which suppresses the spontaneous metastatic ability of highly metastatic Dunning AT6.1 rat prostate cancer cells [237]. Later, Yoshida et al. identified the MKK4/SEK1 (Mitogen-activated protein kinase kinase 4) gene in this chromosomal region as a candidate metastasis suppressor [238]. Ectopic expression of MKK4 in highly metastatic prostate cancer cell line indeed significantly suppressed macroscopic lung metastasis without affecting the primary tumor growth in animals. [238]. Furthermore, Kim et al. examined the status of MKK4 expression in clinical samples of prostate cancer by immunohistochemical analysis and found that the expression of MKK4 was inversely correlated with Gleason score and tumor progression [239]. How MKK4 suppresses metastasis is a crucial question and has been under active investigation. MKK4 belongs to MAP kinase family which plays central roles in cell proliferation, differentiation and apoptosis. It is known that MKK4 is activated in response to a variety of extracellular stimuli including stress followed by activation of JNK(c-Jun N-terminal kinase) and/or p38 MAPK pathways (Fig. 2b) [240]. It is plausible that, when a tumor cell reaches a distant organ site, the expression of MKK gene in cancer cell is suppressed in the stressful environment, and therefore, fails to establish colonization.

A strategy of using monoclonal antibodies has been considered to be an attractive approach for cancer therapy due to their high target specificity. Anti-death receptor antibody such as anti-TRAIL antibodies, 2E12 and TRA-8, have been found to activate the MKK4/JNK/p38 pathway, suggesting a potential utility of the antibodies for anti-metastatic therapy [241]. Furthermore, Ohtsuka et al. reported that the combination of the anti-death receptor antibodies and chemotherapy agents led to a synergistical activation of the JNK/p38 MAP kinase which was mediated by MKK4 (Table 1) [241]. In their studies, agonistic anti-TRAIL antibodies 2E12 and TRA-8, when combined with chemotherapeutic agents such as Adriamycin, were able to increase the release of cytochrome c and Smac/DIABLO from mitochondria in parallel with the profound loss of mitochondrial membrane potential, which resulted in apoptosis in breast, prostate and colon cancer cells [241]. It is interesting to test whether these regimens are able to suppress metastatic potential of MKK-positive cancer cells *in vivo*.
Bisindolylmaleimide VIII was originally developed as a synthetic inhibitor of protein kinase C (PKC) [242,243], and it was later found to promote Fas-mediated apoptosis in a PKC-independent manner [244]. Ohtsuka et al. examined a possible effect of Bisindolylmaleimide VIII on TRA-8 induced apoptosis and found that a combination of Bisindolylmaleimide VIII and TRA-8 induced 50–80% of apoptosis in human astrocytoma cell line (1321N1), while the treatment of the cells with TRA-8 alone induced apoptosis only in up to 20% of the cells [245]. In *in vivo*, either Bisindolylmaleimide VIII or TRA-8 alone partially regressed the xenografted tumor in NOD/SCID mice, while the combination of these two drugs almost completely blocked the tumor growth. However, whether Bisindolylmaleimide VIII enhances TRA-8- induced apoptosis via a role in regulating MKK4/JNK/p38 apoptosis kinase signaling and whether the combination of these drugs indeed suppresses metastasis remains to be examined.

