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Review

# Tissue invasion and metastasis: Molecular, biological and clinical perspectives

W.G. Jiang[a,*], A.J. Sanders[a], M. Katoh[b], H. Ungefroren[c], F. Gieseler[c], M. Prince[d], S.K. Thompson[e], M. Zollo[f,g], D. Spano[g], P. Dhawan[h], D. Sliva[i], P.R. Subbarayan[j], M. Sarkar[j], K. Honoki[k], H. Fujii[k], A.G. Georgakilas[l], A. Amedei[m], E. Niccolai[m], A. Amin[n], S.S. Ashraf[n], L. Ye[a], W.G. Helferich[o], X. Yang[o], C.S. Boosani[p], G. Guha[q], M.R. Ciriolo[r], K. Aquilano[r], S. Chen[s], A.S. Azmi[t], W.N. Keith[u], A. Bilsland[u], D. Bhakta[q], D. Halicka[v], S. Nowsheen[w], F. Pantano[x], D. Santini[x]

[a] Cardiff University, Cardiff, United Kingdom
[b] National Cancer Center, Tokyo, Japan
[c] University Hospital Schleswig-Holstein, Lübeck, Germany
[d] University of Michigan, Ann Arbor, MI, USA
[e] Royal Adelaide Hospital, Adelaide, Australia
[f] Department of Molecular Medicine and Medical Biotechnology (DMMBM), University of Naples Federico II, Naples, Italy
[g] CEINGE Biotecnologie Avanzate, Naples, Italy
[h] University of Nebraska Medical Center, Omaha, USA
[i] Purdue Research Park, Indianapolis, IN, USA
[j] University of Miami, Miami, FL, USA
[k] Nara Medical University, Kashihara, Japan
[l] Physics Department, School of Applied Mathematical and Physical Sciences, National Technical University of Athens (NTUA), Athens, Greece
[m] University of Florence, Florence, Italy
[n] United Arab Emirates University, Al Ain, United Arab Emirates and Faculty of Science, Cairo University, Egypt
[o] University of Illinois at Urbana-Champaign, Urbana, IL, USA
[p] Creighton University, Omaha, NE, USA
[q] SASTRA University, Thanjavur, India
[r] University of Rome Tor Vergata, Rome, Italy
[s] Ovarian and Prostate Cancer Research Trust Laboratory, Surrey, United Kingdom
[t] Wayne State University, Detroit, MI, USA
[u] University of Glasgow, Glasgow, United Kingdom
[v] New York Medical College, Valhalla, NY, USA
[w] Mayo Clinic College of Medicine, Rochester, MN, USA
[x] University Campus Bio-Medico, Rome, Italy

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ABSTRACT

Cancer is a key health issue across the world, causing substantial patient morbidity and mortality. Patient prognosis is tightly linked with metastatic dissemination of the disease to distant sites, with metastatic diseases accounting for a vast percentage of cancer patient mortality. While advances in this area have been made, the process of cancer metastasis and the factors governing cancer spread and establishment at secondary locations is still poorly understood. The current article summarizes recent progress in this area of research, both in the understanding of the underlying biological processes and in the therapeutic strategies for the management of metastasis. This review lists the disruption of E-cadherin and tight junctions, key signaling pathways, including urokinase type plasminogen activator (uPA), phosphatidylinositol 3-kinase/v-akt murine thymoma viral oncogene (PI3K/AKT), focal adhesion kinase (FAK), β-catenin/zinc finger E-box binding homeobox 1 (ZEB-1) and transforming growth factor beta (TGF-β), together with inactivation of activator protein-1 (AP-1) and suppression of matrix metalloproteinase-9 (MMP-9) activity as key targets and the use of phytochemicals, or natural products, such as those from *Agaricus blazei*, *Albatrellus confluens*, *Cordyceps militaris*, *Ganoderma lucidum*, *Poria cocos* and *Silybum marianum*, together

* Corresponding author at: Cardiff-Peking Cancer Institute and Cardiff-Capital Medical University Joint Centre for Biomedical Research, Cardiff University School of Medicine, Cardiff University, Henry Wellcome Building, Heath Park, Cardiff CF14 4XN, United Kingdom. Tel.: +44 29 20687065.
*E-mail address:* jiangw@cf.ac.uk (W.G. Jiang).

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with diet derived fatty acids gamma linolenic acid (GLA) and eicosapentanoic acid (EPA) and inhibitory compounds as useful approaches to target tissue invasion and metastasis as well as other hallmark areas of cancer. Together, these strategies could represent new, inexpensive, low toxicity strategies to aid in the management of cancer metastasis as well as having holistic effects against other cancer hallmarks.


## 1. Introduction

The chain of events leading to the malignant transformation of cells, whether through genetic or epigenetic alterations, is complex. Malignant cells possess key hallmarks, namely, uncontrolled growth potentials and the ability to invade surrounding tissues and metastasize [1]. Cancer cells likely possess these innate abilities to some extent, though the degree and timing of invasion and metastasis may vary due to the genetic and epigenetic heterogeneity within the tumor and further signals from extrinsic factors, such as those within that particular microenvironment [2].

Despite substantial effort dedicated to the early detection and prevention of cancer, most patients are likely to have micro- (not visible using conventional methods) or macro- metastases by the time they come to medical attention [3,4]. Cancer patients, both early and late stage, dependent on life span, are likely to develop metastasis. This metastatic spread of the primary tumor accounts for over 90% of patient mortality associated with solid cancers [1,4,5]. Despite this, research into the field of metastasis, in comparison to other key events such as proliferation, *etc.*, is lagging. This is partly due to the complexity of the metastatic process but also due to a lack of sufficient funding and efforts into this area of research. However, significant progress in this vital area of cancer research has been witnessed over the past decade, though much remains to be elucidated before we fully understand this pernicious condition and a number of significant gaps remain to be filled before we can truly understand this complex process.

Diagnosis and treatment of metastatic disease are vital areas in the constant battle many patients face against cancer, yet effective treatments are limited and substantial morbidity and mortality are still associated with metastatic disease [5,6]. This, together with the complexities surrounding the metastatic process (summarized in Fig. 1) and the complex nature and heterogeneity of metastatic tumors, fully supports and justifies further research dedicated to the discovery of a less toxic means to treat this condition. This is the major mission of getting to know cancer (GTKC). This review aims to discuss key knowledge gaps, explore potential targets in tackling metastasis and also potential methods, including phytochemicals, small molecule inhibitors and natural compounds in devising new strategies for treating metastasis.

## 2. Cellular properties and metastasis

### 2.1. Cell–cell adhesion

In cancers derived from the epithelium, inter-cellular structures and cell–cell adhesion are key factors in maintaining a coherent primary tumor mass [7,8]. Abnormalities in these structures, through mutation or dysregulation, can lead to the dissociation of the primary tumor and an enhanced potential for dissemination and metastatic spread of cancer cells to secondary locations [7–9]. Key structures involved in maintaining this adhesiveness between cells include adherens junctions (including desmosomes), tight junctions (TJ) and gap junctions. While gap junctions confer a weak adhesion structure and TJs, a modest one, the adherens junctions provide the most powerful source of adhesion in epithelial cells. Perhaps one of the strongest and most studied regulators of adhesion is E-cadherin (cadherin-1 or CDH1), a member of the cadherin family of proteins. E-cadherin, together with associated catenins, plays a key role in maintaining cell–cell adhesion and is also involved in the regulation of the cell cycle regulators $p27^{kip1}$ and $p57^{kip2}$, which are involved in cell–cell contact inhibition in normal epithelium, but which are lost or disturbed in cancer cells, mainly due to the loss of E-cadherin in cancer cells [8,10,11]. Hence, reduced cell–cell adhesion not only enhances the potential for metastatic dissemination of cancer cells but also, through loss of contact inhibition, promotes uncontrolled cell growth [7]. E-cadherin has also been established as a key mediator of the epithelial – mesenchymal transition (EMT) process (discussed in Section 2.4). Thus, enhanced expression of key cadherin molecules could offer potential as a strategy to control metastatic dissemination, though realizing this potential has proved difficult; thus far there have been few reports identifying viable treatment options in this regard. However, there are a number of noteworthy options, namely the polyunsaturated fatty acids gamma linolenic acid (GLA) and dihomo-γ-linolenic acid (DGLA), both obtainable through the diet. These have been reported to be key regulators of E-cadherin and desmosomal cadherins in cancer cells and have also been reported to have beneficial effects for patients with several cancer types including pancreatic cancer and breast cancer [12–15]. The desirable effects of these essential fatty acids (EFAs) were blocked by non-EFA, as long chained oleic derivatives on human cell lines [16].

#### 2.1.1. Claudins in cancer

The TJ complex is the apical most junctional complex in most types of epithelial and endothelial cells. TJs are the gasket-like seals that encircle each columnar epithelial cell around its apical pole. They serve two roles: (1) they help to maintain cell polarity by physically separating the apical and the basolateral membrane domains and (2) they prevent free interchange of substances by diffusion along the paracellular pathway between the luminal and antiluminal tissue fluid compartments. TJs and their permeability are important in the formation of the blood brain barrier, blood retinal barrier and blood testis barrier. The TJ proteins can be sub-divided into the integral membrane proteins such as occludin, tricellulin, marvelD3, junctional adhesion molecules (JAMs) and the claudin family (currently 27 members [17]) and the cytoplasmic proteins. The cytoplasmic adaptor proteins are the zonula occludens or ZO proteins, and are designated ZO-1, -2, and -3. These proteins link the membrane proteins to the actin cytoskeleton. Traditionally, research efforts focused on barrier and fence functions, however, there is a new movement in the field, which is to understand how TJ proteins participate in cell proliferation, transformation, and metastasis suppression. Recent studies have demonstrated the role of TJs during epithelial tissue remodeling, wound repair, inflammation, and transformation into tumors. Epithelial multilayering was associated with increased TJ permeability [18], activation of protein kinase C (PKC)-α [19] and phosphorylation of TJ proteins [20].

Studies focusing on the molecular architecture of the TJ have now confirmed that the claudin family of proteins is the integral component of the TJ. Loss of cell–cell adhesion is central to the cellular transformation and acquisition of metastatic potential, however, the role the claudin family of proteins may play in a series of pathophysiological events, including human carcinoma development, is only now beginning to be understood. Several

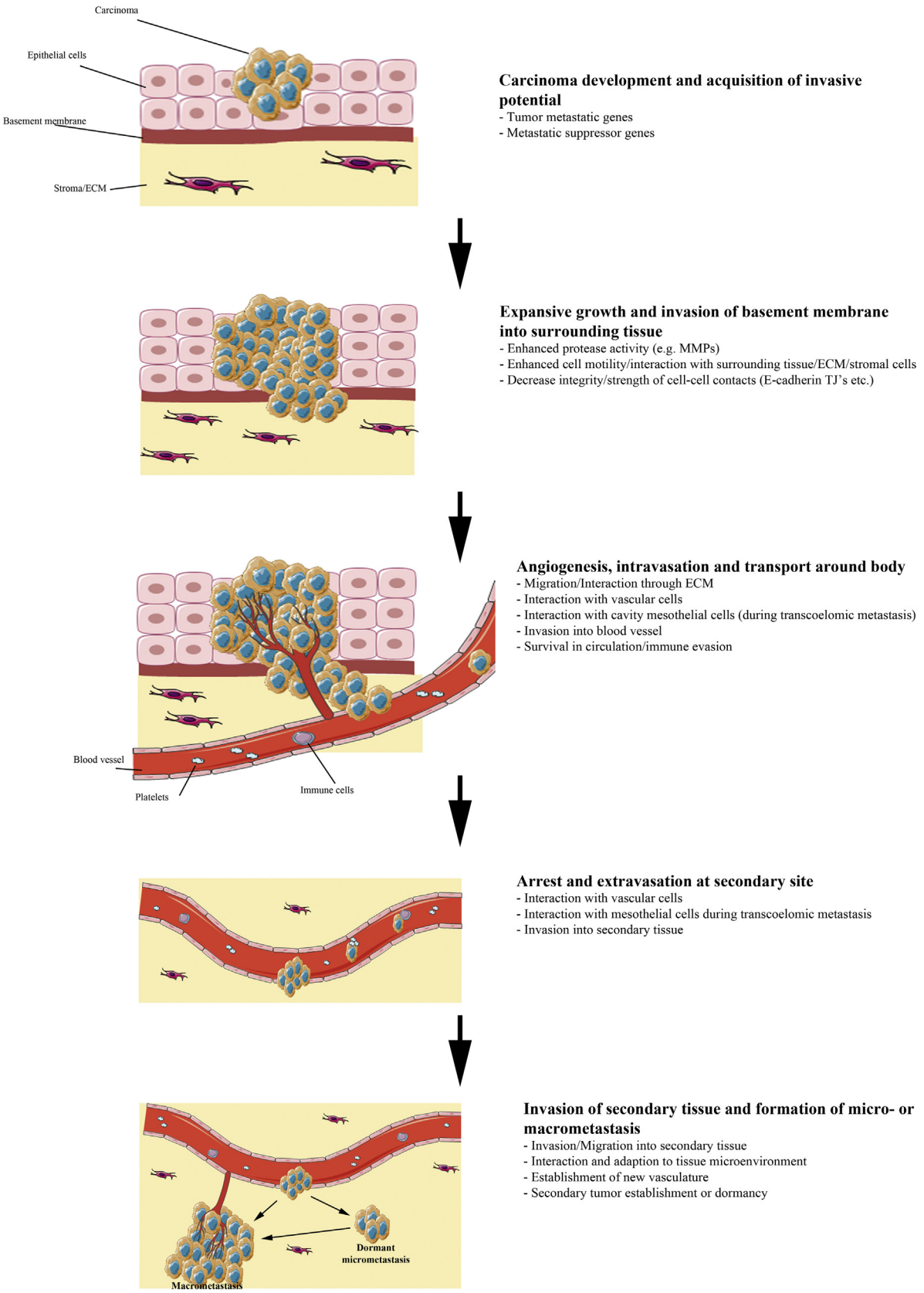


**Fig. 1.** The metastatic cascade and potential for therapeutic interruption. Changes in cellular properties are necessary to allow the development of an invasive phenotype and progression through the metastatic cascade. Key events of the cascade are outlined. Targeting such properties/events or the underlying signaling pathways using low toxicity drugs holds great potential to disrupt cancer cell progression through this cascade.

claudin mouse knockout models have demonstrated their important role in the maintenance of tissue integrity in various organs. The mechanisms of claudin regulation and their exact roles in normal physiology and disease are being elucidated, but much work remains to be done.

There are 27 types of claudins in mammals [17,21] and they are divided in classic and non-classic claudins based on their sequence similarity [21]. Classic claudins include claudins 1–10, 14, 15, 17 and 19 and non classic claudins 11–13, 16, 18 and 20–24 [21]. Claudins are found in epithelial, mesothelial, glial and endothelial cells [22–24] with a molecular weight of around 20 kDa and in cell membranes they are composed of two extracellular loops, EL1 and EL2, four transmembrane domains, one small 20 amino acid long intracellular part between the two extracellular loops and the intracellular aminoterminal and carboxyterminal ends [21,25]. The carboxyterminal end has regions which recognize the PDZ (post synaptic density protein, Drosophila disk large tumor suppressor, and zonula occludens-1 protein) domains of ZO-1, ZO-2 and ZO-3 [25]. The larger EL1 loop influences paracellular charge selectivity whereas the smaller EL2 loop binds to the corresponding claudin of the neighboring cell [25]. Claudin expression and functions are regulated at multiple levels and by diverse mechanisms [26,27]. An important question related to regulation of claudin expression and cancer is the role that claudins may play in the EMT process [28,29]. The paracellular barrier modulated by claudin members can be affected by a wide range of physiological factors including cell signaling pathways, hormones, cytokines, and disruption of the cell–cell contacts. Post-translational modifications, including phosphorylation, lipid modification and removal of claudins by endocytosis, appear to be potential mechanisms for the regulation of claudins. Phosphorylation has been linked to both increases and decreases in TJ assembly and function. Most claudin proteins have putative serine and/or threonine phosphorylation sites in their cytoplasmic carboxyterminal domains. For instance, protein kinase A (PKA) – mediated phosphorylation has been shown to decrease assembly of claudin-3 into TJs [30], yet is necessary for claudin-16 assembly and function [31]. Claudin-3 and -4 can be phosphorylated in ovarian cancer cells by PKA, a kinase frequently activated in ovarian cancer [30]. Claudin phosphorylation associated with TJ disassembly is also enhanced by EPH receptor A1 (EphA1), which is recruited to bind to claudin-4 by forming a complex with ephrin-B1 [32]. Studies have implicated PKC in the regulation of TJs through phorbol ester stimulation [30,33]. Furthermore, modulation of mitogen-activated protein kinase (MAPK) signaling, specifically extra cellular signal-regulated kinase (ERK) 1/2 and p38, as well as phosphatidylinositol 3-kinase (PI3K) have a profound effect on TJ sealing and claudin expression [30]. TJs are also remodeled at a more macroscopic level through strand breaks and reformation [34]. Clathrin-mediated endocytosis plays an important role in this process [35,36]. Claudins are internalized by a unique mechanism, where the tightly opposed membranes of the TJ are endocytosed together into one of the adjoining cells [24]. Host factors and cytokines can also influence TJ turnover and claudin expression [37], for instance, interferon (IFN)-γ increases claudin endocytosis and TJ permeability [38]. Other inflammatory cytokines, such as tumor-necrosis factor (TNF)-α and interleukin (IL)-13, down regulate claudins and induce a marked increase in paracellular permeability by epithelial cells in culture [39,40].

