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REVIEW

# Deciphering molecular mechanisms of metastasis: novel insights into targets and therapeutics

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## Abstract

**Background** The transition of a primary tumour to metastatic progression is driven by dynamic molecular changes, including genetic and epigenetic alterations. The metastatic cascade involves bidirectional interactions among extracellular and intracellular components leading to disintegration of cellular junctions, cytoskeleton reorganization and epithelial to mesenchymal transition. These events promote metastasis by reprogramming the primary cancer cell's molecular framework, enabling them to cause local invasion, anchorage-independent survival, cell death and immune resistance, extravasation and colonization of distant organs. Metastasis follows a site-specific pattern that is still poorly understood at the molecular level. Although various drugs have been tested clinically across different metastatic cancer types, it has remained difficult to develop efficacious therapeutics due to complex molecular layers involved in metastasis as well as experimental limitations.

**Conclusions** In this review, a systemic evaluation of the molecular mechanisms of metastasis is outlined and the potential molecular components and their status as therapeutic targets and the associated pre-clinical and clinical agents available or under investigations are discussed. Integrative methods like pan-cancer data analysis, which can provide clinical insights into both targets and treatment decisions and help in the identification of crucial components driving metastasis such as mutational profiles, gene signatures, associated pathways, site specificities and disease-gene phenotypes, are discussed. A multi-level data integration of the metastasis signatures across multiple primary and metastatic cancer types may facilitate the development of precision medicine and open up new opportunities for future therapies.

**Keywords** Metastasis · Epithelial-mesenchymal transition · Circulating tumour cells · Secondary tumour · Pan-cancer analysis · Precision medicine

## 1 Introduction

Metastasis is a characteristic of malignant tumours driven by the interplay of molecular and physiological processes underlying cellular growth, differentiation and survival. Cancer metastasis is accompanied by an accumulation of somatic mutations, epigenetic changes and signals arising from the tumour microenvironment that alter cell shape and integrity, and promote its aggressive phenotype [1, 2]. It is a highly malicious condition that is initiated by a subpopulation of genetically unstable cells within the primary tumour mass which are compelled towards colonizing the body through dissociation from the tumour followed by invasion to neighbouring tissues and transition through the circulatory and lymphatic system to populate distant organs. Metastasis is the most fatal trait of a malignant cancer, which has presented itself as the biggest clinical hurdle by conferring drug-resistance and recurrence characteristics despite breakthroughs that have been made in drug discovery. It has challenged cancer treatment for many years and is the major cause of cancer fatality. Why tumour cells attain a vagrant nature is not known. However, it is not erroneous to note from the context of tumour progression that metastasis is a survival strategy adopted by cancer cells to escape death signals and immune surveillance. It is also a mechanism to retain their population despite a deficiency of resources and spaces induced by hypoxic conditions, nutritional depletion and mutational aberrations causing overcrowding of cells in the primary tumour site [3]. Metastatic cancer cells develop invasion and motility properties with an advantageous plasticity trait to switch between different

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phenotypes and molecular patterns. In addition, they have the ability to modulate primary and secondary tumour microenvironments by withstanding dynamic biochemical changes and traversing across faltering organs to form micro-metastases and, eventually, colonize them [4].

In 1976, Peter Nowell proposed the clonal evolution of tumour cell populations. The same theory turned out to be evident through the advent of the next-generation single-cell sequencing technology showing that primary tumours do not comprise a population of genetically identical cells but rather subgroups of cells discerned by mutations and somatic variations [5]. However, whether metastatic property arises from a single cell from a pool of genetically diverse subsets of cells, or a cluster of genetically diverse subsets of cells is not clear. When explored deeper for the generation of every subset of cancer cells based on molecular phylogenetics, gene expression patterns and protein interactions that regulate various signalling pathways and mutations, the clonal evolution theory may provide relevant information on metastasis-driving factors. The identification of massive metastasis causing factors, however, remains a hurdle. Since the clinical presentation of metastatic traits is noted only after subsequent generations of tumour cell formation, studying it at the clinically observed stage does not allow the understanding of metastasis at its origin, making it a complicated process to master.