4.4. E-cadherin

The transmembrane protein E-cadherin (also known as CDH 1) was originally isolated as human uvomorulin by screening a cDNA library of the human liver [246]. The E-cadherin is a calcium-dependent adhesion molecule which constitutes the adherence junction in epithelial cells [247,248]. Reduced level of E-cadherin is shown in a variety of human cancers at advanced stages. It is believed that a low level of E-cadherin can give advantage to tumor cells on breaking the adhesion junction and detaching from adjacent cells, so that these cells invade and metastasize to other distant organs. Clinically, several groups have reported that decreased expression of E-cadherin was associated with a poor prognosis in cancer patients [249]. On the other hand, over-expression of E-cadherin in invasive cancer cells has been shown to decrease motility and invasiveness [250]. In addition, using a transgenic mouse model of pancreatic β-cell carcinogenesis (Rip1Tag), Perl et al. showed that tumor incidence or tumor volume was not significantly changed between double-transgenic Rip1Tag2xRip1dnE-cad mice and single-transgenic Rip1Tag2 littermates [251]. However, the double-transgenic mouse developed metastases to the pancreatic lymph nodes, an invasive phenotype that was never observed in single-transgenic Rip1Tag2 mice [251]. Therefore, E-cadherin is considered to function as a metastasis suppressor. Generally, E-cadherin plays an important role in epithelial-mesenchymal transition (EMT) during which epithelial cells lose their cell-cell junctions and acquire mesenchymal characteristics to endow the migratory ability to tumor cells [249]. E-cadherin interacts with β-Catenin to mediate actin binding (Fig. 2b) [252]. Therefore, loss of E-cadherin, in addition to reducing cell-cell adhesion, provides an oncogenic stimulus by freeing β-Catenin from the membrane, so that β-Catenin can travel to the nucleus to activate TCF-regulated genes such as c-Myc and Cyclin D1 [253]. Furthermore, E-cadherin has been recently found to be down-regulated by transcription factors Snail and Slug that are involved in the process of EMT, cell differentiation and apoptosis [254]. Therefore, restoring the function of E-cadherin is considered to be a potential therapeutic option for metastatic disease. PP (pyrazolo [3,4-d]pyrimidines) 1 and PP2 were originally identified as selective inhibitors for Src, and they were shown to be able to block tumor growth and to reduce metastasis in a mouse pancreatic model. However, these compounds have also been found to reactivate the E-cadherin expression in pancreatic and colon cancer cells (Table 1) [255,256]. Therefore, PP1 and PP2 may serve as effective anti-metastatic drugs although they need to be tested more extensively in a clinical trial.

4.5. NDRG1

N-myc downstream regulated gene 1 (NDRG1) was originally identified by differential displays as being significantly up-regulated by induction of *in vitro* differentiation of colon carcinoma cells [257]. The protein encoded by the NDRG1 gene has a molecular weight of 43kDa and possesses three unique 10-amino acids tandem repeats at the C-terminal, among which seven or more phosphorylation sites were predicted and later they were shown to be targets of protein kinase A *in vitro* [258]. The NDRG1 gene is controlled by multiple factors and responsive to various stimuli. The expression of NDRG1 was repressed by C-myc and N-myc/ Max complex *in vitro*, while it was induced by p53, hypoxia and PTEN (Fig. 2b) [259]. NDRG1 has been shown to act as a tumor suppressor as well as a tumor metastasis suppressor depending on cell context [259]. In a clinical setting, NDRG1 was found to be consistently expressed in normal prostate tissue as well as PIN (prostatic intraepithelial neoplasia) and BPH (benign prostatic hyperplasia), whereas the expression was significantly reduced in high-grade tumors [260,261]. In addition, the level of the NDRG1 expression was inversely co-related with the status of metastasis in these patients, supporting the notion that NDRG1 is a tumor metastasis suppressor [260]. In breast cancer, a similar and significant negative correlation of NDRG1 with metastasis has been observed, while the expression of NDRG1 does not show any significant correlation with the size or the histological grade of the primary tumor [261]. These results strongly suggest the negative involvement of NDRG1 in the process of invasion

and metastasis in both prostate and breast cancer. Furthermore, ectopic expression of the NDRG1 gene in a highly metastatic prostate cancer cell line significantly reduced the incidence of lung metastases, suggesting that NDRG1 was able to block the metastatic process without affecting the primary tumor growth [260,261]. Similar metastasis suppressor effect of NDRG1 was also observed in colon carcinoma cells by Guan et al. [262]. In addition, NDRG1 also significantly suppressed the invasive potential of prostate and breast cancer cells as tested by *in vitro* invasion chamber assay [260,261]. Therefore, evidence from both clinical data and the results of *in vitro* as well as animal experiments overwhelmingly support the notion that NDRG1 is a metastasis suppressor gene and that the down-regulation of the gene results in acceleration of tumor metastasis. How NDRG1 suppresses the tumor metastasis is an intriguing question which is under active investigation.