Growth factor receptors that are important in the regulation of cell proliferation and survival including epidermal growth factor (EGF), hepatocyte growth factor (HGF) and insulin like growth factor (IGF) receptors regulate claudin expression and cellular distribution though once again in cell/tissue specific manner [28,29,41]. Claudin transcription can be regulated by the Snail/Slug family [42]. It is well established that overexpression of Snail in epithelial cells induces EMT and the acquisition of migratory and *in vitro* invasive behavior. Snail and Slug bind to the E-box motifs present in the human claudin-1 promoter which play a critical negative regulatory role in breast cancer cell lines that expressed low levels of claudin-1 [42]. Caudal type homeobox 2 (Cdx-2), hepatocyte Nuclear Factor 1-alpha (HNF-α), and GATA binding protein 4 (GATA-4) [43,44] can bind to the promoter regions of various claudin genes and affect their expression. Furthermore, it has been shown that colonic claudin-1 transcripts are regulated by Smad-4, a known tumor suppressor as well as histone deacetylase (HDAC) inhibitors and thus support a complex regulation at multiple levels [45,46]. Collectively, the data provides an emerging picture of the importance of claudin homeostasis in normal and pathological tissue function, but there remains much to be learned, especially regarding whether it may be possible to identify a distinct claudin signature in the initiation and progression of various tumor types.

Alterations in claudin expression profiles during tumorigenesis begs the question of how claudins are regulated in different tissues in both normal and pathological situations. Tan et al. [47] have shown that the expression and distribution of claudin-1 is associated with cell dissociation status in pancreatic cancer cells through MAPK 2 activation. By contrast, claudin-7 has been found to be decreased in invasive ductal carcinomas [48], head and neck cancer [49] and metastatic breast cancer [37]. On the other hand, claudin-3 and -4 are frequently elevated in various cancers including pancreatic ductal adenocarcinoma, prostate, uterine, ovarian cancer [38] and breast cancer [50] while hepatocellular and renal carcinomas expressed lower levels of claudins-4 and -5 [22]. While lower expression of claudin-2 was also seen in breast and prostatic carcinomas, expressions of claudin-1 and claudin-7 that were undetectable in normal cervical squamous epithelium increased in the cervical neoplasia [22,51]. Intriguingly, recent studies have shown that expression of certain claudins, especially claudin-1 and claudin-4, increases during metastasis and genetic inhibition of their expression has a profound effect on the metastatic abilities of cancer cells though in a tissue specific fashion [52–54]. There is the possibility that mutations in claudins may be causal to tumor formation. However, to date there is no systematic sequence data on claudins in any tumor type. On the other hand, gene silencing due to promoter hypermethylation is a common feature of human cancers [55] and it has been suggested to underlie the down-regulation of claudins in certain tumors. For example, a CpG island was identified within the coding sequence of the claudin-4 gene, and treatment with a methyl-transferase inhibitor restored expression of the protein in primary cultures prepared from high-grade human bladder tumors [56]. Furthermore, claudin-4 expression also correlated with its gene methylation profile in healthy and tumoral bladders from 20 patients and claudin-6 expression is partially silenced by promoter CpG island hypermethylation in MCF-7 breast carcinoma cells, while a synergistic effect of a demethylator and histone deacetylase inhibitors upregulates the expression of endogenous claudin-6, and sensitizes the cells for apoptosis [57]. Intuitively, the mechanism by which decreased claudin expression might lead to the compromised TJ function and thus, neoplasia is easy to comprehend, but how increased claudin expression contributes to neoplastic progression is less clear. One plausible mechanism is that upregulation or aberrant tissue expression of certain claudins may contribute to neoplasia by directly altering TJ structure and function. Furthermore, it is postulated that claudins may also affect cell signaling pathways. Claudin proteins are likely involved in signaling pathways *via* binding domains to ZO-1 at their carboxyl terminus [58].

Cell–cell adhesion proteins are known to play an important role in cellular transformation when displaced from their normal membrane localization and could serve as oncogenic molecules, the best studied molecule being β-catenin [59]. A similar functional heterogeneity could be postulated for claudins, however, further

studies are needed to support such a notion. An increase in claudin-1 expression has been reported in human primary colon carcinoma, in metastasis samples and in the cell lines derived from primary and metastatic tumors compared to their normal counterparts [54]. Crucially, there was nuclear localization of claudin-1 in a significant subset of colon cancer samples, particularly among the subset of liver metastatic lesions. Nuclear localization of several cell junction proteins (β-catenin, ZO-1, ZO-2) is known to be correlated with oncogenic transformation and cell proliferation [60]. Mutants of the TJ protein ZO-1 that no longer localize at the plasma membrane induce dramatic EMT in Madin-Darby canine kidney I cells [61]. Similarly, genetic manipulations of claudin-1 expression in colon cancer cell lines induced changes in cellular phenotype, with structural and functional changes in markers of EMT, and had significant effects upon the growth of xenografted tumors and metastasis in athymic mice. Notably, regulation of E-cadherin expression and β-catenin/Tcf signaling emerged as one of the potential mechanism underlying claudin-1 dependent changes and thereby suggested complex interplay between different cell–cell adhesion molecules [54]. Expression of specific claudin family members can be regulated by the wingless-type MMTV integration site family (Wnt) signaling pathway. Claudin-1 and claudin-2 are shown to be target genes regulated by β-catenin signaling [62,63].

Metastasis is a complex phenomenon that requires a number of specific steps such as decreased adhesion, increased motility and invasion, proteolysis, and resistance to apoptosis [64]. Claudin-5 promotes processing of pro-matrix metalloproteinase-2 (pro-MMP-2) by membrane type 1-MMP (MT1-MMP). Expression of claudin-5 not only replaced tissue inhibitors of metalloproteinases (TIMP)-2 in pro-MMP-2 activation by MT1-MMP but also promoted activation of pro-MMP-2 mediated by all MT-MMPs and MT1-MMP mutants lacking the transmembrane domain (DeltaMT1-MMP) [65]. It appears that interaction of MMP with claudins might play an important role in tumorigenesis, invasion and metastasis mediated by claudin expression. It has been observed that overexpression of claudin-1 in colon cancer cells increased activity of both MMP-2 and MMP-9 while inhibition of claudin-1 resulted in a significant decrease in MMP-9 activity [54]. Similarly, overexpression of claudin-3 or 4 in ovarian epithelial cells increased MMP-2 activity [52]. An increase in mRNA transcription and protein expression of MT1-MMP was also observed in claudin-10 overexpressing cells, in which claudin-1, -2, and -4 were also upregulated, suggesting that the expression of claudin-10 in cancer cells may dysregulate the expression of other claudin family members [66].

Most malignant tumors are derived from, and most pathogens invade the body *via* the epithelium. The epithelium is therefore a potent target for improving drug absorption, treating cancer, and curing infectious diseases. Modulation of TJ seals is a popular strategy for improving drug absorption. TJs compartmentalize the apical and basal membrane domains of epithelial cells, leading to the formation of cellular polarity. Loss of cell–cell interaction and cellular polarity, which often occurs in cancer cells during carcinogenesis, leads to exposure of TJ components on the cellular surface. The claudin family of proteins is an attractive target for antitumor therapy considering the epithelium-specific expression and the high specificity of claudin expression patterns in cancer. It is worth mentioning that claudin family members are expressed in a precise tissue-specific manner and thus could serve as tumor specific biomarkers. In this regard, a set of four markers, including claudin-3, was found to be sufficient to accurately identify all 158 ovarian cancers tested, including eight early-stage serous cancers [67]. Furthermore, claudin expression may be used as a prognostic indicator because high claudin-1 expression has been shown to be associated with tumor progression and metastasis in colon cancer [68]. At the same time, in breast cancer, claudin-1 expression is differential between the subtypes and low *versus* high claudin-1 expression helps identify highly aggressive triple negative breast cancer [69]. Similarly, claudin-10 expression has been shown to be an independent prognostic factor for hepatocellular carcinoma recurrence after curative hepatectomy [70]. Regarding the identification of the claudin family of proteins as tools to identify and/or classify tumor types, serial analysis of gene expression (SAGE) studies of the breast [71] and ovarian [72] cancers have allowed for the first time the identification of specific claudin family members as potential biomarkers for these cancers. Although large scale analysis in a clinical setting will be required to establish such potential of claudins, basic research on claudins is likely to remain valuable for providing important insights into normal and neoplastic cellular physiology. Preclinical studies have shown that tumor cells overexpressing claudins can be successfully targeted *via* several approaches, including the use of anti-claudin antibodies as well as the cytolytic enterotoxin from *Clostridium perfringens*. However, most of the studies have concentrated primarily upon claudin-3 and claudin-4 [73]. Both of these proteins have been identified as targets of *C. perfringens* enterotoxin (CPE) and have been reported to be overexpressed in multiple tumor types including ovarian and prostate cancer. Yet another potential approach that has been suggested is the use of claudins as drug delivery system using *Pseudomonas aeruginosa* exotoxin A (PE). PE is widely used in cancer-targeting studies as it binds to the cell surface and is internalized *via* endocytosis. Following this, a PE fragment, protein synthesis inhibitory factor (PSIF), escapes from the endosome to the cytosol [74], where it inhibits protein synthesis by inhibiting elongation factor 2. PSIF lacks the receptor-binding domain of PE, and fusion of a tumor antigen such as claudins with PSIF is a promising strategy for cancer-targeting therapy. Therapies specific to certain claudin family members could also serve as adjuvant therapies. Highly increased and cytosolic/nuclear claudin-1 expression in colon cancer has been reported [54,75] and claudin-1 dysregulation modulates the balance between the Notch- and Wnt-signaling to dysregulate colonocyte differentiation and promotes tumor growth and progression. Since Notch and or Wnt-signaling inhibition carries inherent high toxicity, the use of claudin-1 based therapy may provide an alternative.

### 2.2. Cell–matrix adhesion

Interaction and adhesion between cells and the surrounding extracellular matrix (ECM) classically involves cell surface integrins which interact and bind ECM protein components [76]. Functional integrins consist of a heterodimer structure made up of different α- and β- subunits and different integrin structures possess differing affinities for different matrix proteins [76]. The interaction between integrins and the ECM triggers a series of intercellular events that not only results in the adhesion of the cell to the ECM but also forms a mechanism for communication between intracellular events and the surrounding ECM. This process of cell–matrix adhesion is essential for the attachment of cancer cells to the surrounding matrix and subsequently the degradation of the matrix barrier [9]. A number of integrins have been linked to metastatic likelihood and cancer and/or stromal cells may deposit ECM proteins that again can enhance metastatic progression. Blocking the extracellular part of the cell–matrix adhesion by means of antibodies, small peptides, and other natural- and phytochemicals has been demonstrated and has been covered by another article in this issue. However, blocking the intracellular signaling event has also proved to be a useful approach in inhibiting this important event during cancer metastasis. Key events following the matrix–integrin interaction include activation of the focal adhesion kinase (FAK), paxillin and downstream chain signaling events [77]. Thus, inhibiting FAK and paxillin has become a hotly pursued approach in recent years.

CD44 represents another key cell adhesion molecule that holds potential as an antimetastatic target both through its role in interacting with other cell types and the ECM. The *CD44* gene, located at human chromosome 11p13, encodes the CD44s and CD44v isoforms, which arise through alternative splicing. CD44s and CD44v isoforms share the extracellular globular region that includes binding sites for hyaluronan, collagen, laminin and fibronectin as well as the cytoplasmic tail region that includes binding sites for ERM domain proteins (Ezrin, Radixin and Moesin), Ankyrin and S6 kinase related kinase (SRK). CD44 functions as a hyaluronan receptor, co-receptor for growth factors and as an adhesion molecule [78–82]. CD44 is involved in the malignant phenotypes of cancer stem cells, including EMT, invasion, metastasis, recurrence, resistance to chemotherapy and resistance to radiation therapy [82–85], which clearly indicates that CD44 is a potential target of cancer therapy. Humanized anti-CD44v6 monoclonal antibody BIWA-4 (bivatuzumab), paclitaxel-conjugated hyaluronan prodrug HYTAD1-p20 (ONCOFID-P), SN-38-conjugated hyaluronan prodrug ONCOFID-S, hyaluronan–irinotecan complex and other hyaluronan-conjugated drugs or siRNAs have been developed as cancer therapeutics [86]. Therapeutics targeted to cell–matrix adhesion may represent a useful strategy to block cancer cells from settling on and subsequently penetrating vascular or cavity lining and hence negatively impacting their ability to establish secondary tumors in the new site.

### 2.3. Cellular migration

While essential to normal development and homeostasis, the process of cellular migration is also a trait essential for metastasis. Enhanced migration is key across the metastatic cascade and is involved in the initial scattering of cells and migration from the primary tumor, the penetration of the basement membrane and ECM and intravasation and extravasation of vessels. The migration of cells requires a number of intra- and extra-cellular events such as the detection of extracellular signals by the cells, synthesis of cell surface proteins and the coordination of intracellular signaling and cytoskeleton proteins. Throughout the literature, cell migration has been tightly linked to cancer progression and metastasis. Numerous proteins and pathways have been implicated in altering the migratory potentials of cancer cells and therefore their aggressive nature [87,88]. Hence, given its essential role in cancer progression, treatments that inhibit cell migration or such proteins/pathways involved in enhancing cellular motility represent an attractive strategy for controlling metastatic dissemination. While in normal physiology cellular migration is less active, there are processes where it is essential, such as wound healing, and hence must be taken into consideration. Currently there are many compounds that inhibit cellular migration, although very few have been tested in a clinical setting.

### 2.4. EMT

The process through which epithelial cells undergo a series of morphological and biochemical changes to take on a more mesenchymal phenotype is known as epithelial–mesenchymal transition. EMT is widespread throughout normal development but has also been linked to the establishment of a more invasive, motile cancer cell phenotype facilitating detachment and dissemination away from the primary tumor [89–92]. EMT involves the loss of cell–cell adhesion and the polarized epithelial morphology through the characteristic loss of epithelial cell junctional proteins such as E-cadherin, claudins and ZO-1, and a subsequent increase in mesenchymal markers such as N-cadherin, vimentin and fibronectin and cytoskeletal reorganization [91,93]. Indeed, the loss of E-cadherin and subsequent replacement with N-cadherin ('cadherin switching') is a characteristic of EMT, seen in many cancer types and is thought to account somewhat for the enhanced invasive and motile properties of cancer cells [8]. Unsurprisingly, alterations in cell adhesion molecules (CAM) such as E-cadherin, impact the processes of cell–cell adhesion and cell–matrix adhesion and subsequently their metastatic potential. E-cadherin plays an essential role in the adhesion of cells and tissues and together with other members of the adhesive complex, such as β-catenin, regulates cell adhesion, signaling and transcription in cancers and control metastatic progression [94]. Indeed, studies have demonstrated an association between loss of E-cadherin and α-catenin expression with enhanced tumor cell invasiveness [95]. Other work has demonstrated an inverse correlation between E-cadherin expression and tumor cell invasion and motility and similarly with metastatic disease in cancer patients [96]. The translocation of β-catenin from the adhesive structure to the nucleus, an event leading to transcriptional activation of a number of target genes has also been demonstrated to correlate with development of a mesenchymal phenotype [97,98].