Increasing evidence indicates that metastasis employs intrinsic cell motility mechanisms like chemokine signalling, metabolic and immune pathways. These mechanisms are well studied in bone metastasis of breast and prostate cancer (RANKL/RANK/OPG) [6, 7]. On the other hand, the establishment of metastatic colonies is mediated by employing phenotypic switching between epithelial and mesenchymal cell types, similar to what is observed during embryogenesis. However, this is just one aspect and a much more complex molecular interplay is involved. Anderson et al. stated that irrespective of the route of migration, the subset of cells that colonize the secondary site is molecularly and behaviourally distinct from the primary tumour cells, and acquires the ability to localize, adapt and proliferate within a new tissue microenvironment [8]. This notion opens up the scope for interrogating the detailed biological and mechanical aspects of molecular diversity and phenotypic heterogeneity of the many steps involved in the metastatic cascade.

In 1889 Paget proposed the "Seed and Soil" hypothesis while investigating autopsy reports of 735 women with breast cancer. He observed that metastatic cells follow a specific affinity towards the organ to which they migrate. As breast cancer is, for example, commonly observed to metastasize to the bone, in addition to exploring how breast cancer cells adapt to the bone microenvironment, the question of precisely how the bone microenvironment contemplates the growth of breast cancer cells still awaits to be answered [9]. The "Seed and Soil" hypothesis remains one of the most promising blueprints for revealing the mechanisms underlying the organ specificity of metastasis. The physical viability, vascular support and stromal constitution of every organ set the criteria for the migrating cancer cells to inhabit. Indeed, the biological pathways and genes associated with the establishment of the bone microenvironment reveal much about a cancer cell's bone specificity. Moreover, the multiple steps involved in the metastatic process demand to decipher and trace every step of the cascade holistically to comprehensively understand the biology of tumour outgrowth and progression.

There are two modes of targeting metastasis. One is halting the metastatic cascade from occurring at the primary tumour site and preventing dissemination to other sites. The other is to sabotage the already established metastatic colonies. A new class of drugs, referred to as migrastatics, prefers to target the distinct mechanisms liable for cell invasion, motility and migration [10]. Current cancer treatments mainly focus on targeting the primary tumour mass and often include kinase inhibitors and immunotherapies. These treatment modalities mostly elicit cytotoxic effects on cancer cells and block immune checkpoints, respectively, but fail to explicitly attack the dispersal of cells, tumour circulation and dormancy. Early detection of metastasis is also a clinical shortcoming, which is mostly due to limitations in implementing acquired knowledge into metastasis diagnostics. Unfolding molecular knowledge on invasion, migration and signalling pathways involved in specificity and colonization will help the identification of druggable metastasis-related targets and enhance the therapeutic effectiveness. Amply available data linked to genetic mutations, pathway anomalies, transcriptional irregularities and protein structures involved in metastasis are being explored to understand their functional roles in cancer cell environments. Additionally, several inhibitors targeting these molecules are being studied by various methods (*in vitro, in vivo* and *in silico*) to understand their biological significance. In the following sections we will discuss in detail the interplay of molecules driving the metastatic cascade and their therapeutic status. We will also lay emphasis on insights from pan-cancer analyses and how multi-level data integration can guide metastasis treatment decision making and overcome the limitations of current therapies.

## 2 The metastatic cascade

The process of metastasis involves a series of dynamic steps occurring in parallel. The primary tumour associated with its tumour microenvironment comprises T cells, cancer-associated fibroblasts (CAFs), mesenchymal stem cells and tumour-associated macrophages (TAMs) embedded in a rich collagenous extracellular matrix. The microenvironment is triggered by nutrient deprivation, hypoxia, accumulation of mutations and transcriptional alterations. Once a cancer cell

attains the intrinsic metastatic genotype, it is further enhanced to progress by multiple intracellular and extracellular changes. Upon receiving the appropriate molecular signals, the cell starts to attain a more fibrillar and spindle-shaped motile nature with a mesenchymal phenotype, pushing through the endothelial layer and the basement membrane and generating paths to surrounding blood vessels for escape. This process is called intravasation. Some tumour cells are limited to local invasive properties while others are capable of distant migration and invasion, which is deterministic for metastasis [11, 12]. Next, the tumour cells start to flow through the blood system and are referred to as circulating tumour cells (CTCs). Subsequently, they start scanning for a compatible niche (landscape) to establish a metastatic colony (secondary tumour) by passing through the basement membrane, invading the extracellular matrix and establishing a suitable microenvironment, a process called extravasation (Fig. 1).