Recently, Fe chelators, desferrioxamine (DFO) and 311 were shown to be able to up-regulate the NDRG1 expression in human breast cancer cell line MCF7 [263]. In the past years, dietary Fe restriction has been shown to markedly decrease tumor growth in rodents [264–266], and Fe chelators such as Triapine and desferrioxamine (DFO) were reported to be potentially useful for cancer therapy (Table 1) [266–268]. More recently, Whitnall et al. examined the effect of another Fe chelator, di-2-pyridylketone-4,4,-dimethyl-3-thiosemicarbazone (Dp44mT), on tumorigenesis in xenografted mice models of lung carcinoma, neuroepithelioma and melanoma and found that Dp44mT strongly inhibited the growth of all tested human xenografts in nude mice [269]. Notably, Dp44mT significantly augmented the expression of the NDRG1 gene in the tumor compared to that of control group, suggesting a promising utility of this compound as an anti-cancer as well as anti-metastatic drug [269].

5. Conclusion and Future direction

Despite significant improvement in surgical techniques and chemotherapy for cancer treatment in general, none of the current medical technologies “cure” the metastatic disease, and the patients who have already acquired metastatic cancer are left virtually with no options. Therefore, there is an urgent need for developing a novel approach of target-specific therapy to metastatic tumor cells, which requires more comprehensive understanding of the molecular mechanism of metastases. The goals of anti-metastatic therapy are three folds. Firstly, we need to develop a specific drug that blocks secondary metastasis to treat patients who have already acquired metastatic disease but are still at an early stage. Secondly, a drug should also be developed to treat patients who underwent surgical resection of their primary tumors in order to prevent a possible recurrent disease. However, the ultimate goal is to develop a non-toxic agent which can be taken as diet for prevention of metastasis. In the past decade, the major effort of anti-cancer research has been focused on the development of drugs that can block the proliferation of tumor cells. They take advantage of the fact that tumor cells are more actively proliferating than other normal cells, and therefore, “selectively” kill the cancer cells. However, this “selectivity” has narrow margins and these agents inevitably cause severe side effects even when they are used in combination to lower the toxicity. From these experiences, we have learned an important lesson that the most critical issue for anti-cancer drugs is their specificities. Therefore, to develop an anti-metastatic drug, it is crucial to define a target molecule which is specifically expressed in metastatic cells. Ideally, an agent which can attack the molecule is inactive (pro-drug) when given to patients, and is activated only in the tumor cells. In theory, monoclonal antibodies and siRNA are highly specific to target genes, and active investigations are underway to utilize these technologies for the development of anti-metastatic drugs. If a target is well defined and specific, these agents are considered to be very effective, although there are still many unknown technical questions such as stability and delivery method of these agents. However, recent advancement of bio-technology such as nano-particles has provided us with a hope that we can eventually overcome these problems.

We have learned a great deal of the metastasis cascade, and many new genes and signal pathways involved in this process have been identified. Some genes hold great promises as potential druggable targets. The genes that control EMT and cell motility as well as their signal pathways are rational candidates for the drug development. Although a clinical trial of the drugs that block MMP resulted in a rather disappointing outcome, these molecules are still considered to be excellent targets. The fact that metastatic cells are the only epithelial cells in circulation may provide us with a window of opportunity to attack such cells. In addition, tumor cells are often attracted by various types of chemokines to the distant organ sites, and these chemokines may also serve as molecular targets for anti-metastatic therapy. Reactivation of metastasis suppressor genes and their signal pathways such as MKK/JNK, PTEN/Akt and NDRG/ATF are also a rational strategy. Recent finding that KAI1 blocks metastasis by inducing senescence upon interaction with endothelial cells also suggests an interesting possibility to develop an effective drug to activate the KAI1 pathway. Perhaps, genome wide shRNA library screening and comprehensive proteomics approach may reveal more suitable targets for metastatic therapy in the near future. The use of computer-driven strategies such as automated determinations of the structures of target molecules and computer-aided design of drug molecules followed by a high-throughput screening has already begun to set this trend into motion.