Initiation signals, such as HGF, EGF and transforming growth factor β (TGF-β) are believed to onset the EMT process, resulting in upregulation of EMT-inducing transcriptional factors such as Snail, Slug and Twist [99–102]. Slug, Snail and Twist have been implicated in influencing the expression of EMT proteins and are hence linked to metastasis [103–105]. For example Slug and Snail are involved in the down-regulation of E-cadherin [99,106] and the expression between Snail and E-cadherin is inversely correlated in a number of cancers including breast cancer [107]. Similarly, as discussed in Section 2.1.1, Snail exerts regulatory effects over members of the TJ such as the claudins. These initiation factors also act on other effector molecules to bring about EMT, such as the MMP family. Members of this family of proteinases play key roles in matrix-degradation, invasion, motility and adhesion and are frequently dysregulated in cancer progression. Slug and Snail have both been implicated in the upregulation of MMP-2 and MMP-9 and subsequent EMT initiation [108].

The process of EMT and subsequent acquisition of an invasive, motile phenotype with enhanced likelihood of invasion and dissemination represents a key interest in cancer research. Therapeutic strategies that can specifically target this process in cancer cells are likely to be effective in reducing the metastatic potential of tumor cells.

### 2.5. Molecular networks in the tumor microenvironment

It is now well established that solid tumors are not simply aggregates of replicating neoplastic cells but are also living entities, composed of numerous cell types, whose complexity approaches, and may even exceed, that of normal healthy tissues. Many non-malignant cell types, referred to as the stroma, populate, at majority, the solid tumors. These non-malignant cells include fibroblasts, resident epithelial cells, pericytes, myofibroblasts, vascular and lymphovascular endothelial cells and infiltrating cells of the immune system. During malignant progression, neoplastic cells acquire the ability to recruit, incorporate and reprogram the biology and the function of these healthy host cells, thus providing them with support, essential nutrients and weapons to hamper antitumor immune activity. In turn, the recruited non-malignant cells respond by enhancing the neoplastic phenotype of the nearby cancer cells, which again feed signaling back to the stroma to continue its reprogramming. Thus, the previous idea that the malignant phenotype of tumor cells was exclusively determined by cell-autonomous genetic and epigenetic alterations is now replaced by the hypothesis that the malignant progression of cancer not only depends on tumor cells' genetic aberrations but also on the bidirectional, dynamic and intricate network of interactions between

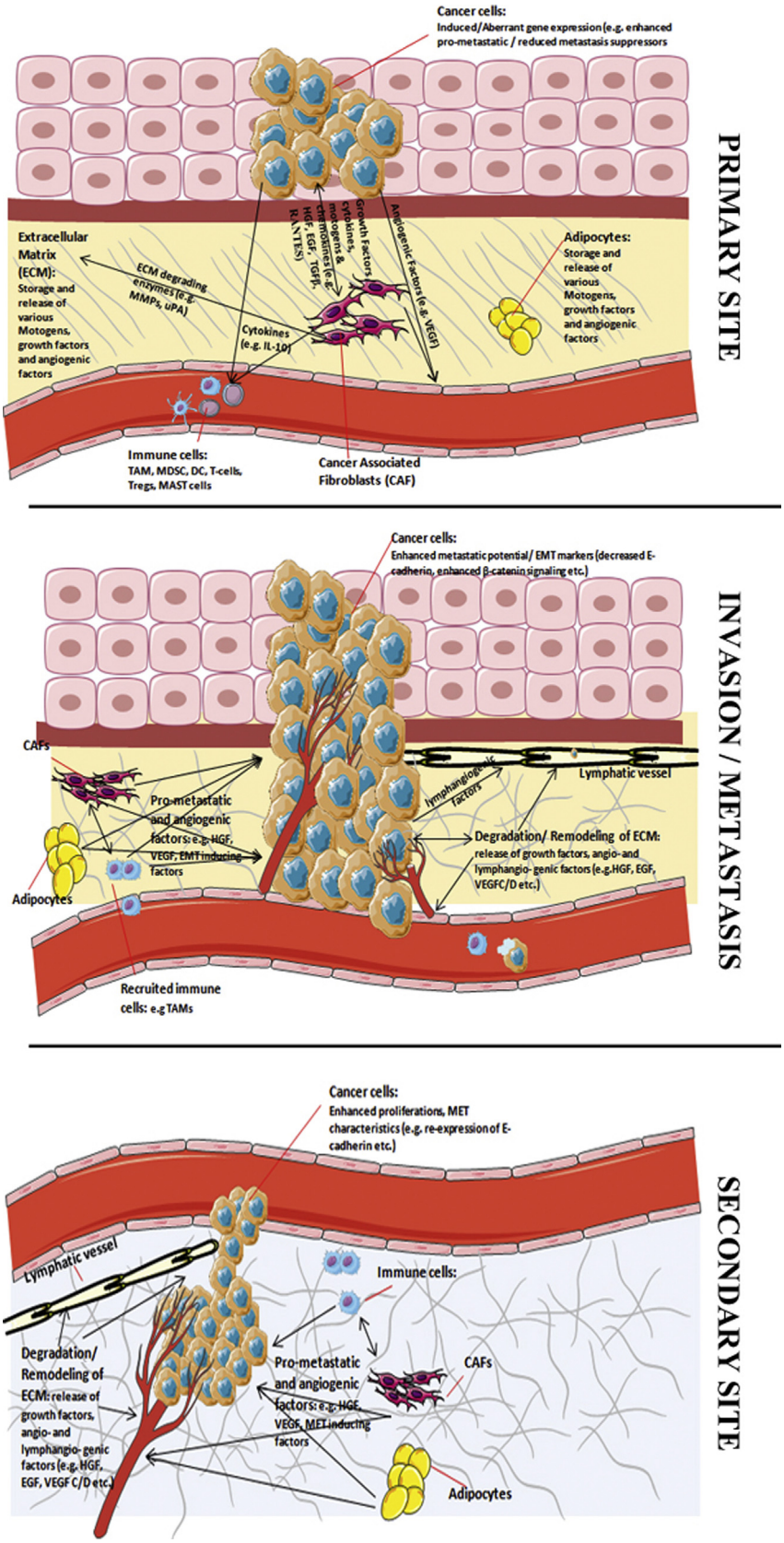


**Fig. 2.** Cellular interactions within the tumor microenvironment. Numerous interactions between cell types are involved throughout tumor progression and metastasis. Communication between main components of the surrounding microenvironment play vital roles in enhancing metastatic potential, epithelial to mesenchymal transition (EMT), immune-evasion, mesenchymal to epithelial transition (MET) and angio- and lymphangio-genesis.

the cells of the stroma and cancer cells within the tumor microenvironment [109,110] (Fig. 2).

Among the non-malignant cells that inhabit the tumor microenvironment, cancer-associated fibroblasts (CAFs) and tumor infiltrating-immune inflammatory cells are noteworthy because of the roles they play in tumor development and malignant progression. CAFs secrete factors that act on tumor cells in both paracrine and autocrine fashions, thus resulting in a more aggressive cancer phenotype. Across most cancers, activated CAFs secrete a wide variety of growth factors, chemokines, collagens, and ECM-modifying enzymes, which collectively supply a communication network and an altered three-dimensional ECM scaffold that together govern proliferation of cancer cells and tumor invasion and metastasis across tissue types. They also contribute to tumor progression by recruiting tumor-promoting immune cells and supporting angiogenesis. The tumor infiltrating-immune cells include the tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), dendritic cells (DCs), tumor infiltrating T cells, regulatory T cells (Tregs) and mast cells [109]. Tumor cells secrete chemokines and cytokines that are able to recruit mast cells, DCs, TAMs and MDSCs. Tumor cells also activate mast cells, promote the expansion of the MDSCs and the polarization of TAMs. Furthermore tumors both inhibit DC maturation through IL-10 secretion, thus leading to antigen-specific anergy, and reprogram the DCs, inducing them to exert immunosuppressive or angiogenic functions, thus resulting in an immunosuppressive and inflammatory tumor microenvironment. Once recruited to the tumor microenvironment, these immune cells can contribute to the malignant progression of the cancer-cell phenotype by supporting tumor proliferation, survival, invasion, metastasis, angiogenesis and ECM remodeling.

In cancer cells, the constitutive activation of various signaling pathways (including MAPK, signal transducer and activator of transcription 3 (STAT3) and β-catenin pathways) results in the secretion of cytokines which modulate the recruitment and function of the stromal cells. In particular, the tumor-derived regulated on activation, normal T cell expressed and secreted (RANTES)/Chemokine (C–C motif) ligand 5 (CCL5) cytokine stimulates CAFs to externalize the S100A4 protein, which stimulates tumor-cell survival and migration, up-regulation of the MMPs, down-regulation of TIMPs, activation of the nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB) and MAPK pathways, infiltration of T cells and finally, up-regulation of RANTES, thus generating a signal amplification loop. RANTES also induce angiogenesis and act as chemoattractants for additional effector immune cells. Tumor-derived stem cell factor (SCF) promotes the recruitment and activation of mast cells and the MDSC expansion. Tumors also secrete the thymic stromal lymphopoietin (TSLP) and bone marrow stromal cell antigen 2 (BST2). TSLP induces DCs to express OX40 ligand, which directs $CD4^+$ T cells to generate TH2 cells secreting IL-4 and IL-13. These cytokines prevent tumor cell apoptosis and indirectly promote the proliferation of tumor cells by stimulating TAMs to secrete EGF. BST2 is a ligand of immunoglobulin-like transcript 7 (ILT7), which is expressed on DCs surface. The interaction of ILT7 on DCs with BST2 on tumor cells results in inhibition of IFN-α and pro-inflammatory cytokines production by DCs with immunosuppressive effects.

Oncogene activation and subsequent signal activation in cancer cells trigger multiple cascades thus resulting in the secretion of several immunosuppressive molecules, including TGF-β, IL-10, IL-6, vascular endothelial growth factor (VEGF), CCL2/monocyte chemoattractant protein 1 (MCP1), cyclooxygenase-2 (COX2), that induce the immunosuppressive immune cells. Production and secretion of these factors by both cancer and surrounding cells enhance tumor cell proliferation, migration and invasion. Furthermore it enhances the production of immunosuppressive cytokines and chemokines, including TGF-β itself, IL-10 and CCL2/MCP1. TGF-β and the potential for targeting this signaling pathway in cancer is discussed in Section 4.2. A plethora of recent reports has painted a consistent picture of how stromal cells (CAFs and inflammatory cells) can promote malignant progression. Indeed, within the primary tumor microenvironment, the stromal cells provide potent oncogenic signals, such as TGF-β, HGF, EGF, Wnt, and basic fibroblast growth factor (bFGF), which stimulate cancer-cell proliferation, survival and invasion, thus facilitating metastasis. Moreover, these cells produce several angiogenesis-modulating enzymes, such as VEGF, thymidine phosphorylase, MMP-2, MMP-7, MMP-9, MMP-12, COX2, urokinase plasminogen activator (uPA) and cathepsins B and D, which together degrade the ECM, again promoting metastasis. TAMs promote carcinoma-cell motility and invasion through a paracrine signaling loop between the tumor cells and the TAMs. Within this loop the macrophages express EGF, which promotes formation of elongated protrusions and cell invasion by carcinoma cells. In addition, EGF promotes the expression of colony stimulating factor 1 (CSF-1) by the carcinoma cells, which further promote the expression of EGF by macrophages generating a positive-feedback loop. The secretion of stromal-cell-derived factor 1 (SDF1), also known as chemokine (C–X–C motif) ligand 12 (CXCL12), by TAMs and CAFs at a tumor site can enhance the invasion, intravasation, metastasis formation and recruitment of MDSCs, TAMs and endothelial cells to the primary tumor. This enhancement of invasion and intravasation depends upon chemokine (C–X–C motif) receptor 4 (CXCR4) signaling, and it is most likely to occur through activation of CXCR4 on macrophages, which results in increased paracrine interactions with tumor cells in the tumor microenvironment. Increased CXCL12/SDF1 secretion also gives rise to an increased microvessel density, which might also be mediated by TAMs and might contribute to an altered tumor architecture, thus resulting in increased intravasation through the presence of a higher density of entrance sites into the blood, with a corresponding increase in the formation of metastases. The significance of CXCL12/CXCR4 signaling in breast cancer invasion and metastasis is widely appreciated. CXCR4 expression in breast cancer cells has been shown to increase metastasis through the homing of tumor cells to sites of increased CXCL12 expression, such as the lymph nodes. Similarly, the interaction of CXCL12/SDF1 and CXCR4 expressed on mammary adenocarcinoma MTLn3 cells increases the chemotactic and invasive behavior of these cells to CXCL12/SDF1, as well as their motile behavior within the primary tumor and their ability to intravasate. TAM-derived CCL17 and CCL22 chemokines preferentially attract T cell subsets that are devoid of cytotoxic functions, such as Tregs and Th2 lymphocytes. TAM-derived CCL18 recruits naïve T cells, which induce T cell anergy. Within the tumor microenvironment IL-10, secreted not only by immune cells, but also by CAFs and tumor cells, is the main cytokine responsible for the establishment of the immunosuppressive milieu. Furthermore, IL-10, together with IL-4, IL-6 and IL-13, induces monocyte differentiation toward a mature M2-polarized phenotype that is characteristic of TAMs. At the tumor site, the IL-1β and IL-6 cytokines, S100A8 and S100A9 pro-inflammatory proteins and the chemoattractant molecules CCL2/MCP1, CXCL12/SDF1 and CXCL5 are the main factors that are responsible for the recruitment and the induction of MDSCs. VEGF is one of the main factors responsible for the expansion of MDSCs, while IL-4, IL-13, IFN-γ, IL-1β and TGF-β turn on their suppressive functions. MDSCs produce high levels of IL-17, which further exacerbates the inflammatory tumor microenvironment.

The growing body of evidence regarding the roles played by non-malignant cells of the tumor microenvironment in promoting tumor progression indicate that it is conceivable that these cells can serve as novel therapeutic targets in the cancer treatment. For this purpose, several therapeutic approaches that use small

**Table 1**
Effects of approved and experimental targeted agents on tumor cells and tumor microenvironment stromal cells.