## 2.1 Molecular interplay in the tumour microenvironment leading to intravasation

### 2.1.1 Remodelling the cytoskeleton

The cytoskeleton components - actin, microtubules and intermediate filaments - are tightly associated with maintaining cellular shape and integrity. Metastasis requires dynamic remodelling of the cytoskeleton to facilitate invasion and motility. Cell migration is a complex biological process that begins with the protrusion of small actin-rich outward projected

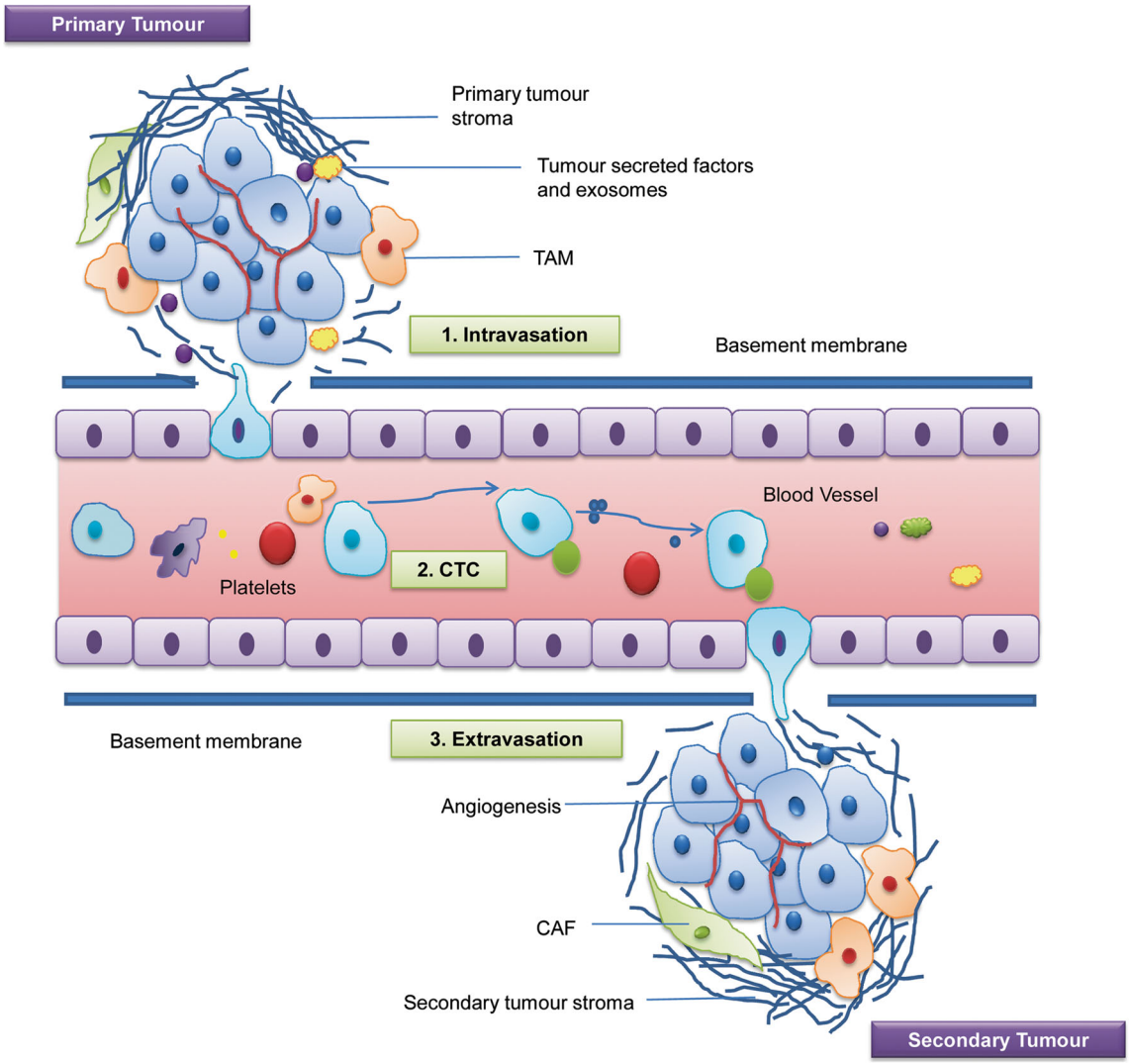


**Fig. 1** The metastatic cascade: (1) Intravasation: The tumour cells break out through the basement membrane and tumour stroma to the surrounding blood vessels and lymph nodes. (2) CTC: The tumour cells then enter the circulation system and are called circulating tumour cells; they scan across the body for suitable secondary niches. (3) Extravasation: Upon reaching the secondary organ, the tumour cells colonize by pushing through the capillary bed, establishing angiogenesis, and a new tumour microenvironment

structures called filopodia, lamellipodia, invadopodia or podosomes (Fig. 2c). The process of actin polymerization initiates the formation of these cellular projections. The molecular regulators of actin remodelling and cell migration are Rho GTPases, RAC and CDC42, and