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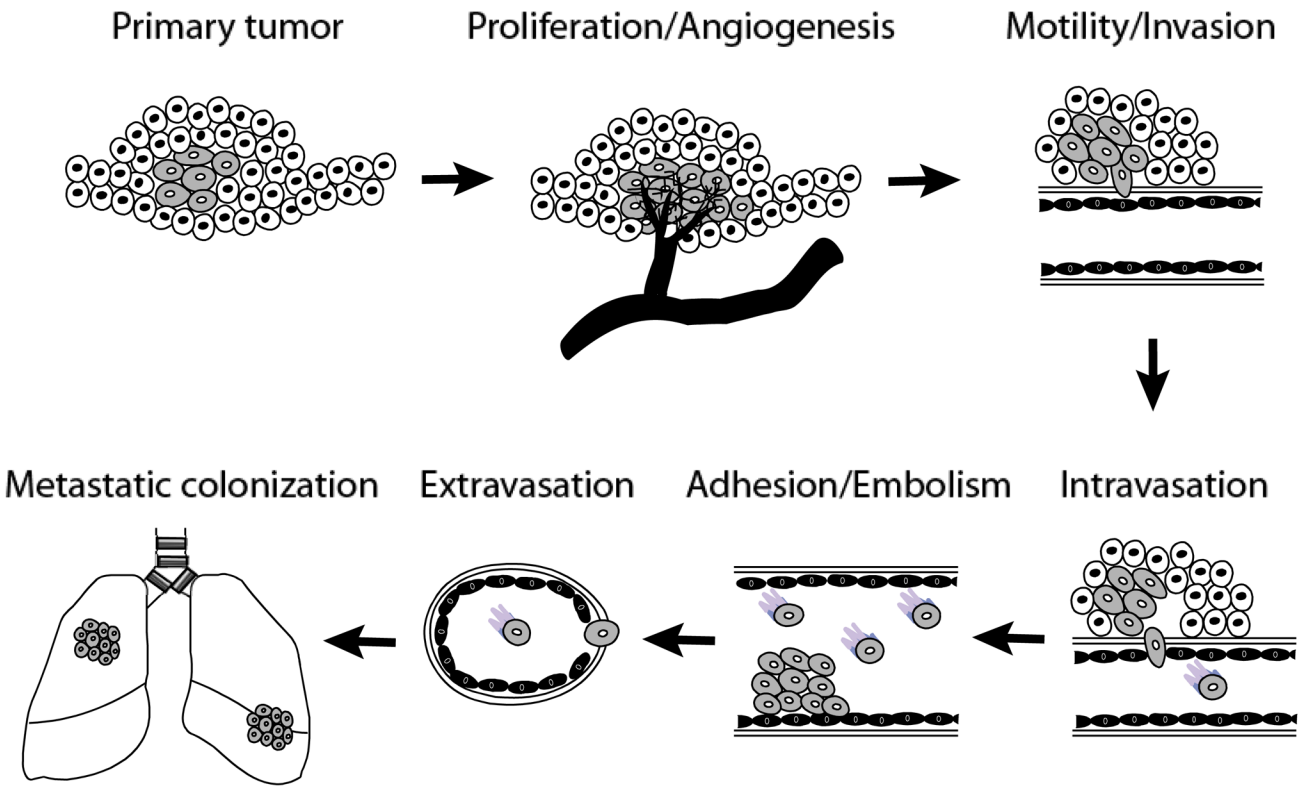
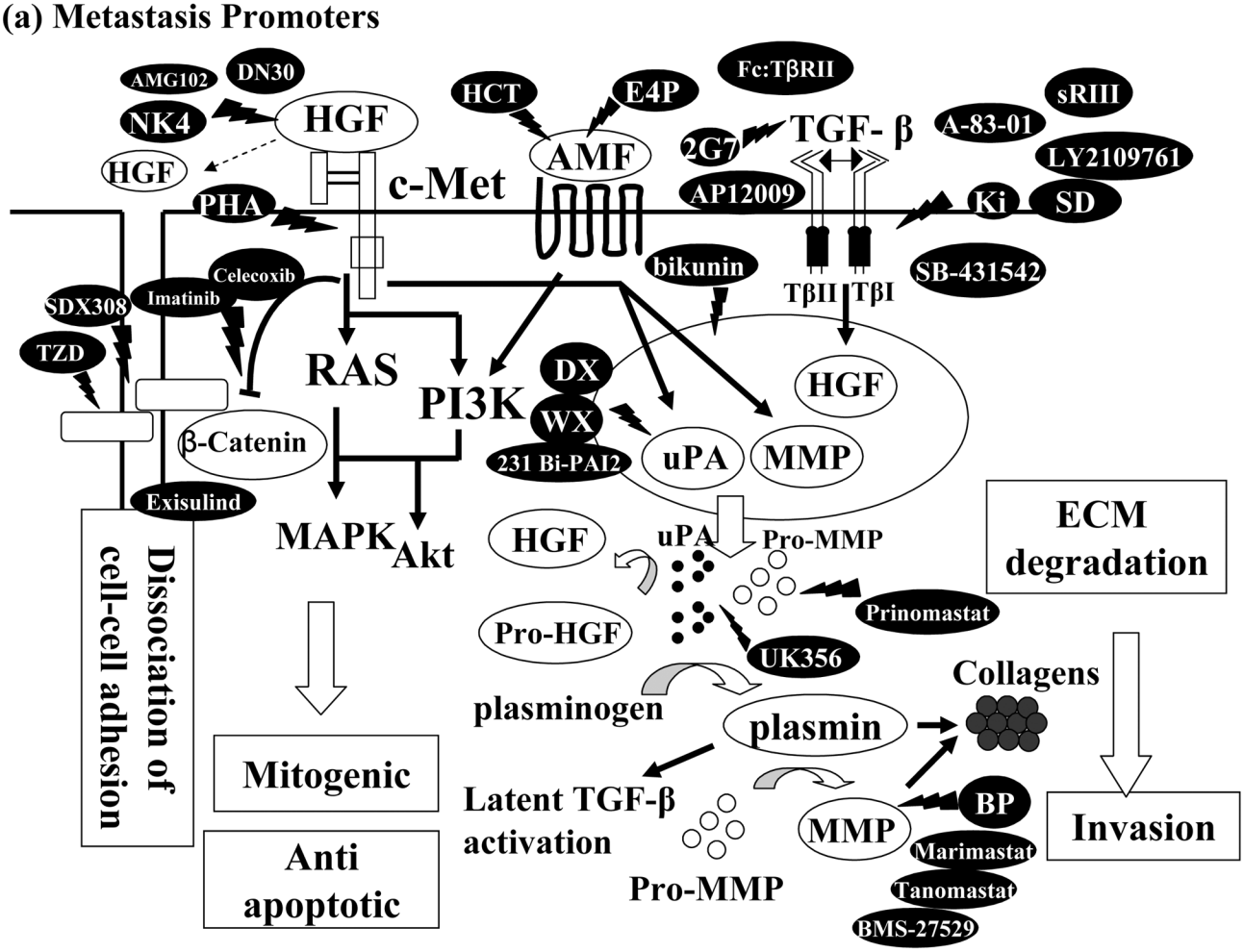