| Drug | Drug class | Target | Effect on tumor | Effect on the immune system | References |
|---|---|---|---|---|---|
| STI571 (Gleevec or imatinib mesylate) | Small molecule inhibitor | PDGFR and c-Kit | Reduces microvessel density | Prevents mast cell proliferation and survival | [283,284] |
| Bevacizumab | Monoclonal antibody | VEGF | Blocks angiogenesis | Increases DC maturation, shifts DC differentiation toward mature DCs instead of MDSCs and increases DC priming of T cells | [285,286] |
| IM-2C6 | Antibody | VEGFR | Blocks angiogenesis | NA | [287] |
| SU5416 | Small molecule inhibitor | VEGFR | Reduces vascular density | NA | [288] |
| MMI-166 | Small molecule inhibitor | MMP-2 and MMP-9 | Suppresses MMP-2 and MMP-9 activities; inhibits angiogenesis and tumor growth | NA | [289] |
| S-3304 | Small molecule inhibitor | MMP-2 and MMP-9 | Inhibits MMP-2 and MMP-9 | NA | [290] |
| Dasatinib | Small molecule inhibitor | c-Kit, ABL, SRC, PDGFR | Induces apoptosis in leukemic cell | Induces apoptosis in mast cell | [117] |
| Dipyridamole | Small molecule | Wnt, MAPK and NF-κB pathways | Decreases tumor growth and metastasis | Decreases TAM and MDSC infiltration | [118] |
| Bindarit | Small molecule | CCL2/MCP1 | Decreases tumor growth and metastasis | Decreases TAM and MDSC infiltration | [119] |
| Upanap-126 | RNA aptamer | uPA | Delays the proteolitic conversion of pro-uPA to active uPA; inhibits tumor cell invasion; reduces the tumor cell intravasation and dissemination | NA | [111] |
| ATN-658 | Monoclonal antibody | uPA receptor (uPAR) | Decreases tumor cell invasion and migration and tumor volume | NA | [112] |
| L2G7/Rilotumumab | Monoclonal antibody | HGF | Inhibits the tumor growth | NA | [113,291,292] |
| Trastuzumab | Monoclonal antibody | HER2 | Blocks growth signals | Primes antitumor CTLs, boosts NK secretion of IFN-γ and mediates potent antibody-dependent cellular cytotoxicity | [293] |
| Cetuximab | Monoclonal antibody | EGF receptor (EGFR) | Blocks growth signals | Immune activating: increases MHC class I and MHC class II expression; augments DC priming of tumor-specific CTLs. | [294] |
| MGA271 | Monoclonal antibody | B7-H3 | NA | Mediates potent antibody-dependent cellular cytotoxicity | [295] |
| AMD3100 | Small molecule | CXCR4/CXCL12 (SDF1) signaling | Sensitizes cancer cells to chemotherapy: inhibits tumor growth | Reduces the recruitment of bone-marrow derived cells | [114–116] |
| Celecoxib | Small molecule inhibitor | COX2 | NA | Decreases both MDSC numbers and function | [296] |
| 5-Fluorouracil | Small molecule | Thymidylate synthase | Promotes the cytotoxicity of tumor cells | Induces MDSC apoptotic cell death | [297] |
| All-trans-retinoic acid (ATRA) | Vitamin A derivative | NA | NA | Reduces MDSCs | [298] |
| Sclareol | Phytochemical | NA | Decreases the tumor size | Decreases the number of Tregs | [120] |
| Temozolomide | Small molecule | DNA | Promotes the cytotoxicity of tumor cells | Reduces the number of Tregs | [121] |

molecule inhibitors, antibodies or phytochemicals that specifically target molecules and signaling pathways involved in the recruitment, activation and function of tumor infiltrating non-malignant cells have been tested in both animal models and human. Table 1 summarizes the most up-to-date drugs available with potential use in cancer therapy, known effects on tumor cells and activity against tumor-stromal microenvironment communications. Several strategies to inhibit either CAF activation or CAF-derived factors (*e.g.* HGF, uPA, CXCL12/SDF1) have been applied in preclinical studies of cancer therapies and the results have shown efficacy in the inhibition of tumor growth and invasion [111–116]. Similarly, several immunotherapeutic approaches have been developed to target immune cells that infiltrate the tumor. Some anti-angiogenic agents impair proliferation and survival of mast cells and induce DC maturation and their antitumor activity (*e.g.* STI571 and bevacizumab). The impairment of the stem cell factor (SCF)/c-Kit signaling pathway by dasatinib induces apoptosis of both tumor cells and mast cells [117]. Several immuno-therapeutic strategies that target MDSCs and that can neutralize their immunosuppressive effects have been reported in both animal models and human. These strategies include approaches that are aimed at the induction of differentiation of these immature cells [*e.g.* all-trans-retinoic acid (ATRA)], or of a decrease in their number and tumor infiltration (*e.g.* dipyridamole and bindarit), or at interfering with their immunosuppressive functions (celocoxib), or killing MDSCs (5 fluorouracil- or 5FU). Interestingly, dipyridamole [118] and bindarit [119] decrease the infiltration not only of MDSCs but also of TAMs in breast and prostate cancer proof of concept animal model studies. Finally sclareol and temozolomide reduce tumor growth and the number of tumor infiltrating Tregs [120,121]. Therefore, although further studies will be needed to determine which cell(s) is/are the best therapeutic target(s) and which drugs are the most efficient

and selective, there is no doubt that the therapeutic targeting of tumor microenvironment cells represents a valuable strategy to complement conventional anticancer strategies.

### 2.6. Cancer stem cells (CSC)

Cancer stem cells (CSC) present an exciting yet somewhat controversial field in cancer research. In the cancer stem cell model of carcinogenesis there is a hierarchical organization of cancer cells. The CSC represent a highly tumorigenic sub-population of cancer cells that can be isolated from other cancer cells in the same tumor. These highly tumorigenic cells have been proposed as crucial to the growth and development of primary tumors and are believed to be resistant to conventional therapy and therefore likely to be responsible for disease recurrence and treatment failures. CSC were first isolated in acute myelogenous leukemia (AML) and subsequent investigations of solid tumors have revealed the presence of highly tumorigenic cancer cells (CSC) in essentially every solid cancer type including breast, lung, colon, pancreas, head and neck [122–127].

The critical characteristics of a CSC require these cancer cells to be: tumorigenic, able to reproduce the original tumor heterogeneity including both the tumorigenic and non-tumorigenic subpopulations of cancer cells, self-renewing, and separable from the other cancer cells. CSC typically represent only a small subpopulation (<10%) of the entire cancer cell population. A variety of cells surface markers and biological markers have been used to isolate the CSC population from other cancer cells, including CD24, CD44, CD26, CD133, epithelial specific antigen (ESA), and aldehyde dehydrogenase activity (ALDH) [128]. So far, no single marker or combination of markers has proven useful for isolating CSC from every tumor site. The expression of CSC markers in primary tumors has been found in some studies to be associated with tumor stage, prognosis and response to therapy. It is a logical extension of the CSC theory to hypothesize that, as CSC are the only cancer cells that can produce a primary tumor, CSC must also be essential for the development of metastatic disease.

In breast cancer, cells characterized by high CD44 expression and low levels of CD24 expression (CD44+CD24−/low) have been shown to encompass the CSC subpopulation of cancer cells [125]. Another marker for the identification of CSC in breast cancer is high levels ALDH expression, also a marker for many normal stem cell populations [123]. By comparing gene expression profiles between ALDH+ and ALDH− breast cancer cells, a 413-gene breast CSC signature was determined. Among the differentially expressed genes, the gene encoding for the IL-8 receptor CXCR1/IL-8RA, previously described to be involved in the regulation of cancer growth and metastasis, was found. The ALDH + CSC derived from breast cancer cell lines were shown to be significantly more metastatic than ALDH− cells by intracardiac injection in Nonobese diabetic/severe combined immunodeficiency (NOD-SCID) mice indicating a possible role for CXCR1/IL8-RA in the metastatic potential of breast CSC. Additionally the same CSC-enriched populations gave rise to extra-pulmonary metastases in the pancreas, liver, spleen and kidney [129]. Similar results have been shown in head and neck cancer where the CSC collected based on CD44 expression were shown to be essential for metastatic formation in a tail vein model and in an orthotopic head and neck cancer model [130,131]. This data supports the concept that CSC are critical to the development of metastasis.

There is accumulating evidence of cellular heterogeneity within the CSC compartment with some CSC exhibiting an enhanced potential for the development of metastasis. Evidence for metastatic and non-metastatic CSC was first raised in cancer of the pancreas. The pancreatic CSC population is defined by CD133 expression. *In vivo* studies of the CD133 + CSC revealed a subpopulation of migrating CSC defined by surface expression of CXCR4. CXCR4 is a protein that has previously been implicated in cancer cell metastatic potential [132]. Using an orthotopic model of pancreatic cancer only the migrating CSC population, expressing CD133+CXCR4+, were able to establish liver metastasis. Inhibition of CXCR4 significantly reduced the CSC metastatic potential [133]. These results are significant as the first observation of the importance of the CSC phenotype to their metastatic potential as well as the role of CXCR4 in regulating this behavior in CSC [134].

Additional evidence of the existence of different CSC subtypes responsible for specific behaviors exists in colon cancer. It has been reported that colon CSC have three distinct phenotypes; self-renewing long-term (LT-TICs), tumor transient amplifying cells (T-TAC), and delayed contributing (DC-TICs). Interestingly the self-renewing LT-TICs were the only subpopulation of CSC able to contribute to metastasis formation [135]. More recently, CD26 was confirmed as a marker for metastatic CSC in colon cancer [136]. None of the patients without CD26-expressing cancer cells in their primary tumors developed metastases, while the majority of those whose tumors contained CD26+ cells did. In animal models, both CD26+ and CD26− cells were capable of giving rise to primary tumors, however, only CD26+ cells had the capacity to produce metastasis [137]. These reports confirm the importance of CSC, and more specifically the migratory subpopulations of CSC, to the development of metastasis in colon cancer.

EMT has been implicated as an important mechanism by which cancer cells gain metastatic potential. Many cancer types have been shown to exhibit EMT. EMT is believed to represent a crucial step toward cancer cells acquiring invasiveness and the potential to produce metastasis [138]. There is accumulating evidence that CSC undergo EMT and this ability has important regulatory functions related to CSC behavior. Studies have revealed that EMT can induce apparently differentiated cancer cells to gain a CSC-like phenotype increasing their tumorgenicity and their ability to migrate to and invade tissues distant from the primary tumor. Additionally, cancer cells undergoing EMT have been shown to be enriched for CSC [139]. CSC express many EMT regulating factors including TWIST, Snail and Slug suggesting these genes play an important regulatory role in CSC behavior [140].

The preferential location of EMT cells along the invasive front of tumors and the association of EMT with high Wnt signaling levels have been demonstrated. This includes the nuclear accumulation of β-catenin, evidence of Wnt activation, observed in cells undergoing EMT at the invasive front [134]. Signals from the tumor microenvironment have been demonstrated to elicit Wnt signaling in colon cancer cells, inducing EMT and allowing for their detachment and spread from the primary tumor site. Observations regarding EMT and Wnt expression in locations within primary tumors where CSC typically reside led to the concept of migrating CSC as proposed by Brabletz et al. in 2005 [134].

EMT has been shown to be a reversible process in that mesenchymal-to-epithelial transition (MET) can transform mesenchymal cells back to their epithelial state. Similarly metastatic CSC may respond to local cues to revert from their mesenchymal state back to their original epithelial state. Once the CSC have returned to their original epithelial condition they can form growing metastatic deposits. It is highly likely that CSC lead to the development of metastases by acquiring mesenchymal properties that enhance their ability to migrate and invade and then transition back to their epithelial phenotype to form a metastasis. The factors that regulate EMT in cancer cells are being studied in multiple cancer types but are not yet fully understood. The tumor microenvironment has been proposed as an important regulator of EMT in CSC including local factors such as hypoxia, cytokines including IL-6 and cancer associated cells including tumor associated fibroblasts, mesenchymal stem cells, and lymphocytes able to secrete diffusible EMT-inducing signals [141,142].

Although attractive and in agreement with many genetic analyses and with the evaluation of CSC in primary tumors and animal models, to date the causative role of CSC in metastasis formation has not been formally proven. Metastases represent one of the key factors in treatment failures in patients with cancer. Recognition that CSC play a critical role in the development of metastasis is an important step toward increasing our understanding of how metastasis develop. The factors that modulate the CSC metastatic phenotype have not yet been fully elucidated and require more intensive investigation. This work will lead to more effective anticancer therapy and improved outcome for patients.

## 3. Cancer cell dissemination and the metastatic cascade

### 3.1. Organ specific metastasis

The predisposition of certain body sites or organs to house metastatic cells and establish secondary tumors has been apparent for centuries and has been famously explained in Paget's 'seed and soil' theory of metastasis [143]. This theory dictates that a specific tumor cell (the seed) will only establish in a particular suitable organ or location (the soil). Indeed, many cancers have an increased propensity to establish secondary metastasis at certain sites. For example, breast and prostate cancers appear to be predisposed to metastasizing to the bone environment whereas gastrointestinal cancers frequently metastasize to the lung and liver [2,144]. Indeed a few organs represent the main secondary destination for most cancers, namely, the liver, lung, bone and brain, while organs such as the spleen and heart rarely host metastasis. The factors underlying this organ specific predisposition for metastatic dissemination by many cancer types are largely unknown and are widely being studied within the scientific community. Establishment of such factors may again yield intuitive strategies to limit metastatic disease.

#### 3.1.1. Targeting bone metastasis

Bone metastases are a common complication of several types of cancers, including breast, prostate and lung cancer. The occurrence of these bone metastases deeply impact the prognosis and the quality of life of patients and are responsible for significant morbidity. Bone metastases are often osteolytic (due to significant bone destruction), sometimes sclerotic (due to an excess of bone formation) or mixed. Numerous mechanisms and factors are involved in the invasion, colonization and establishment of tumor cells in the bone microenvironment. The complex sequence of events that lead to the onset of bone metastases not only involves processes common to any other metastasis but also processes that are more specific to the bone tissue (tumor cell invasion in the bone environment, implantation of tumor cells in bone marrow, osteomimicry, deregulation of osteoblast\osteoclast activity) [145]. The current section illustrates progress made toward the understanding, treatments and management of this particular form of metastatic disease.

The spread of metastatic cells from the bloodstream to the bone marrow involves factors that are produced by osteoblasts and stromal cells in the bone marrow. Among these factors, a key role is played by chemokines (CXCL12, CXCL13, chemokine (C-X3-C motif) ligand 1 (CX3CL1), CCL22) that stimulate cancer cell migration to the bone marrow, since they express membrane receptors corresponding to these chemokines [145]. For example, CXCL12 and its receptor CXCR4 play an important role in bone tropism of cancer cells and treatment with inhibitors of CXCR4 (AMD 3100, T140) or CXCL12 (OTP-9908) has demonstrated efficacy in decreasing the formation of bone metastases in experimental models of breast cancer or prostate cancer [145,146]. In addition, some proteins (bone sialoprotein, osteonectin, osteopontin, collagen) can stimulate bone matrix invasion by binding the surface of tumor cells through specific membrane receptors such as integrins αVβ3 and α2β1 [145] and it has been demonstrated that breast cancer cells expressing αVβ3 integrin and prostate cancer expressing the αVβ2, have higher incidence of bone metastases [147]. There is preclinical evidence that αVβ3 integrin inhibition is able to prevent bone colonization by αVβ3 expressing human breast cancer cells [148]. Several ongoing clinical trials are evaluating the anticancer effect of integrin antagonists in advanced refractory and metastatic cancers, but only one phase I clinical trial is evaluating integrin antagonists (GLPG0187) in cancer patients with bone metastasis (NCT01313598) [149,150].

It has been demonstrated that the tyrosine kinase c-MET promotes stemness phenotype, tumor growth, invasion, and metastasis in several malignancies. In particular, c-MET is overexpressed in prostate cancer cells and is associated with tumor progression and metastatic invasion to bone [151]. Cabozantinib (XL184) is an oral small molecule inhibitor of multiple kinase signaling pathways including c-MET and vascular endothelial growth factor receptor 2 (VEGFR2). A recent phase II "randomized discontinuation" trial in patients with metastatic castration resistant prostate cancer (mCRPC) included 171 men with castration-resistant prostate cancer (CRPC from a larger phase II randomized discontinuation trial that included multiple tumor types treated with cabozantinib). The randomization was stopped after 122 patients because of improvements in bone scans and a decrease in pain. At the time the study was halted, a group of 31 patients had been randomly assigned. In this group, there was a marked improvement in the primary end point of progression-free survival (PFS) in the patients receiving cabozantinib compared with placebo ($p < 0.001$) [152]. Phase III trials are currently on ongoing.

Cathepsins are a class of globular lysosomal proteases that belong to the papain-like cysteine protease family expressed in a wide variety of tissues including the bone, where they appears to be a key enzyme in bone matrix degradation [153]. Different cancers express cathepsin K, including prostate and breast cancers [154]. Until recently, a role for cathepsin K in bone metastasis had been mainly attributed to its ability to degrade native collagen I, a process necessary for the expansion of the tumor within the bone. For example, the human breast, bone seeking, cancer cell line MDA-MB-231/B02 secreted cathepsin K and treatment of these cells with a cathepsin K antagonist can inhibit tumor invasion [155]. Due to its selectivity, odanacatib is the only cathepsin K inhibitor in clinical development. A phase II controlled study on women with breast cancer and established bone metastasis, randomized to receive daily administration of odanacatib or a single dose of zoledronic acid, showed reduced bone remodeling markers (urinary NTx) after 4 weeks treatment, demonstrating that odanacatib is as effective as zoledronate to reduce bone resorption markers [156].