Rho-associated protein kinase (ROCK) [13]. The initiation of invasion represents a composite phase of signals arising from the extracellular matrix (ECM) passed to cytoskeleton components and *vice versa*. Growth factor receptors, cytokine receptors, cadherins and integrins lead to downstream activation of RhoA, RAC and CDC42. RhoA recruits ROCK which phosphorylates LIM kinase, myosin light chain (MLC) and MLC phosphatase, thereby generating actin stress fibres and contractile force [14, 15]. CDC42 and CDC42-interacting protein (CIP4) lead to activation of Wiskott-Aldrich syndrome protein (WASP), N-WASP and the Arp2/3 complex to form filopodia and invadopodia [16]. RAC1 induces a cascade that governs the formation of lamellipodia and branched actin structures. There is another class of proteins called WAVE1 and WAVE2 that mediates a pathway downstream of the GTPases and leads to the formation of lamellipodia by association with the Arp2/3 complex in the same way as WASP [17–20] (Fig. 3). Arp2 also physically interacts with PIK-4, causing phosphorylation at the Arp2 T237/T238 activation site and mediating PIK-4 driven cell movement [21].

CDC42 and RAC1 activate LIMK via phosphorylation, and LIMK inhibits cofilin while Phospholipase C (PLC) activates cofilin. This type of cofilin regulation is useful for