Fig. 1. Process of tumor metastasis

As primary tumor grows, tumor cells induce angiogenic factors to promote vessel formation which facilitates tumor growth and cell invasion into the circulatory system. Some tumor cells gain an invasive ability by expressing motility factors and proteases followed by breaching the basement membrane. Tumor cells then enter the blood vessel where they often aggregate with the platelets and cause embolize. When cells migrate to a distant organ, they adhere to endothelial cells and extravasate by inducing proteases. Cells then colonize and establish metastasis at the distant organ site where appropriate growth factors are provided.



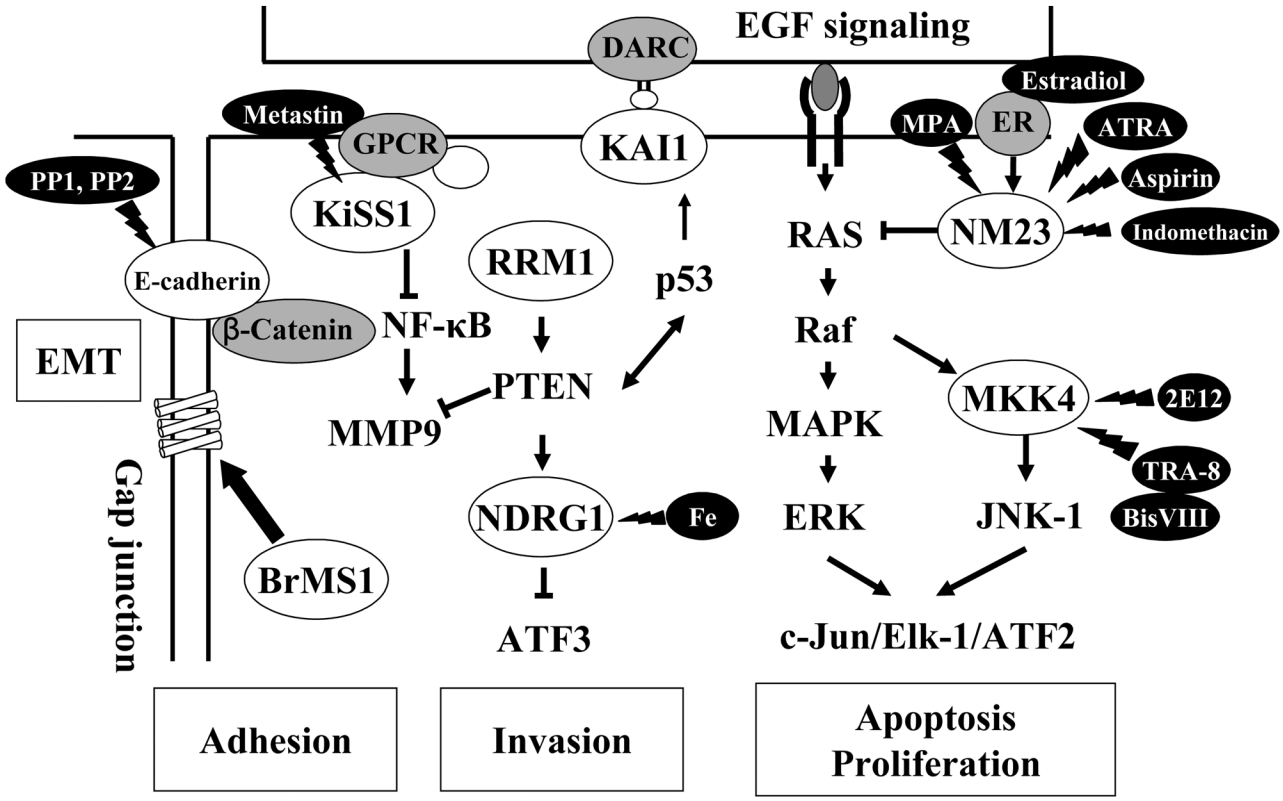


Fig. 2. Signal pathway of tumor metastasis

Tumor metastasis is a result of complex interplay of both positive (a) and negative (b) factors. These pathways and their factors are potential targets for anti-metastatic therapy. The drugs currently under development are shown as black oval shapes.