Receptor Activator of Nuclear Factor-κB Ligand (RANKL), the Receptor Activator of Nuclear Factor-κB (RANK) and the decoy receptor osteoprotegerin (OPG) are members of the TNF and TNF-receptor superfamily, which are able to induce proliferation, differentiation, activation and apoptosis of osteoclastic cells. Bone remodeling is mediated by the interaction of RANKL expressed on the osteoblasts, RANK expressed on the osteoclast surface and OPG which prevents osteoclast activation [157]. Murine models have shown that RANKL is able to act as chemoattractant and as a pro-migratory factor in RANK-expressing breast and prostate cancer cell lines and that RANKL inhibition is able to reduce bone lesions and tumor burden in a melanoma model of bone metastasis [158]. It has also been demonstrated that RANK primary tumor expression levels correlate with the occurrence of bone metastases and that RANK-expressing cancer could be found in up to 80% of bone metastases originated from solid tumor [159,160], suggesting that RANK enables cancer cells to migrate to bone where RANKL

is abundantly expressed by osteoblasts. Some tumor cells may directly express RANKL, whereas others further enhance RANKL expression by cell-to-cell contact of tumor cells with osteoblastic cells. This leads cancer cells to enter a vicious cycle where they stimulate osteoclasts that express RANK. Bone degradation by osteoclasts creates further space for expansive tumor growth within the bone microenvironment which releases a variety of growth factors and cytokines stored in the bone matrix that further boost the proliferation of cancer cells [161]. Recently, it has been shown that DCs also express RANK, and therefore can be stimulated by RANKL, responding by upregulation of co-stimulatory molecules CD86, CD205 and cytokines such as TNF-α, IL-6, and IL-10 leading to Tregs lymphocyte expansion and subsequent local and systemic immunosuppression [162]. Together, these result in enhanced bone resorption, tumor invasiveness and evasion of the immune system by cancer cells. Denosumab (AMG 162) is a noncytotoxic IgG2 monoclonal antibody with an extremely high affinity for human RANKL. It was developed to treat patients with skeletal pathologies mediated by osteoclasts, such as bone metastasis, and cancer treatment-induced bone loss (CTIBL). Three large phase III randomized clinical trials were carried out in order to assess the efficacy of denosumab. In breast cancer bone metastatic cancer patients, denosumab was shown to be superior to zoledronic acid in delaying the time-to-first and time-to-first-and-subsequent skeletal related event (SRE) by 18% and 23%, respectively [163]. Moreover in castration resistant prostate cancer patients who suffered from bone metastasis, denosumab treatment significantly prolonged the median time to first on-study SRE (21 months) compared with zoledronic acid (17 months) ($p = 0.008$) [164]. In a third trial, a total of 1776 patients with osteolysis due to myeloma and solid malignancies other than breast and prostate cancer were enrolled showing a median time to first on study SRE of 21 months in the denosumab group compared to 16 months in the group receiving zoledronic acid, and demonstrating a non-inferiority ($p = 0.0007$) but neither a superiority for denosumab over zoledronic acid after adjustment for multiple comparison ($p = 0.06$) nor an advantage in overall survival [165]. Finally, in a recent phase III trial conducted in men with non-metastatic castration-resistant prostate cancer with a high risk of developing bone metastasis (NCT00286091), denosumab significantly prolonged bone-metastasis-free survival by a median of 4 months compared to placebo ($p = 0.028$), potentially confirming the role of RANK\RANKL in mediating cancer cell homing in to the bone [166].

The proto-oncogene Src (encoded by the *c-src* gene) is a non-receptor, membrane-associated tyrosine kinase that belongs to Src family kinases modulating key physiological and pathological processes such as cell proliferation, migration and the propensity of cancer cells to metastasize to the bone [167]. Moreover, Src coordinates both osteoclast and osteoblast activities; it positively regulates osteoclast survival and resorbing activity. Conversely, Src may negatively regulate osteoblast maturation through inhibition of runt-related transcription factor 2 (Runx2) regulated genes [168]. Thus, Src kinase is essential for osteoclast activation and osteoblast inhibition. Saracatinib is an orally active small-molecular-weight inhibitor of c-Src and breakpoint cluster region – c-abl oncogene 1, non-receptor tyrosine kinase (BCR-Abl) able to inhibit androgen-dependent and androgen-independent prostate cancer cell proliferation, *in vitro* migration and *in vivo* tumor growth [169,170]. Saracatinib also inhibited human osteoclast differentiation and osteoclast-mediated bone resorption *in vitro* [171]. In a phase II trial of saracatinib in patients with advanced CRPC, treatment was well tolerated and five patients displayed a slight reduction in prostate specific antigen (PSA) levels. Two phase II studies currently ongoing will compare the efficacy of saracatinib or zoledronic acid plus standard of care on bone turnover in patients with bone metastatic breast or prostate cancer (NCT00558272) and patients with metastatic hormone receptor-negative or locally advanced unresectable breast cancer (NCT00559507).

Tumor cells not only stimulate osteoclast activity, but also inhibit osteoblast activity. Inhibition of osteoblast activity has been linked to the production by the tumor cells of a soluble protein, namely Dickkopf-1 (DKK-1). DKK-1 protein was initially discovered as a protein secreted by tumor plasma cells in patients with multiple myeloma, but is also produced by tumor cells that induce osteolytic lesions *in vivo*. DKK-1 inhibits osteoblast activity by blocking the action of Wnt proteins on osteoblasts [145]. Wnt signaling in osteoblasts upregulates OPG expression and downregulates RANKL expression [172], suggesting a mechanism by which Wnt signaling in osteoblasts indirectly regulates osteoclastogenesis. Data from several tumor phenotypes suggest that DKK1 promotes osteolytic metastases, and may facilitate the conversion of osteoblastic metastases to an osteolytic phenotype. In prostate cancer cells, DKK1 was found to block osteoblastic metastases without affecting tumor growth, while inhibition of DKK1 in osteolytic prostate cancer cells switched bone metastases from osteolytic to osteoblastic. Anti-DKK-1 therapy on bone metabolism and tumor growth was recently experimented in mice. DKK-1-neutralizing antibodies restored the bone mineral density (BMD) of the implanted myelomatous bone, increased the numbers of osteocalcin-expressing osteoblasts and reduced the number of multinucleated tartrate-resistant acid phosphatase (TRAP)-expressing osteoclasts. Furthermore, anti-DKK-1-treated mice showed reduced tumors burden [173]. BHQ880, a fully human anti-DKK-1 neutralizing antibody, is currently under evaluation in phase I and II trials for patients with multiple myeloma (NCT00741377, NCT01302886 and NCT01337752).

Recent advances show that the adaption of metastatic cancer cells to the bone environment and the subsequent crosstalk between tumors and host tissue underpin their involvement in the development of skeletal metastasis. The development of therapeutics to interfere with processes involved in cancer cell colonization and establishment in the bone, as illustrated in this section, is key in the management of this metastatic disease. Further research to identify and establish such compounds is vital.

### 3.2. Metastatic routes

To successfully metastasize from the primary site, cancer cells need to successfully overcome a number of barriers. Transportation of cancer cells throughout the body to distant sites will occur through one or more of several routes, namely vascular, lymphatic and transcoelomic routes.

#### 3.2.1. Vascular spread

Vascular dissemination is perhaps one of the best studied routes of cancer spread. Here cancer cells breach the basement membrane and invade nearby blood vessels, either pre-existing or newly formed (angiogenesis), to gain access to the circulation and are subsequently transported to a distant site. Through either generic or specific mechanisms, cancer cells will arrest on the vascular bed and extravasate through the vessel wall to their secondary location. How cancer cells manage to evade and survive immunosurveillance and how angiogenesis can act as target for anticancer treatment has been covered by another article in the same issue [174]. The following section will focus the other two routes.

#### 3.2.2. Lymphatic spread

Lymphatic dissemination of cancer cells involves the transport of the cancer cells through the lymphatic system. Here, cancer cells invade into the lymphatic system and are transported to regional and distant lymph nodes and throughout the body to secondary sites.

Accurate staging is critical in predicting prognosis and tailoring therapy for almost every type of cancer. In the preface of the 7th edition of the American joint committee in cancer (AJCC) cancer staging manual, the editors state that the anatomic extent of disease remains the key prognostic factor in most cancers [175]. However, the current staging system has 2 major limitations. First, there are no preoperative investigations that can predict lymph node involvement with satisfactory accuracy. Similarly, targeted biopsy (*via* radiology guidance, endoscopic ultrasound, *etc.*) has an acceptable sensitivity (>85%) for accurately diagnosing positive regional lymph nodes, but only those which are completely replaced by tumor cells (*i.e.* metastatic lymph nodes, not micrometastic disease). Precision in the preoperative detection of lymph node metastases is of great importance as the trend toward individualized cancer care and minimally invasive surgery gathers momentum. Second, the not infrequent observation of later tumor recurrence in patients who have seemingly had a complete resection of their tumor suggests that clinically undetectable tumor deposits must be present at the time of operation and the fact that lymph nodes are a frequent site of tumor recurrence indicates that this compartment must be an important site for occult disease. Recent studies indicate that 1–17% of histologically negative lymph nodes, and 11–50% of pathologically node negative patients have nodal metastases that were missed by routine pathologic examination [176,177]. This therefore means that a patient's pN designation is often incorrect and results in suboptimal treatment decisions. Robust, sensitive immunohistochemical techniques using antibodies to detect epithelial tumor cells in lymphatic tissue have been in use since the mid-1990s [178]. It is therefore surprising that no consensus exists regarding the prognostic significance of immunohistochemically identified isolated tumor cells (ITCs) in many tumor types [179–194]. The main reason for this is the lack of unequivocal results showing their prognostic significance in various solid tumors. However, many studies suffer from small numbers of patients, limited analysis of existing paraffin blocks, and, most importantly, varying definitions for both isolated tumor cells and micrometastases. As per the 7th edition of the AJCC cancer staging manual, micrometastases are occult metastases greater than 0.2 mm but not greater than 2.0 mm in size, while ITCs are defined as small clusters of cells not greater than 0.2 mm, or nonconfluent or nearly confluent clusters of cells not exceeding 200 cells in a single histologic lymph node cross section [175]. In esophageal cancer, it was found that the distinction between an isolated tumor cell and a micrometastasis was not important [195]. Patients with either of these in one or more lymph node(s) had significantly reduced overall survival compared to patients who remained node negative after serial sections and immunohistochemistry. The importance of isolated tumor cells in lymph nodes has been reported not only in esophageal cancer [181,184–187,189], but also in several studies of gastric cancer [196,197], melanoma [198], breast cancer [199–202], colorectal cancer [203–205], and non-small-cell lung cancer [206]. Lymph nodes containing isolated tumor cells should not be designated pN0(i+) as per the AJCC's breast cancer staging system. Whether these cells represent tumor cells in transit is uncertain, but they are associated with a worse prognosis compared to more likely true node negative (pN0) patients. It is likely that these cells represent microscopic tumor cell dissemination, but practical and economic constraints often prevent their routine detection.

Although we often categorize cancer as either localized or metastatic, this simplistic thinking might be misleading. According to Klein et al. [207], "the true nature of the disease might be better conceptualized within an evolutionary model, in which the continuous selection of genetically unstable variant cells and their expansion determines disease course and risk of dying from cancer". In this model, a dynamic process of mutation, selection, clonal expansion, and genetic diversification occurs at several primary and secondary sites simultaneously. Disseminated or occult tumor deposits (OTDs), such as those found in lymph nodes, may therefore display quite different chromosomal aberrations from the primary tumor, and require specific targeting in adjuvant therapy settings. The sentinel lymph node (SLN) concept, first described by Morton in the early 1990s [208], depicts the preferential drainage of a primary tumor to a regional lymph node(s). It is the gold standard in cancer care for patients with breast cancer and melanoma, but remains controversial in other solid tumors with continued debate regarding its role, if any, in staging and treatment algorithms [209]. However, there are 2 reasons why all cancers should adopt the SLN concept. First, SLN biopsy is the only practical method in today's economic climate to identify the most important nodes for detailed histopathological analysis. And second, adoption of this technique will promote the development of novel sentinel lymph node tracers, which are capable of non-invasive lymph node staging, and delivery of chemotherapeutic agents to disseminated tumor cells within the nodes. Many novel nanomaterials have been proposed in recent years for medical applications [210] and they are rapidly progressing toward clinical medicine. Nanoparticles (10–30 nm) with high binding affinity for lymphoid cells are ideal imaging agents of the SLNs. Working toward this hypothesis, conjugated anti-CD45 antibodies with gold nanoparticles (18 nm), through an optimal polyethylene glycol (PEG) coating, have been constructed and injected into mice [211]. Analysis confirmed rapid uptake and transport of the nanoparticles in the lymphatics, as well as significant retention in the lymph nodes. Taking this application one step further, Weissleder et al. [212] have shown that lymphotrophic superparamagnetic iron oxide nanoparticles, injected systemically as exogenous contrast, can discriminate healthy *versus* tumor-burdened nodes by the degree of accumulation of particles in the nodes. At present, there are limitations to the accuracy of this approach, as false negative results may occur in the case of lymph nodes less than 5 mm in diameter, and by extension, those with early micrometastatic disease. An alternative approach, which may offer better accuracy when dealing with micrometastatic disease, is to inject the tracer *via* the interstitial route rather than systemically. Recent studies have shown that mammaglobin-A or carbonic anhydrase specific mAbs conjugated to near infrared fluorescent dyes can detect as few as 1000 cancer cells in the lymph nodes after interstitial injection [213,214]. Although this technique is currently limited to animal studies, the application of this approach to other imaging modalities holds promise for the future development of reliable, accurate non-invasive lymph node staging.

If clonal divergence does indeed exist between occult tumor deposits in lymph nodes and the primary tumor, a logical solution would be to deliver anticancer drugs directly to lymphatic tissues, which would optimize response and limit nonspecific organ toxicities of systemic chemotherapeutic agents. Carrier systems for targeted lymphatic delivery include liposome-based, polymer-based, and immunotherapy-based [215]. Perhaps the most promising of these are the polymer-based drug delivery systems as particle size can be carefully standardized to achieve the desired effect. As an example, Liggins et al. [216] loaded a synthetic polymer microsphere with paclitaxel (PTX), and injected them *via* an intraperitoneal route into a rat model with intraperitoneal tumor cells. Rats treated with PTX microspheres showed no evidence of tumors in the peritoneal cavity, while those without, all died within 4 weeks. With recent advances in nanotechnology and a better understanding of the lymphatic anatomy and function, targeted chemotherapeutic delivery *via* polymeric nano-/microparticles may greatly improve efficacy of anticancer treatments.

#### 3.2.3. Transcoelomic metastasis

Spreading and formation of metastatic tumors in the body cavities (mostly in peritoneal and pleural cavities) is a common feature in certain malignant conditions and are broadly referred to as transcoelomic metastasis. Trancoelomic spread occurs mostly from tumors to adjacent tissues/organs. Transcoelomic peritoneal metastasis arise mostly from pancreatic cancer, colorectal cancer and ovarian cancer, followed by gastric cancer and cervical cancer [217]. The mesothelium is the lining of cavities in the body, mainly the peritoneal cavity, pleural cavity, and pericardiac cavity. The main cell type that forms the mesothelium is the mesothelial cells. Malignant transformation of mesothelial cells results in mesothelioma, an aggressive malignant condition against which there is little effective treatment. It has been reported that mesothelial cells may be a privileged site for tumor cells to attach [218]. This was thought to be due to the layer of hyaluronan, a molecule released by mesothelial cells and which, together with other proteins, forms a protective surface on the mesothelium. Peritoneal metastasis occurs *via* one of two main routes: systemic spreading and local implantation after invasion of local tissues. Tumors away from the cavities are likely to develop transcoelomic metastasis *via* the systemic route, for example, peritoneal metastasis from breast cancer and lung cancer. Perhaps most peritoneal metastases come from tumors originated from organs adjacent to the peritoneal cavity, namely tumors from the stomach, colon, pancreas, ovaries, and bladder. Cancer cells from these tumors invade surrounding tissues, breach the peritoneal lining and spread by way of seeding in the peritoneal cavity, although trans-serosal, inter-serosal and sub-serosal spread can also be seen. It is clear that in most forms of peritoneal spreading, tumor-mesothelial interactions are an essential step in establishing a metastatic tumor in the cavity.