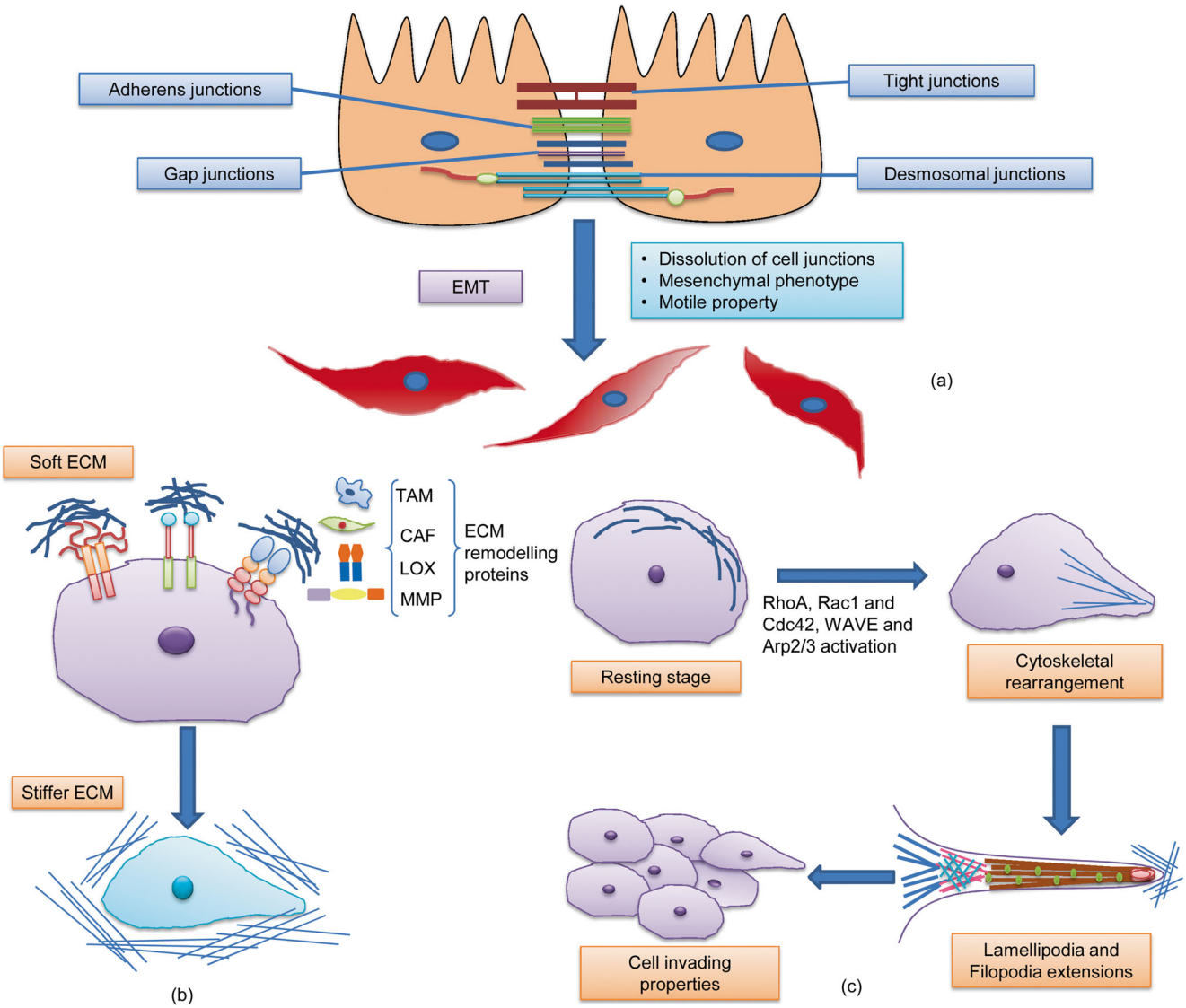


**Fig. 2** Molecular rearrangements leading to phenotypic changes during intravasation. **a** Cells undergo EMT by switching off the gene encoding E-cadherin, CDH1 and other junctional proteins, and upregulating the expression of N-cadherin causing a shift to a mesenchymal phenotype with enhanced motility. **b** Cell-matrix remodelling is mediated by integrins, DDR-2, TAM, CAF, LOX and MMPs to reorganize a stiffer tumour stroma and generate paths for escape to nearby tissues. **c** Rapid polymerization of actin filaments in the cytoskeleton results in leading edges, called lamellipodia and filopodia, to generate contractile force and push the cells through the different migration modes