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Table

Metastasis promoter	drug	Original target	action	animal	Clinic trial	Reference
AMF	carbohydrate phosphate compounds (E4P,M6P,5PA)	AMF	Inhibit AMF cytokine enzymatic activity		Pre-clinical studies	[25,26]
	Herceptin	EGFR2	Down-regulates AMF protein and promoter activity	Increase the tumor progression time in mice model of xenograft tumor of Her2 over-expression	In clinical use	[27,270]
HGF/c-Met	NK4	HGF	competitive antagonist for HGF binding to the c-Met receptor	Inhibited tumorigenesis, angiogenesis and metastases in mouse tumor xenograft models	Pre-clinical	[46,49]
	uncleavable HGF	HGF	Prevent maturation of pro-HGF and compete with HGF to bind to c-Met receptor	Inhibited tumor growth, angiogenesis and metastases in tumor xenograft models	Pre-clinical	[50]
	AMG102	HGF	Neutralizing anti-HGF antibody	pharmacokinetic and safety profile are passed through in cynomolgus monkeys test	Phase II	[53]
	DN30	c-Met	Binds to extracellular domain of c-Met and prevent its activation	inhibited growth and metastatic spread to the lung of tumor xenograft mouse model	Pre-clinical	[61]
	PHA-665752 SU11274 K252a	Kinase inhibitors	inhibit c-Met phosphorylation	Inhibition of tumor growth in c-Met-dependent lung and gastric carcinoma xenograft animal model	Pre-clinical	[55–60]
TGF-β	SD-208 SD-093 SB-431542 A-83-01 LY2109761	TGFβ1 receptor	TGF-β typeI receptor kinase inhibitor	Inhibited primary tumor growth, angiogenesis and metastasis of xenograft animal model	Pre-clinical studies	[68,73,87–92]

Metastasis promoter	drug	Original target	action	animal	Clinic trial	Reference
	2G7	TGFβ	Neutralizing antibody of TGFβ	Inhibited abdominal and lung metastasis of xenograft animal model	Pre-clinical	[69]
	β- glycan (sRIII)	TGFβ	Soluble extracellular domain of TGF-β type III receptor	Inhibited lung metastasis in human breast tumor xenograft model	Pre-clinical	[96]
	Fc:TβRII	TGFβ	Dominant negative TGF-β typeII receptor	Inhibited lung metastasis in human melanoma xenograft model and MMTV-Neu model	Pre-clinical	[94,95]
	AP12009	TGFβ	Oligonucleotide against human TGFβ2		PhaseI/II (high grade glioma)	[97]
MMP	Marimastat (BB-2516)	MMPs	Pharmacologically developed MMPs inhibitor		PhaseII,III,IV (Pancreatic cancer) phaseIII Non-small-cell lung cancer)	[122,271]
	Prinomastat (AG3340)	MMPs	inhibitor with selectivity for MMPs 2, 3, 9, 13, and 14	enhance tumoricidal activity after Photodynamic therapy in a mouse mammary tumor model	Phase III, IV (NSCLC) phaseII (advanced esophageal cancer)	[122,272]
	Tanomastat(BAY12-9566)	MMPs	Pharmacologically developed MMPs inhibitor		PhaseIII (Small-cell lung and pancreatic cancer)	[122,271]
	BMS-275291Neovastat	MMPs	Pharmacologically developed MMPs inhibitor		PhaseIII, IV (Non-small-cell lung and Renal	[122,271]

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Metastasis promoter	drug	Original target	action	animal	Clinic trial	Reference
					cell carcinoma)	
	Bisphosphonates (BP)	for use in disorders of bone metabolism	Inhibit proteolytic activity of MMPs	Increase bone mineral density in animal model	In use (osteolytic metastases)	[129]
uPA	WX-UK1 WX-671	uPA	Protease inhibitor		Phase I,II	[148]
	231 Bi-PAI2	uPA	Recombinant PAI-2 (uPA inhibitor-2)	Inhibited micrometastasis in human breast cancer xenograft models	Pre-clinical studies	[160–162]
	1-Isoquinolinylguanidines(UK-356,202) and its derivatives	uPA	Reversibly competitive inhibitors of uPA enzymatic activity	Inhibit exogenous uPA in human chronic wound fluid and in the porcine excisional wound model	Pre-clinical studies	[273]
	Bikunin	Trypsin and plasmin	Down-regulate uPA gene and protein expression	once-daily oral administration of bikunin against ovarian carcinoma in nude mice	Phase I	[153–156]
	DX-1000 PEGylated DX-100	plasmin	Down-regulate uPA expression	Inhibited tumor proliferation and vascularization in human tumor xenograft model	Pre-clinical	[157,158]
β-catenin	Celecoxib	COX-2	Induce degradation of β-catenin via a COX-2-independent mechanism	Diet treatment significantly reduce tumor development without signs of metastasis in TRAMP mice	phase II (advanced colorectal cancer)	[182,183,274]
	R-Etodolac and its analog (SDX-308)	enantiomer of Etodolac	Down-regulates protein and promoter activity, increase β-catenin and E-cadherin complex at the membrane	inhibited tumor development and metastasis in the transgenic mouse adenocarcinoma of the prostate (TRAMP) model	phase II (chronic lymphocytic leukemia)	[182,183]
	Thiazolidinedione (TZD)	PPARs	cause localization shift to cytoplasm,	Inhibited lymph node and	Pre-clinical	[185]