When tumor cells disseminate through and develop a metastatic lesion in the pleural or peritoneal cavity, the cancer cells need to adapt themselves to the environment and interact with the mesothelial cells. Certain tumors have a far higher incidence of developing peritoneal metastasis. Of course, peritoneal metastasis does not occur alone, and can be seen as locoregional issues of a wider spread of cancer cells. For example, 70% of ovarian cancers which have local regional lymphatic metastases also have peritoneal metastases [219]. Similarly, 50% of patients with gastric cancer which has invaded serosa have peritoneal metastasis [220,221] and the majority of patients with pancreatic cancer have peritoneal metastasis [222]. Sadly, patients with wide spread peritoneal metastasis survive no longer than 6 months [223]. Treatment options for peritoneal metastasis are rather limited. Management involves surgical procedure to remove the primary tumor. However, this has little impact on established peritoneal metastasis. In the case of systemic metastasis, systemic chemotherapy and intraperitoneal chemotherapy have been attempted to treat the primary cancer and peritoneal metastasis with limited benefit. Management of peritoneal metastasis also involves prevention and early intervention. A critical opportunity is during the surgical debulking of the primary tumor, a key procedure. However, this procedure may also introduce tumor cells into the peritoneal cavity, although this has been a consequence that surgeons have tried to avoid. In addition, peritoneal metastases or tumor cell seeding are likely to exist at the time of operation. Surgery itself presents an excellent opportunity to prevent peritoneal metastasis and to act early on the metastasis before it becomes full scale carcinomatosis. Yet, there are very few options for this intervention. Apart from techniques to prevent artificial seeding, during surgery (for example the use of padding/isolation materials to avoid contact between tumor and surrounding tissues), a widely practiced approach is peritoneal washing/irrigation following the surgical procedure with the aim to remove any debris and possibly existing tumor cells. This is hardly a satisfactory solution, and further research is essential in order to develop alternatives.

The contact of cancer cells to mesothelial cells is followed consequently by adhesion, invasion and growth of tumor cells at such a new site. The exact role of mesothelial cells in tumor cell adhesion and growth is unclear. Many studies have demonstrated that traumatized mesothelial surfaces are privileged sites for tumor cell adhesion possibly due to the binding of tumor cells to the hyaluronan coat of mesothelial cells [218], upregulation of adhesion molecules on mesothelial cells in response to inflammatory mediators and exposure of underlying ECM. However, hyaluronan in conditioned medium from cultured mesothelial cells prevented tumor cell attachment to mesothelial cells, possibly by binding to CD44 molecules on the tumor cells and preventing their interaction with hyaluronan on the mesothelial cell surface [224]. On the other hand, factors released from tumor cells or adjacent stroma may also provide a favorable environment for the interaction between cancer cells and mesothelial cells. For example, IL-1β or TGF-β1 from cancer cells can act on the mesothelial cells and/or adjacent stroma to promote peritoneal dissemination [225,226]. Further investigation into this particular interaction will shed light on the mechanism(s) of cancer cell dissemination in pleural and peritoneal cavities, and may also provide novel therapeutic opportunities.

## 4. Therapeutic approach to cancer metastasis

### 4.1. Natural products with antimetastatic properties

To date, surgery remains the primary cancer treatment option for patients who are deemed curative at diagnosis. Existing surgical procedures are successful in removing the majority of tumors, however, cancer cells that were missed during surgical removal or cells that had already migrated out of the primary tumor sites are important sources for metastasis. The migrated cells later impair the function at the newly metastasized organ sites. Eventually, the functional impairment at metastatic sites results in cancer related mortality [227].

Tremendous advancements have been made in cancer screening, early diagnosis and development of novel chemo(radio)therapy regimens. However, little progress has been made in cancer prevention or containment of primary tumors from metastasizing. Therefore, there is a need for a multipronged approach to prevent the primary tumor from spreading. Two essential properties of new anticancer drugs are to stop tumor growth as well as inhibit metastasis. Historically, chemotherapy drugs were developed to manage primary tumors. We have advanced from using single drugs to combination chemotherapy regimens. In the past decade, several new agents have been added to the chemotherapy arsenal. These new drugs target specific cell signaling pathways. Some of the new targeted agents include monoclonal antibodies and kinase inhibitors. However, these new agents are not effective by themselves, but only in combination with other antimetabolite drugs like 5-fluorouracil. This highlights the need for more drugs that could target primary tumors [228].

Only 5% of small molecules (investigational drugs) with medicinal properties enter human clinical trials. Several investigational drugs are taken off clinical studies due to toxicity or lack of efficacy. Invariably, the trial drugs are seldom used as single agents. Instead, they are added on to existing drugs. Moreover, clinical trials focus on reducing primary tumor burden. On the other hand, these investigational drugs may potentially inhibit metastasis. Therefore, we must rededicate our efforts to go beyond reducing primary tumor burden. For example, EMT, while important in development and wound healing, is detrimental in cancer patients as it is a hallmark

of metastasizing cancer cells. Thus, focus on how to prevent EMT in cancer cells represents a key area of research toward treatment development. A good approach could be to develop a set of markers affected in EMT. Such a panel could serve as a benchmark for small molecular screens to select molecules with anti-EMT properties. Usually, these small molecules alone are not cytotoxic but may have antimetastatic activity through their interactions with key EMT regulators, *etc.* The small molecules selected through such screens may be combined with the standard of care drugs to benefit cancer patients.

Many anticancer agents in use were originally developed from natural products. Plants, fungi and marine organisms are the major sources for new drug discoveries. About 60–85% of chemotherapy agents in use are natural product derivatives [229]. However, not all the bioactive molecules isolated from a natural product are introduced to the clinic because these molecules may be either toxic or do not inhibit cancer cell growth *in vitro*. However, the isolated small molecules may inhibit specific signaling factors involved in tumor promotion. Current anticancer drug discovery efforts focus on tumor cell toxicity. Such approaches will miss specific inhibitory activities of the test compounds. Therefore, we need to systematically screen small molecules for their antimetastatic properties as well. Molecules thus identified, may be combined with other cytotoxic drugs to inhibit metastasis.

Drug discovery from natural products has two important challenges. The first is technical. The second, and equally important, is biodiversity (governmental) regulations. Synergism of multiple molecules in crude preparations is an important technical obstacle. Even when a single active molecule has been identified, *in vitro* synthesis of natural products has proved difficult. Understandably, environmental concerns and intellectual property (IP) issues affect acquiring natural products nationally and internationally. Viewed from a broader perspective, the Biodiversity convention rules and regulations are essential. Despite these obstacles, the identification and generation of new therapeutic strategies to target cancer metastasis has great potential. Research into this area is ongoing and numerous compounds have demonstrated anticancer activity.

This review outlines and has discussed key factors and pathways involved in the establishment of an invasive cancer cell phenotype and metastatic dissemination. The ability to understand this process fully is an invaluable tool in combating this process. Furthermore, identification of suitable, low-toxicity compounds which interfere with these processes in cancer cells is paramount in generating a new generation of cheap, readily available compounds. Ideal compounds would have low inherent toxicity with cancer specific effects, would have low cost and be readily available, have effects across a broad range of cancer types rather than specific subsets and be free of intellectual properties. In respect to this, this review outlines a number of possible targets that may represent key areas to target metastatic spread combined with potential therapeutic approaches to interfere with such targets (Table 2). Given the complex nature of cancer which extends beyond just that of tissue invasion and metastasis, these target approaches and potential strategies have also been explored and their efficacy investigated in the key hallmark areas of cancer development and progression (outlined in the other articles of this journal edition) to highlight potential overlaps and further illustrate how these targets (Table 2) and approaches (Table 3) may have beneficial holistic approaches to cancer. Together these tables summarize key data across the literature. This cross-validation is a very important exercise for cross-target and cross dicipline verification. It provides useful information as to whether these approaches and targets have complementary or contrary interactions with the other hallmark areas and, thus, their likelihood of resulting in pro-carcinogenic or tumor-stimulating effects.

#### 4.1.1. Silibinin

One such example is seen in the antimetastic properties of silibinin, a plant derived anticancer agent. Silibinin is a mixture of two flavonoids, silibinin A and silibinin B. It is derived from the milk thistle plant *Silybum marianum*. This plant is known for its hepatoprotective properties. *In vitro* and *in vivo*, silibinin is demonstrated to inhibit cancer cell migration, invasion and metastasis [230]. Tumor necrosis factor related apoptosis inducing ligand (TRAIL/Apo2L) is an important mediator of intrinsic apoptotic pathway. However, some tumors fail to respond to TRAIL mediated cell death signals. Silibinin induces apoptosis in TRAIL-resistant tumor cells by inducing the caspase cascade [231,232]. Yousefi et al. [233] demonstrated that silibinin inhibited the growth of neuroblastoma cell line SK-N-MC by downregulating Akt-mediated NF-κB1. Silibinin is shown to inhibit metastasis by inhibiting the expression of mRNA levels of GDP dissociation inhibitor (D4-GDI) and cell division cycle 42 (Cdc42) in the highly metastatic breast cancer cell line, MDA-MB-231 and protein levels of CD31, nestin, VEGF, VEGFR1, VEGFR2, phospho-Akt and hypoxia-inducible factor-1α (HIF-1α), the signaling molecules involved in neovascularization [234].

#### 4.1.2. Yangzheng Xiaoji

Traditional chinese medicines (TCMs) represent another source of potential antimetastatic agents. Cancer cell adhesion and invasion are key traits in the metastatic cascade. While relatively few TCMs have been reported to influence these steps, the formulation 'Yangzheng Xiaoji' (YZX) has demonstrated an efficacy in inhibiting cancer cell adhesion, migration and angiogenesis *in vitro* and *in vivo* [235–237]. YZX capsules consist of 16 herbs. An YZX extract, DME25, did not show a significant effect on the growth of cancer cells though it markedly suppressed cell adhesion and migration. It has been demonstrated that YZX inhibited the cell adhesion of gastric cancer cells (HGC27) in a concentration dependent manner, colorectal cancer cells (HRT18), breast cancer cells (MCF7), lung cancer cells (A549) and osteosarcoma cells (MG-63) and the migration of lung cancer cells and colorectal cancer cells. In addition, it was verified that the inhibitory effect of YZX on the adhesion of cancer cells is related with PI3K signal pathway. Wortmannin, an inhibitor of PI3K activity, can suppress PI3K/AKT signaling and consequently reduce adhesiveness of cancer cells. DME25 which also targets the AKT pathway can enhance such inhibitory effect. The influence of DME25 on the PI3K pathway may not depend on only one signal pathway, namely the AKT pathway [235,237]. Another study has demonstrated that YZX can suppress the formation of canaliculus of vascular endothelial cells, and indicated that cell matrix adhesion and migration could be inhibited in a concentration dependent manner [236]. Cell adhesion and migration are critical during angiogenesis, particularly canaliculus formation by vascular endothelial cells when they adhere to the cell matrix and subsequently migrate in the ECM.

The FAK signaling pathway is a key pathway involved in cell–matrix adhesion [238–240]. DME25 has been reported to have an inhibitory effect on the phosphorylation of the FAK pathway, while treatment with a FAK inhibitor significantly enhanced the effect of DME25 on the FAK pathway [241]. YZX has also demonstrated the ability to not only inhibit the growth of colorectal cancer cells and lung cancer cells but also to suppress the formation of mouse peritoneal tumor nodules *in vivo*. The significant inhibition of tumor growth could be observed in both oral administration and intraperitoneal injection. FAK and phospho- FAK immunofluorescent staining indicated that YZX lowered the expression of FAK and could inhibit the activation of FAK through treatment with a combination of DME25 and FAK inhibitor. Hence, YZX demonstrates potential as a novel antimetastatic agent, targeting key traits in the metastatic cascade and demonstrating efficacy using *in vivo*

**Table 2**
Priority targets for tissue invasion and metastasis.

| Priority targets for tissue invasion and metastasis other cancer hallmarks | Upregulation of E-cadherin | Promotion of formation of tight junctions (claudins, *etc.*) | Suppression of synthesis, secretion and/or activity of the urokinase plasminogen activator (uPA) | Inhibition of PI3K/AKT signaling | Inhibition of FAK signaling | Inhibition of AP-1 activity | Inhibition of NF-κB | Suppression of synthesis, secretion and/or activity of MMP-9 expression | Inactivation of β-catenin/ZEB1 signaling | Inhibition of TGF-β signaling |
|---|---|---|---|---|---|---|---|---|---|---|
| Genomic instability | + [299] | 0 | 0 | + [300] | 0 | 0 | + [301–303] | 0 | 0 | 0 |
| Sustained proliferative signaling | +/− [304,305] | + [306,307] | + [308,309] | + [310,311] | + [311,312] | + [313] | + [314–316] | + [317,318] | + [319] | + [278,320] |
| Tumor-promoting inflammation | +/− [321,322] | + [323,324] | 0 | + [325–327] | + [328,329] | + [330,331] | + [332,333] | + [334] | + [335] | + [336,337] |
| Evasion of anti-growth signaling | +/− [304,338–340] | + [341,342] | 0 | + [343] | + [344,345] | + [346,347] | 0 | + [348,349] | + [350] | +/− [255,351–353] |
| Resistance to apoptosis | + [354] | + [355] | + [356] | + [357] | + [358] | + [359] | + [360] | + [361] | + [362] | + [262] |
| Replicative immortality | 0 | 0 | − [363] | +/− [364–366] | + [367] | − [368] | +/− [369–371] | + [367] | 0 | − [372,373] |
| Dysregulated metabolism | + [374,375] | 0 | + [376] | + [377] | + [378] | 0 | + [379] | 0 | 0 | + [380] |
| Immune system evasion | 0 | 0 | + [381] | +/− [382,383] | 0 | 0 | + [384] | 0 | 0 | + [385] |
| Angiogenesis | − [386] | − [387] | + [388] | + [389] | + [390,391] | + [392–394] | +/− [395,396] | + [397] | + [398] | +/− [399] |
| Tumor microenvironment | +/− [400,401] | + [402] | + [309] | + [403] | + [404,405] | + [406,407] | + [408] | +/− [409,410] | + [319] | + [411] |

Key targets identified in tissue invasion and metastasis were also examined in the other hallmark areas of cancer. Targets relevant to other hallmarks are listed as complementary (+) if they display anti-carcinogenic effects, contrary (−) if they display pro-carcinogenic effects or controversial (+/−) if they display both anti- and pro-carcinogenic affects, or identified as having no known relationship (0).

**Table 3**
Possible approaches to impact priority targets for tissue invasion and metastasis.

| Approaches other cancer hallmarks | Gamma linolenic acid | Eicosapentanoic acid | β-(1–6)-D-glucan (*Agaricus blazei*) | Grifolin (*Albatrellus confluens*) | Cordycepin (*Cordyceps militaris*) | Polysaccharides (*Ganoderma lucidum*) | Ganoderic acids (*Ganoderma lucidum*) | Pachymic acid (*Poria cocos*) | Silibinin | 5,6-dihydro-4H-pyrrolo[1,2-b]-pyrazoles |
|---|---|---|---|---|---|---|---|---|---|---|
| Genomic instability | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + [412–414] | 0 |
| Sustained proliferative signaling | + [415,416] | + [417] | + [418] | + [419] | + [420–423] | +/– [424,425] | + [426] | + [427] | + [428–431] | 0 |
| Tumor-promoting inflammation | 0 | + [432,433] | + [434] | 0 | + [435,436] | + [437,438] | + [439] | + [440] | + [441] | 0 |
| Evasion of anti-growth signaling | + [442,443] | + [444–446] | 0 | + [447] | + [422,448] | + [449–451] | + [439,452] | + [427] | + [453–455] | 0 |
| Resistance to apoptosis | + [456] | + [457] | + [458] | + [419] | + [459] | + [460] | + [461] | + [462] | + [463] | 0 |
| Replicative immortality | 0 | + [464,465] | 0 | 0 | 0 | 0 | 0 | 0 | + [466–468] | 0 |
| Dysregulated metabolism | + [469] | 0 | + [470] | + [471] | + [472] | + [473] | 0 | 0 | + [429,474] | 0 |
| Immune system evasion | 0 | 0 | 0 | 0 | 0 | + [475] | 0 | 0 | + [476] | 0 |
| Angiogenesis | + [416] | + [477] | 0 | 0 | + [478] | + [479] | + [439] | 0 | + [230] | 0 |
| Tumor microenvironment | + [480] | + [481,482] | + [483,484] | + [419] | + [485] | + [486] | + [487] | + [488] | + [414,489] | + [490] |

Potential approaches to impact priority targets were cross-validated across other hallmark areas of cancer. Approaches relevant to other hallmarks are listed as complementary (+), contrary (−), controversial (+/−) or as having no known relationship (0).

metastasis models. Further research into this compound and other TCM as viable, new antimetastatic agents is fully warranted.