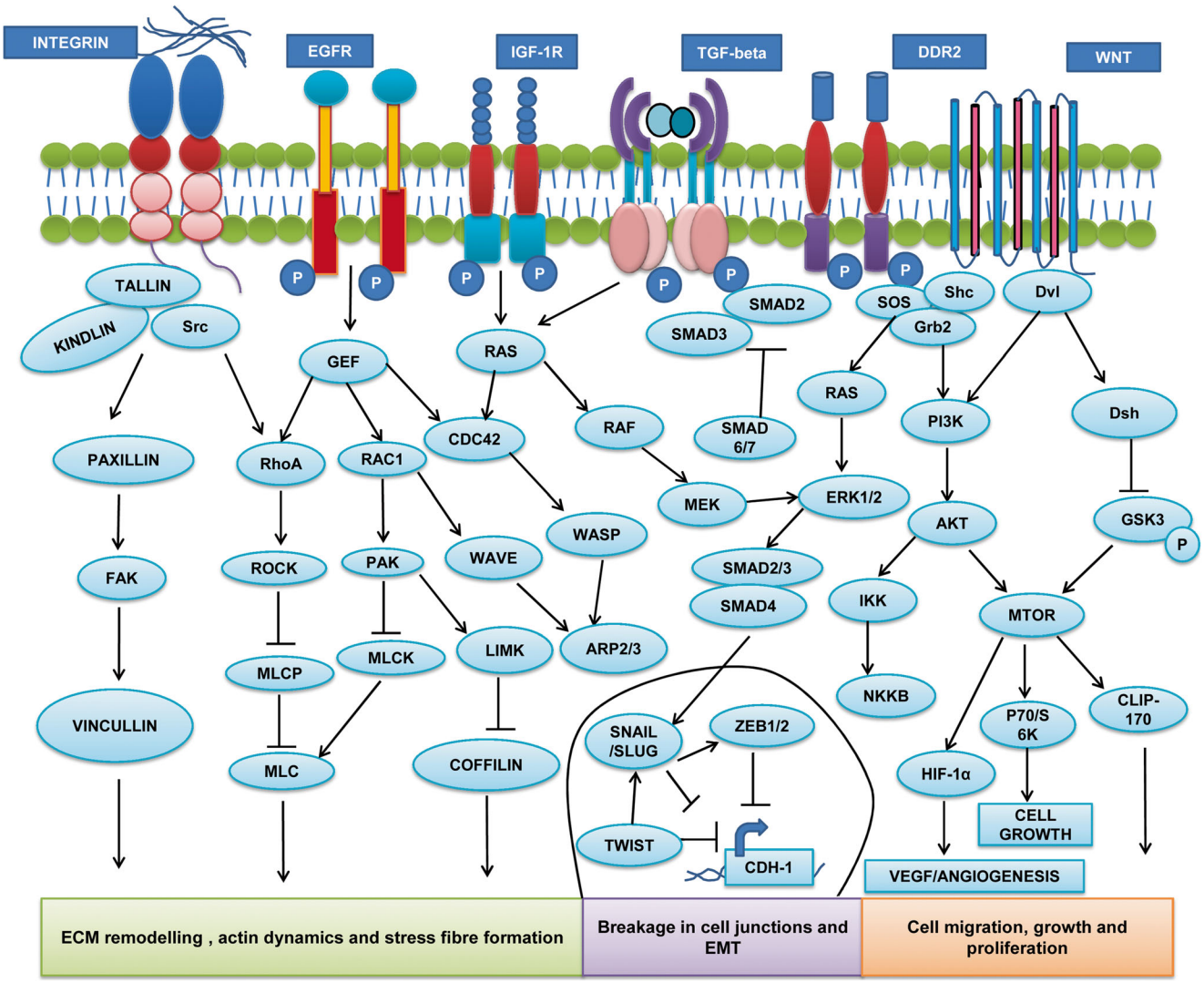


**Fig. 3** Molecular mechanism of metastasis. Interplay between several factors in the tumour microenvironment responsible for causing stromal disintegration, cytoskeleton reorganization and cell migration, along with pathways regulating cellular growth and proliferation, are depicted