Metastasis promoter	drug	Original target	action	animal	Clinic trial	Reference
			reduced tyrosine phosphorylation of beta-catenin	lung metastases in the xenograft animal model	studies	
	Exisulind(Aptosyn) CP461 CP248	SAANDs	Down-regulate β-catenin and cyclin D1 via PKG- mediated signalling	Inhibited tumor growth and metastasis of human lung cancer xenograft in athymic nude rats.	Phasae I,II,III	[164,172,173]
	Imatinib (Gleevec)	PDGF receptor	Inhibits tyrosine-phosphorylation of β-catenin and resultant cell migration		In use (chronic myelogenous leukemia (CML), gastrointestinal stromal tumors (GISTs) etc)	[176]
Metastasis Suppressor						
NM23	Medroxyprogesterone acetate (MPA)	Progesterone receptor	MPA elevated NM23 expression and inhibited soft agar colonization	Inhibited lung cancer metastasis in the experimentally metastasis mice model	Phase III(metastatic breast cancer)	[206,275,276]
	Estradiol	Estrogen receptor	Up-regulates NM23-H1 in ERa+ breast cancer cell lines. Inhibits invasion *in vitro*.	Suppression of lung metastasis *in vivo* model of chemically induced hepatocellular carcinoma.	Phase II (metastatic breast and prostate cancer)	[212,277]
	Aspirin	Cox1/2 inhibitor	Up-regulates NM23. Decreased metastatic phenotype *in vitro*.		Phase III (esophageal cancer)	[217,277]
	Indomethacin	Cox1/2 inhibitor	Up-regulates NM23 expression in breast cancer cell lines	Inhibited lung tumor metastasis in the experimental metastasis mice model	Phase II (head and neck cancer)	[219,220,277]

Metastasis promoter	drug	Original target	action	animal	Clinic trial	Reference
	All-trans retinoic acid (ATRA)	Retinoid receptors	Up-regulates NM23 in hepatocarcinoma cells. Increased adhesion to ECM *in vitro*	Inhibits the growth of xenograft tumors and gastric cancer cell metastasis to liver.	Currently in clinical use, (acute promyelocytic leukemia)	[226–228,278]
KiSS-1	Metastin	orphan G-protein coupled receptor	Regulate the NFκB signaling pathway		Pre-clinical studies	[279]
MKK4	Anti-death receptor antibody (2E12, TRA-8)	death receptor	Induce apoptosis *in vitro.* Activate MKK4/JNK/p38 pathways		Pre-clinical studies	[241]
	Bisindolylmaleimide VIII	PKC inhibitor	Enhances affects of anti-death receptor antibodies		Pre-clinical studies	[245]
E-cadherin	pyrazolo [3,4-d]pyrimidines (PP)1, PP2	Src family inhibitor	Reactivate the E-cadherin expression. Reduced migration ability of breast cancer cells	Decrease in pancreatic tumor growth and metastasis in nude mice	Pre-clinical studies	[255,256,280]
NDRG1	Fe chelator (DFO, 311)	Fe	NDRG1 was specifically up-regulated by Fe chelation.	Delay or regression of tumor cell growth in athymic nude mice.	Phase II (Neuroblastoma)	[263,266,281,282]