#### 4.1.3. Medicinal mushrooms as a source of anticancer agents

The beneficial anticancer properties of a variety of food and natural compounds have been known for millennia. Isolation and characterization of specific compounds combined with advances in new molecular biology techniques have helped to identify new specific targets. For example, certain phytochemicals including alkaloids, carotenoids, and flavonoids demonstrated anti-invasive, antimetastatic and antiangiogenic activities in cell culture and animal experiments (reviewed in [242,243]). In addition to the typical dietary phytochemicals from soy, green tea, berries, spices and other dietary plants, edible and medicinal mushrooms contain a variety of specific compounds which can target signaling molecules/pathways involved in cancer progression, metastasis and angiogenesis. Different mushroom components also modulate immune system resulting in the secretion of a variety of cytokines and stimulation of natural killer cells which are responsible for their anticancer activities. Table 4 lists a number of mushroom components that have direct targets in cancer cells. We should redouble our efforts on these natural products for their antimetastatic properties.

### 4.2. Targeting TGF-β in cancer using small molecule inhibitors

TGF-β, discovered in the early 1980s, has been recognized as a pivotal cytokine involved in a broad range of physiological processes. It is also well known for playing a crucial role in tumor cell behavior, regulating cell growth/proliferation, angiogenesis, EMT, tumor cell migration, invasion and metastasis. Studies have described dual functions for TGF-β in tumorigenesis, that of a tumor suppressor in normal cells and in cancer cells in early stages of tumor development and that of a tumor promoter in late stages of tumor progression, enhancing immune suppression, angiogenesis, migration, invasion and metastatic dissemination [244–246]. In humans, three isoforms have been identified in the TGF-β superfamily (TGF-β1–3), all of which signal through a heterotetrameric complex consisting of two transmembrane receptor serine/threonine kinases, one of type I (TGF-βR1 or activin receptor-like kinase 5, ALK5) and one of type II (TβRII) (Fig. 3). Signal transduction is initiated by binding of TGF-β to TβRII resulting in the recruitment of ALK5 into the complex and its activation through phosphorylation in the GS region (glycine/serine rich domain). This triggers the phosphorylation of intracellular mediators, the receptor-regulated Smads (R-Smads) Smad2 and Smad3 by ALK5. Phosphorylated Smad2 or Smad3 then complex with Smad4 and the resulting hetero-Smad complex is translocated to the nucleus to activate the transcription of various TGF-β-responsive genes [247] (Fig. 3). Given the involvement of TGF-β in tumorigenesis and in particular tumor promotion, the targeting of the TGF-β signaling pathway for therapeutic purposes appeared to be a promising strategy. Up to now, several inhibitors have entered clinical trials, from phase I to III. A comprehensive list of therapeutic TGF-β signaling inhibitors used in pre-clinical and clinical studies has recently been published [248]. Based on the TGF-β signaling pathway, four major strategies of interfering with TGF-β expression, function, or signaling have emerged.

The first and clinically most advanced strategy relies on direct or indirect inhibition of TGF-β1 or TGF-β2 secretion including blocking the generation of the TGF-β ligand using antisense oligonucleotides to TGF-β mRNA. Silencing oligonucleotides have been clinically validated with the anti-TGF-β2-specific phosphorothioate antisense oligonucleotide AP12009 (trabedersen, Antisense Pharma) [249] demonstrating a significant increase in survival rate for patients with recurrent refractory high-grade

**Table 4**
Effective anticancer agents present in medical mushroom.

| Mushroom | Cell line(s) | Compound(s) | Biological effects | Molecular targets | Ref |
|---|---|---|---|---|---|
| *Agaricus blazei* | HRA (ovarian)<br>LL3 (lung) | β-(1–6)-D-glucan | Inhibition of cell proliferation, induction of apoptosis, suppression of metastasis | ↑ p38 MAPK, caspase-9<br>↓ uPA | [418] |
| | S180 (sarcoma) | Linear | Inhibition of tumor growth, angiogenesis | ↓ VEGF | [491] |
| | BEL-7402 (liver) | β-(1–3)-D-glucan | | ↓ MMP-9, ↑ nm23-H1 | [492] |
| | B16 (melanoma) | | Inhibition of invasion through matrigel, regression of metastatic tumors | | |
| | U937 (leukemia) | Agaritine | Induction of apoptosis | ↑ caspase-3, -9 | [458] |
| | Hep3B (liver) | Blazeispirol | Induction of apoptosis | ↑ caspase-3, -9, ↓ Bcl-2, ↓ Bcl-xL | [493] |
| *Albatrellus confluens* | CNA1 (nasopharyngeal)<br>HeLa (cervix) | Grifolin | Induction of apoptosis | ↑ caspase-3, -8, -9, ↓ Bcl-2 | [494] |
| | MCF-7 (breast)<br>SW480 (colon) | | Induction of apoptosis | ↑ PARP, caspase-3, -9,↓ Akt, FOXO, GSK3 | [419] |
| | K567 (leukemia) | | Cell cycle arrest at G1 | | [447] |
| | Raji, B95-8 (lymphoblast) | | | ↓ ERK1/2, ERK5, ↑ p19 | [495] |
| | U2OS, MG63 (osteosarcoma) | | | ↑ DAPK1, p53<br>↑ p21 | [496] |
| | CNE1 (nasopharyngeal) | | | | |
| *Antrodia camphorata* | HT-29, HCT116, SW-480 (colon) MDA-MB-231 (breast) | Triterpenes (zhankuic acids) | Induction of apoptosis | ↑ PARP, ↓ Bcl-2, ↓ pro-caspase-3 | [497] |
| | Huh7, HepG2, Hep3B (liver) | Methyl antcinate A | Induction of apoptosis | ↑ Bax, Bak, PUMA, ↓ Bcl-2, Bcl-xl, | [498] |
| | OEC-M1, OC-2 (oral) | | Induction of apoptosis | ↑ caspase-2, -3, -9<br>↑ Bax, PARP, caspase-3, ↓ Bcl-2, Bcl-xl | [499] |
| | HepG2 (liver) | Methylancinate B<br>Antcin B | Induction of apoptosis, enhancing oxidative stress | ↑ caspase-2, -3, -8, -9, ↑ Fas, FasL Bax, ↓ Bcl-2, Bcl-xl | [500] |
| | MDA-MB-231 (breast) | Antrocin | Induction of apoptosis | ↓ Bcl-2, Bcl-xl, surviving, ↓ mTOR, GSK-3β, NF-κB | [501] |
| | H441, H1975 (lung) | | Induction of apoptosis | ↓ JAK2, STAT3, mcl-1, ↑ caspase-3 | [502] |
| | A549 (lung) | Antroquinol | Induction of apoptosis | ↓ PI3K, mTOR, Bcl-2, ↑ PARP, caspase-3 | [503] |
| | PANC-1, AsPC-1 (pancreas) | Antroquinol | Induction of apoptosis, autophagy, accelerated senescence | ↓ Akt, mTOR, ↑ p21, K-ras | [504] |
| | HL-60 (leukemia) | Dehydroeburicoic acid | Induction of apoptosis | ↑ PARP, caspase-3, ↓ topoisomerase II | [505] |
| *Cordyceps militaris* | MDA-MB-231,<br>MCF-7 (breast) | Cordycepin | Induction of apoptosis,<br>Induction of autophagy | ↑ caspase-3, -9, Bax<br>↑ LC3 | [506]<br>[507] |
| | PC-3 (prostate) | | Induction of apoptosis | ↑ caspase-3, -9, Bax/Bc-2, ↓ IAP | [508] |
| | LNCaP (prostate) | | Inhibition of cell motility and invasiveness | ↓ MMP-2,-9, ↑ TIMP-1,-2, ↓ PI3K/AKT | [509] |
| | SK-N-BE(2)-C (neuroblastoma) | | | | |
| | SK-MEL-2 (melanoma) | | Induction of apoptosis | ↑ caspase-3, PARP | [422] |
| | 5637, T-24 (bladder) | | Growth inhibition, cell cycle arrest at G2/M<br>Inhibition of TNF-α-induced migration and invasion | ↑ p21, p-JNK<br>↓ MMP-9, NF-κB, AP-1 | [510] |
| *Cordyceps sphecocephala* | HepG2 (liver)<br>SK-N-SH (neuroblastoma) | Polysaccharide peptide | Induction of apoptosis | ↑ caspase-3, Bax, ↓ Bcl-2 | [511] |
| *Cordyceps sinensis* | B16 (melanoma) | Exopolysaccharide | Inhibition of tumor growth in lungs and liver | ↓ c-myc, c-fos, VEGF | [512] |
| | HepG2 (liver) | Ergone | Induction of apoptosis, cell cycle arrest at G2/M | ↑ caspase-3,-8, 9, PARP, ↑ Bax, ↓ Bcl-2 | [513] |
| | CGTH W-2 (thyroid) | Cordycepin | Induction of apoptosis | ↑ caspase-7, PARP | [514] |
| *Ganoderma lucidum* | THP-1 (leukemia) | Polysaccharides | Induction of apoptosis | ↑ DR3, DR4/5<br>↑ caspase-3, -7, -8, -9, p53 | [515]<br>[516] |
| | S180 (sarcoma) | Polysaccharides | Cell cycle arrest at G2/M, inhibition of tumor growth | ↑ Bax, ↓ Bcl-2 | [517] |
| | NTUB1, N/P(14)<br>N/As (0.5) (urothelial) | Polysaccharides | Enhancing apoptosis in therapy resistant cancer cells | ↑ Fas, caspase-3, -9, ↑ Bax, Bad, ↓ Bcl-2, Bcl-xL | [518] |
| | HUVECs (endothelial) | Polysaccharide-peptide | Induction of apoptosis, inhibition of angiogenesis | ↓ Bcl-2/Bax, VEGF | [519] |
| | SGC-7901 (gastric) | Protein Lz-8 | Induction of autophagy | ↑ ATF4, CHOP | [473] |
| | THP-1 (leukemia) | Lipids | Induction of apoptosis | ↓AKT, Erk1/2, ↑ JNK1/2, caspase-3, -8, -9 | [520] |

Table 4 (*Continued*)

| Mushroom | Cell line(s) | Compound(s) | Biological effects | Molecular targets | Ref |
|---|---|---|---|---|---|
| | Huh-7 (liver) | Triterpenes | Inhibition of growth, cell cycle arrest at G2 | ↓ PKC, ↑ p38MAPK, ↑ JNK | [521] |
| | HT-29 (colon) | | Induction of autophagy | ↑ Beclin-1, LC3, ↓ p38 MAPK | [522] |
| | HepG2 (liver) | Ganoderic acid A | Sensitizing to cisplatin-induced apoptosis | ↓ STAT3, JAK1, JAK2 | [523] |
| | MDA-MB-231 (breast) | Ganoderic acid A, H | Inhibition of cell growth, cell adhesion, migration and invasion | ↓ CDK4, uPA, ↓ NF-κB, AP-1 | [426] |
| | MCF-7 (breast) | Ganoderic acid DM | Inhibition of cell growth, induction of apoptosis | ↓ CDK2, CDK6, c-Myc, ↓ cyclin D1, ↑ PARP | [452] |
| | 95-D (lung) | Ganoderic acid Me | Inhibition of cell adhesion and migration | ↓ MMP-2/9 | [524] |
| | HeLa (cervix) | Ganoderic acid Mf, S | Induction of apoptosis | ↑ caspase-3,-9, Bax/Bcl-2 | [525] |
| | HCT-116 (colon) | Ganoderic acid T | Inhibition of cell adhesion and migration | ↓ uPA, MMP-2/9, NF-κB | [526] |
| | 95-D (lung) | | Induction of apoptosis | ↑ p53, Bax | [527] |
| | LNCaP (prostate) | Ganoderol B | Binding to androgen receptor | ↓ 5α-reductase | [528] |
| | MDA-MB-231 (breast) | Ganodermanontriol | Inhibition of cell growth, cell adhesion, migration and invasion | ↓ CDC20, uPA, uPAR | [308] |
| | HCT-116, HT-29 (colon) | | Inhibition of tumor growth | ↓ cyclin D1, β-catenin | [529] |
| | HepG2 (liver) | Lucidenic acid B | Inhibition of invasion | ↓ MMP-9, NF-κB, AP-1 | [530] |
| *Grifola frondosa* | SGC-7901 (gastric) | Polysaccharide-peptide | Cell cycle arrest at G2/M, induction of apoptosis | ↑ caspase-3, Bax, ↓ Bcl-2 | [531] |
| | MCF-7 (breast) | β-Glucan | Induction of apoptosis | ↑ BAK-1 | [532] |
| | PC-3 (prostate) | β-Glucan | Cell cycle arrest at G1, induction of apoptosis | ↓ CDK2,4,6, cyclin D1, E | [533] |
| *Lentinus edodes* | HepG2 (liver) | Mycelia | Induction of apoptosis | ↑ caspase-3,-8 | [534] |
| | KB, HSC3 (oral squamous) | β-D-glucan (lentinan) | Inhibition of growth, induction of apoptosis | ↓ TS, DPD, ↑ OPRT | [535] |
| *Pleurotus abalones* | MCF-7 (breast) | Polysaccharides | Inhibition of proliferation, cell cycle arrest at S, induction of apoptosis | ↑ caspase-3,-9, PARP, ↑ Bax/Bcl-2, p53 | [536] |
| *Pleurotus ostreatus* | HT-29 (colon) | α-Glucan | Inhibition of cell proliferation, induction of apoptosis | ↑ Bax | [537] |
| *Pleurotus pulmonarius* | Normal colon | Glucans | Inhibition of colon carcinogenesis in mice | ↑ Bax, ↓ Bcl-2, NF-κB | [538] |
| | Huh7, Hep3B (liver) | Polysaccharide-protein complex | Inhibition of proliferation and invasion, inhibition of tumor growth | ↓ PI3K/AKT, VEGF | [539] |
| *Poria cocos* | MCF-7 (breast) | β-Glucan | Inhibition of cell proliferation, cell cycle arrest at G1, induction of apoptosis | ↓ cyclin D1, cyclin E, ↑ Bax/Bcl-2 | [540] |
| | BxPc-3 (pancreas) | Triterpenes Pachymic acid | Inhibition of cell proliferation and invasion cell cycle arrest at G0/G1 | ↓ KRAS, MMP-7 | [427] |
| | MDA-MB-231, MCF-7 (breast) | Pachymic acid | Inhibition of cell invasiveness | ↓ MMP-9, NF-κB | [488] |
| | DU145 (prostate) | | Inhibition of cell proliferation, induction of apoptosis | ↑ p21, ↓ Bad, ↑ Bcl-2, ↑ caspase-3,-9 | [462] |
| | NUGC-3 (gastric) | Dehydroebryconic acid | Inhibition of cell proliferation, cell cycle arrest at G1 | ↓ DNA topoisomerase II | [541] |
| | A549 (lung) | Polyporenic acid C | Inhibition of cell proliferation, induction of apoptosis | ↓ PI3K/AKT, ↑ p53, ↑ caspase-8 | [542] |
| | HL60 (leukemia) | Poricotriol A | Induction of apoptosis | ↑ caspase-3,-8,-9, Bax/Bcl-2 | [543] |
| | A549 (lung) | | Induction of apoptosis | ↑ AIF, Bax/Bcl-2 | |

glioma compared to standard chemotherapy in phase IIb clinical trials [250]. AP12009 is currently undergoing phase III clinical trials for patients with anaplastic astrocytoma and phases I/II clinical trials for patients with pancreatic neoplasms, melanoma, and colorectal neoplasms. AP11014, another oligonucleotide from Antisense Pharma targeting TGF-β1, is also under investigation at an advanced preclinical stage.

The second strategy relies on inhibition of TGF-β receptor binding, including the use of monoclonal antibodies to block specific TGF-β isoforms and thus receptor-ligand interactions. This strategy comprises three compound groups: highly specific anti-ligand monoclonal antibodies, soluble TGF-β receptors (fusion constructs like soluble TβR2:Fc fusion proteins) and synthetic peptides. The three most advanced antibodies are GC-1008, CAT-152, CAT-192. The synthetic peptides, P17, P144, have also been used to modulate the TGF-β pathway, of which the most advanced is P144 (DigNA Biotech). P144 blocks the binding of TGF-β1 to ALK5 and TβRII. Systemic treatment of mice with either P17 or P144 significantly reduced tumor burden induced by TGF-β1 and in metastatic nodules consistently reduced mitotic/apoptotic ratio, mesenchymal traits and angiogenesis induced by TGF-β1 [251].