initiating actin depolymerization causing an increase in actin filaments, so that they can be recycled for another round of polymerization to form cell protrusions in response to chemotaxis [22, 23]. Amyloid precursor-like protein 2 (APLP2) is involved in pancreatic cancer cell migration and invasion. It interacts with cytoskeleton components through the mammalian homolog of the Drosophila Enabled gene (Mena or Enah), which directs actin arrangement [24, 25]. Actin remodelling also ensures escape from immune recognition. A group invasion of cancer cells is often observed in cancer caused by epithelial cells such as e.g. in breast cancer, melanoma and colorectal cancer. In this type of cell migration, the cell-cell junctions remain intact. A cumulative cellular polarity and cytoskeletal reassembly maintain the cell's response to trajectory force, after which degradation of the basement membrane takes place. The leading cell in this type of migration exhibits a mesenchymal phenotype. It establishes sharp extended edges by Rho-mediated actin polymerization forming the invadosomes and filopodia, whereas the rest of the cells in the mass still maintain their epithelial connections. A major connection between the ECM and the cytoskeleton is established through a structure called Focal adhesion (FA) made up of bundles of F-actin tethered to myosin. It mediates several extracellular stimuli and can perceive mechanical forces experienced by a migrating cell.

Syndecans constitute another class of transmembrane receptors that can interact with various growth factors to eventually activate the PI3K pathway and RhoA to modulate cell motility. Syndecan-1 is expressed in head and neck cancer, ovarian cancer and breast cancer, and syndecan-4 is expressed in breast cancer. It interacts with growth factor receptors (GFRs; EGFR, IGF-IR, HER2) and integrins ($\alpha 3\beta 1$, $\alpha 6\beta 4$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha 5\beta 1$). It is crucial for mediating cellular functions such as proliferation, adhesion, angiogenesis and survival [26, 27]. Integrins ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$, $\alpha 11\beta 1$), representing a class of cell surface receptors, bind to specific

motifs in a wide range of collagens and transduce bidirectional signals. Binding of collagen I, III, IV, IX, XIII and XVI to integrin $\alpha1\beta1$ and collagen I, III, IV, V and XI to integrin $\alpha2\beta1$ leads to activation of focal adhesion kinases (FAK) followed by downstream activation of ERK, which induces phosphorylation of the myosin light chain (MLC) by myosin light chain kinase (MLCK) leading to F-actin stress fibre formation and cytoskeleton reorganization [28, 29]. Additionally, it activates PI3K, RAS and AKT signalling, leading to cellular proliferation, transcription, apoptosis and metastasis [28]. As collagens bind to the extracellular domains of integrins, cytosolic proteins like Talin and Kindlin are recruited to the cytoplasmic tail of integrins, which directs interactions between actin in the cytoskeleton and integrins in the membrane leading to the trading of proteins associated with cell-cell junctions and cell-matrix junctions called Vinculin, Paxillin and FAK [30] (Fig. 3). These molecular events again highlight activation of GTPases (RhoA, RAC and CDC42), modulating cytoskeletal activity [31]. FAK activation is also key to actomyosin contractility and invasion of cells in collagen 3D matrices. Suppression of FAK reduces transforming growth factor-β (TGF-β)-mediated tumour cell migration and invasion [32].

#### 2.1.2 Transforming the extracellular matrix

Tumour cells are embedded in a dense network of extracellular matrix made up of collagen, laminin, fibronectin, elastin and glycoproteins (hyaluronan and proteoglycans). These molecules are proteolytically degraded and remodelled during metastasis by enzymes like hepsin, cathepsin, heparanase and matrix metalloproteinases (MMPs) [33]. Collagen, a major component of the extracellular matrix known for tissue support and structural stability, is degraded by MMPs during metastasis. A major class of MMPs, encompassing MMP-1, MMP-8 and MMP-13 are referred to as collagenases, is involved in proteolytic cleavage of a group of collagens (Collagen-I, II, III, VII, VIII, X) and laminins, which induces remodelling of the ECM during induction of the metastatic cascade [34]. MMPs in the tumour microenvironment serve as predictive biomarkers of tumour progression and metastasis. Another enzyme called lysyl oxidase (LOX) mediates cross-linking of fibrillar collagen with fibronectin and elastin and induces tumour stroma stiffening through proteolytic cleavage. This creates a path that regulates a directional movement of tumour cells through the tumour stroma to extravasate to the nearest vasculature. LOX is an important contributor to tumour invasion, migration and stroma coordination [35, 36] (Fig. 2).