The third strategy is based on inhibition of TGF-β receptor activation and involves small molecules that block the ALK5 kinase and hence all Smad and non-Smad signaling pathways originating

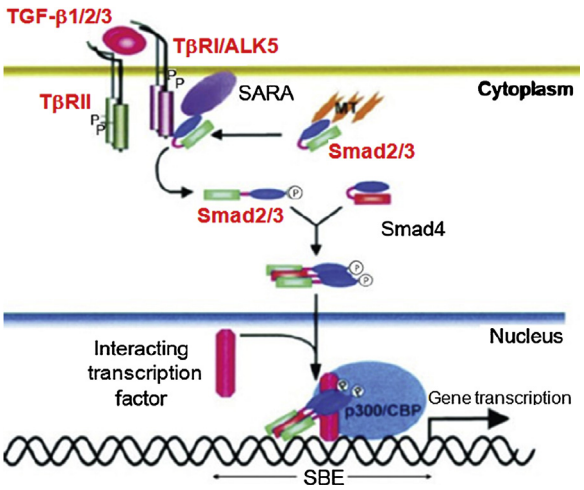


**Fig. 3.** Canonical TGF-β Smad signaling pathway from initiation to nucleus. The active TGF-β ligand can bind TβRII (or the accessory receptor TβRIII, not shown), which results in bridging of TβRI/ALK5 in to the complex, and allows TβRII to phosphorylate ALK5. Following recruitment to ALK5 (a process that can be facilitated by auxiliary proteins such as SARA), the R-Smads Smad2 and Smad3 are phosphorylated by the ALK5 kinase. These activated R-Smads form a complex with the Co-Smad Smad4 and this complex is imported into the nucleus where in association with interacting transcription factors and p300/CBP it binds to the Smad binding element (SBE) in target gene promoters to drive transcription from these genes.

from TβRII and ALK5. In contrast to the large molecule strategies highlighted above, small molecules offer the possibility of intracellular modulation of the TGF-β pathway. The extensive knowledge of the ALK5-dependent Smad2/Smad3 phosphorylation pathway has made ALK5 and TβRII attractive targets for the pharmaceutical industry and has focused the research toward the development of small molecules ALK5 inhibitors which act as competitive inhibitors for the catalytic adenosine triphosphate (ATP)-binding site of the ALK5 kinase. The TGF-β signaling pathway offers many different avenues for therapeutic intervention such as the intracellular inhibition of the ALK5 kinase and/or dual inhibitors of both the ALK5 and TβRII kinases with small molecule inhibitors. An overview on these agents and their pre-clinical and clinical use has already been given in excellent reviews [248,252–255].

Inhibition of ALK5 kinase with compounds such as SB431542, LY573636, SD-208, SM16, SX-007, IN-1130 and YR-290 has illustrated their anticancer efficacy. Treatment of glioma cultures with SB431542 inhibited proliferation, TGF-β-mediated morphologic changes and cellular motility [256], and attenuated the tumor-promoting effects of TGF-β, including TGF-β-induced EMT, cell motility, migration and invasion and VEGF secretion in human cancer cell lines [257]. LY573636 is currently being assessed in phase I and phase II studies of patients with advanced solid tumors (unresectable or metastatic malignant melanoma, metastatic soft tissue sarcoma, metastatic non-small cell lung cancer (NSCLC), and ovarian cancer) [258,259]. Another compound, SD-208, inhibits growth and invasiveness of murine and human glioma cells *in vitro* and *in vivo*, resulted in increased infiltration of the tumor with immune effector cells and prolonged survival in mice bearing intracranial SMA-560 gliomas [260]. Treatment of syngeneic R3T or 4T1 tumor-bearing mice with orally administered SD-208 inhibited primary tumor growth as well as the number and size of metastases and also resulted in a decrease in tumor angiogenesis [261]. In pancreatic adenocarcinoma, SD-208 inhibited TGF-β-stimulated invasion *in vitro* and reduced primary tumor growth and incidence of metastasis in an orthotopic xenograft mouse model [262]. In melanoma cell lines, SD-208 blocked TGF-β induction of Smad3 phosphorylation, Smad3/4-specific transcription, Matrigel basement membrane invasion and expression of TGF-β target genes and also prevented the development of osteolytic bone metastases and significantly reduced the size of osteolytic lesions in mice with established bone metastases [263]. Similarly, SM16 showed potent activity against established AB12 malignant mesothelioma tumors using an immune-mediated mechanism and was found to significantly prevent tumor recurrence after resection of bulky AB12 malignant mesothelioma tumors [264]. Blockade of TGF-β signal transduction in 4T1 tumor cells by SM16 prevented TGF-β-induced morphological changes and inhibited TGF-β-induced invasion *in vitro*, inhibited Smad2 phosphorylation in cultured 4T1 tumor cells as well as in primary and metastatic 4T1 tumor tissue and inhibited the growth of primary and metastatic 4T1 tumors *in vivo* [265]. The combination of SM16 with anti-OX40 elicited a potent antitumor effect against established poorly immunogenic, highly metastatic, TGF-β-secreting primary 4T1 mammary tumors, with a 79% reduction in tumor size, a 95% reduction in the number of metastatic lung nodules, and a cure rate of 38% [266]. SX-007, an orally active, pyridopyrimidine ALK5 kinase inhibitor, was also evaluated for its therapeutic potential in cell culture and in the syngeneic, orthotopic glioma model SMA-560, where it exerted a therapeutic effect by reducing TGF-β-mediated invasion, reversing immune suppression and improving survival in this model [267]. The IN-1130 inhibitor has also displayed antitumor effects in mice injected subcutaneously with the murine prostate cancer cell line Tramp C2. Here the treatment group demonstrated a dramatic decrease in tumor volume in association with an enhanced immune response [268]. YR-290 has also been shown to inhibit the TGF-β-mediated downstream signaling pathway, metastasis-associated genes, and TGF-β-dependent cell migration and invasion in breast cancer cells. In tumor metastasis mouse models, YR-290 almost completely blocked cancer metastasis, reducing lung tumor nodules in comparison with control animals and significantly prolonged the survival of tumor-bearing mice [268].

Dual inhibitors of the ALK5 and TβRII kinases have also been studied, again demonstrating potential anticancer effects. Examples of such compounds include LY2157299 and LY2109761. One compound, LY2157299, that can be orally administered, has entered phase I trials for advanced/metastatic cancer. Daily oral administration of LY2157299 was safe and well tolerated [269]. In triple negative breast cancer (TNBC) cell lines and mouse xenografts, the chemotherapeutic drug paclitaxel increased autocrine TGF-β signaling and IL-8 expression and enriched for CSCs, as indicated by mammosphere formation and CSC markers. LY2157299 blocked paclitaxel-induced IL-8 transcription and CSC expansion. Moreover, treatment of TNBC xenografts with LY2157299 prevented reestablishment of tumors after paclitaxel treatment. These data suggest that chemotherapy-induced TGF-β signaling enhances tumor recurrence through IL-8-dependent expansion of CSCs and that TGF-β pathway inhibitors prevent the development of drug-resistant CSCs and argue for testing a combination of TGF-β inhibitors and anticancer chemotherapy in patients with TNBC [270]. Similarly LY2109761 has displayed efficacy as an anticancer agent. LY2109761 decreased liver metastases and prolonged survival in mouse models of colon metastasis [271] and in decreased metastasis in a mouse model of pancreatic cancer model [272]. LY2109761 inhibited TGF-β-mediated activation of Smad and non-Smad pathways in CT26 colon adenocarcinoma cells having V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (K-Ras) mutation and attenuated the oncogenic effects of TGF-β on cell migration, invasion and tumorigenicity of CT26 cells. These findings highlight the therapeutic value of LY2109761 for metastatic colorectal cancer [271]. Both LY2157299 and LY2109761 inhibited TGF-β-stimulated *in vitro* migration and invasiveness of MDA-MB-231 subclones and significantly reduced the metastatic burden to either lungs or bones *in vivo*. Besides

inhibiting metastasis in a tumor cell autonomous manner, the TGF-β antagonists inhibited angiogenesis associated with lung metastases and osteoclast number and activity associated with lytic bone metastases [273]. A large series of studies with LY2157299 and LY2109761 have been completed in the hepatocellular carcinoma (HCC) model. LY2157299 and LY2109761 inhibited HCC cell migration on laminin-5, fibronectin, vitronectin, and collagen-I and invasion through Matrigel [274] both constitutively invasive and with acquired invasive properties [275]. This inhibition is associated with the decreased phosphorylation of Smad2, FAK and β1-integrin (intracytoplasmic tail), and with increased levels of E-cadherin. Finally, in a xenograft model of HCC, LY2109761 strongly inhibited tumor growth, intravasation and metastasis [276]. These studies support the use of LY2157299 in clinical trials. The anti-tumor activity of LY2109761 was also associated with inhibition of molecular pathways involved in neo-angiogenesis and tumor growth of HCC. This anti-angiogenic effect was more effective than that of bevacizumab, which specifically targets VEGF. LY2109761 blocked the paracrine cross-talk between HCC and endothelial cells, inhibiting blood vessel formation. This effect was mediated by Smad2/3 and affected the secretion of VEGF. Of note, LY2109761 did not show significant effects on physiological angiogenesis [277]. LY2109761 also interrupted the cross-talk between cancer cells and CAFs, leading to a significant reduction of HCC growth and dissemination. Preclinical results also indicate that LY2109761 targets the cross-talk between HCC and the stroma and provide a rationale for future clinical trials [278]. Zhang and colleagues [279] have reported that LY2109761 inhibited radiation-induced invasion, reduced tumor microvessel density, and attenuated EMT in glioblastoma. However, there is also evidence of acquired resistance to LY2109761. Therefore, TGF-β inhibitors might be clinically useful for applications requiring acute administration, but long-term patient exposure to such drugs should be undertaken with caution [280].

Other studies have also shown that the Src family kinase inhibitors PP1 and PP2 are powerful inhibitors of ALK5. In *in vitro* kinase assays with recombinant ALK5, PP1 and PP2 displayed an $IC_{50}$ of $5.0 \times 10^{-08}$ M and $5.6 \times 10^{-07}$ M, respectively, with PP1 being more potent and PP2 being nearly as potent as SB431542 ($IC_{50}$ of $2.25 \times 10^{-07}$ M). PP2, but not PP1 also weakly inhibited the TβRII kinase. In pancreatic carcinoma cells, PP1 and PP2 effectively inhibited TGF-β1-induced phosphorylation of Smad2/3 and p38 MAPK, gene expression, and EMT *in vitro* [281]. Together, these data show that PP1 and PP2 strongly inhibit the ALK5 kinase and can block TGF-β/Smad signaling in a Src-unrelated fashion. Both agents may be useful as dual TGF-β/Src inhibitors in experimental therapeutics of late stage metastatic disease.

The fourth strategy is based on inhibition of Smad activation and uses pseudo-substrate inhibitors that mimic Smads and block intracellular Smad signal pathways. While research has focused in recent years on the generation and therapeutic evaluation of inhibitors targeting the ALK5 ATP-binding site, another approach to target the kinase on the substrate-binding site has been reported [282]. This novel strategy aims to inhibit signaling by blocking the substrate-binding site of the ALK5 kinase with peptides mimicking Smad2 (Smad pseudo-substrate inhibitors). This new class of inhibitors acts as "dominant negative inhibitors" which occupy the Smad2-binding pocket and prevent Smad2 phosphorylation, and hence its activation. This idea should by definition allow a high specificity that some ATP-mimicking inhibitors (such as SB431542) do not possess. The results have shown that Smad mimetics can indeed impede TGF-β signaling by blockage *in vivo* and *in vitro* of ALK5-dependent phosphorylation of endogenous Smad2, as well as downstream events such as gene expression. Finally, these pseudo-substrates have shown higher efficiency with the ALK5 kinase than with other type I receptor kinases of the TGF-β/bone morphogenetic protein (BMP) family [282]. The development of pseudo-substrate inhibitors is a promising approach that may lead to new therapeutics. It is widely accepted that the Smad pathway mediates tumor-suppressor functions of TGF-β, while the tumor-promoting effects of TGF-β are largely controlled by non-Smad pathways. Therefore, the use of compounds that selectively inhibit Smad signaling may not be as efficient as ALK5 inhibitors unless the Smad-independent pathways are activated downstream of Smad signaling. Hence, tumors that harbor a non-functional Smad pathway, such as tumors with mutations in *DPC4* (encoding Smad4), might not be amenable to treatment with this kind of inhibitors. The various targets and inhibitors discussed above are summarized in Table 5.

**Table 5**
Overview of TGF-β signaling inhibitors used in pre-clinical and clinical studies. The mechanism of action involves (i) direct or indirect inhibition of TGF-β secretion, (ii) inhibition of TGF-β-T β R binding, (iii) inhibition of TβR activation, or (iv) inhibition of Smad activation. ASON, antisense oligonucleotide; mAb, monoclonal antibody. See text for specific details.

| Target protein | Inhibitor (type) | Mechanism |
|---|---|---|
| TGF-β1 | AP11014 (TGF-β1-specific ASON) | (i) |
| | CAT-192/Metelimumab (rec. | (ii) |
| TGF-β2 | human IgG4 anti-TGF-β1 mAb) | |
| | AP12009, | (i) |
| TGF-β1–3 | Lucanix/Belagen-pumatucel-L | |
| | (TGF-β2-specific phosphorothioate | (ii) |
| | ASONs) | (ii) |
| | CAT-152 (rec. human IgG4 | (ii) |
| | anti-TGF-β2 mAb) | |
| | GC-1008 (human anti-panTGF-β | |
| | mAb) | |
| | 1D11, 2G7 (anti-panTGF-β mAbs) | |
| TβRII | Soluble TβR2:Fc fusion protein | (ii) |
| | (soluble TGF-β receptors) | (iii) |
| | LY2109761, LY2157299 (small | |
| | molecules) | |
| TβRI/ALK5 | A-83-01, Antp-Sm2A, EW-7195, | (iii) |
| | EW-7203, IN-1130, Ki-26894, | |
| | LY2109761, LY2157299, | |
| | LY364947/HTS-466284, LY550410, | |
| | LY573636, LY580276, NPC30345, | |
| | PP1, PP2, SB431542, SB505124, | |
| | SD-093, SD-208, SKF104365, SM16, | |
| | SM305, SX-007, YR-290 (small | |
| | molecules) | |
| TβRIII/Betaglycan | P17, P144 (synthetic peptides) | (iii) |
| Smad2 | Peptides mimicking Smad2 (Smad | (iv) |
| | pseudo-substrate inhibitors) | |
| Smad3 | SIS3 | (iv) |

## 5. Perspectives

This article has provided a summary of some of the recent progress in the area of cancer invasion and metastasis. As highlighted in this special issue, although progress in this area over the past two decades has been rapid, when one considers the severity of cancer metastasis, the damaging impact on patient's longevity and quality of life, and the lack of successful treatment regimens to combat cancer metastasis, these triumphs are far from satisfactory, as partly echoed in the capstone article. A great deal more investment and research is required to provide breakthrough treatments, which will benefit late stage cancer patients. These achievements can be seen as early 'sweeteners' in this fundamental area of cancer research; however, currently there are very limited options for effective intervention of cancer metastasis. Patients suffering with metastatic disease commonly have a poorer general condition and health than those at an early stage. Conventional options, such as chemotherapy and radiotherapy, generate collateral adverse effects, which are hard to bear for these patients. Development of

new, better tolerated, treatment approaches is essential. It is pleasing to see that anti-angiogeneic therapies, such as those discussed in an earlier article of this issue [174], have been shown to be effective while presenting with far fewer adverse effects. Nevertheless it is disappointing that similar options for anti-metastasis are not yet available.

The other main focus of this current article was to explore the opportunities that reside in phytochemicals, conventionally reported as having some antimetastatic effects, yet with fewer adverse effects. However, there are significant challenges with phytochemicals. First a lack of IP protection; second, insufficient clinical efficacy data largely due to the lack of IP protection; Third, a lack of continued thorough investigation into their specific mechanisms of action; and finally, the relatively weaker effect of these novel agents in comparison with traditional, high toxicity chemotherapeutic agents. However, these chemicals may well hold great potential within this fragile group of patients with poor general condition.

Thus, the current article has called for more investment and research into the mechanism(s) of cancer invasion and metastasis. At the same time, it is urged that the phytochemical approach should be revisited and emphasized. This area of research may well present an attractive option to this large group of patients with late stage cancers.

## Conflict of interest

None declared.

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