Discoidin domain receptor 1 (DDR1), a membrane-bound receptor tyrosine kinase associated with communication with the stroma, is highly involved in remodelling the ECM. Collagens, upon binding with DDR1, activate the extracellular regulated kinase PI3K, as well as NOTCH and STAT3. This promotes reactivation of dormant cancer cells at metastatic sites and further downstream by inducing cell division and migration, respectively [37]. DDR1 has been reported to be an essential contributing factor to breast cancer metastasis [38]. Interaction between cancer cells and their new stromal environment is a critical factor determining metastatic fate. Vascular adhesion molecule 1 (VCAM1) is expressed in breast cancer and interacts with integrins ($\alpha4\beta1$) and tumour-associated macrophages (TAMs) to activate the PI3K pathway, thereby promoting cancer cell survival, growth and angiogenesis [39, 40]. It is regarded as a potential therapeutic target of metastasis.

The interstitial spaces of cancerous tissues contain an array of cells, including cancer-associated fibroblasts (CAFs) which often represent dysregulated fibroblasts or myofibroblasts, tumour-associated macrophages (TAMs) and tumour-associated neutrophils (TANs) [41, 42]. A desmoplastic condition of a tumour activates CAFs. It is a condition of continuously receiving inflammatory signals from interleukins (ILs), TGF-β, hypoxia and reactive oxygen species (ROS), which leads to changes in the ECM and disturbs homeostasis. CAFs actively produce collagen and proteases that remodel the ECM. TAMs accumulate in abundance in the tumour microenvironmental edges. It is evident that TAMs are linked with TGF-β signalling and confer immune resistance and epithelial to mesenchymal transition (EMT) properties [43]. Vascular endothelial growth factor A (VEGFA) expressing macrophages confer intravasation properties to tumour cells through vascular permeability. TAMs reduce $CD8^+$ T cell cytotoxic effects by expressing inhibitors like PDL-1, B7-H4 and indoleamine-2,3-dioxygenase [44]. The decreasing level of $CD8^+$ T cells and natural killer (NK) cells increases breast cancer metastasis without affecting primary tumour growth. It produces a higher number of proteolytic enzymes such as MMPs (MMP-2, MMP-7, and MMP-9), cathepsins that mediate ECM degradation and cell-ECM biochemical interactions. TAMs produce a metastatically important protein called SPARC that regulates ECM decomposition and produces a traction force by allowing interactions between fibronectin and vitronectin. This eventually pulls the cancer cells through the stroma to the nearest vasculature. They are also associated with chemoresistance [45–48]. Together, TAMs, tumour cells and endothelial cells have been referred to as tumour microenvironment of metastasis (TMEM) and found to be associated with a poor breast cancer prognosis [49].

Chemokine signalling is one of the most important factors carrying tumour cells across to their metastatic sites. CCR7 released from breast tumour cells signals its migration and metastasis through a CCL19 or CCL21 stimulus [50]. CXCR5 and CXCL13 support bone metastases in prostate cancer. In several tumours, CXCR4 expression allows tumour cells to metastasize into organs secreting high levels of

CXCL12. It is also specifically associated with the metastasis of cancer cells to lymph nodes, lung and bone. Lung metastasis in breast cancer is associated with metastasis-associated macrophages (MAMs). Various cytokines and chemokines have been reported to regulate the recruitment of MAMs to the lungs. In prostate cancer, CXCR6-CXCL16 interactions lead to Ezrin and integrin activation, causing leading-edge extension through FAK/PI3K/PKC, thereby increasing cytoskeletal dynamics and invasion [51].

#### 2.1.3 Loss of cell adherence and EMT

For a cancer cell to migrate to distant organs, it should dissociate itself from the mass of clumped cells in a tumour microenvironment consisting of a rich ECM network of collagen and dense blood supply. One way by which a cancer cell accomplishes this is by undergoing a biochemically driven phenotypic shift called EMT. Cells make contact with each other through tight junctions, adherens junctions, desmosomes and gap junctions. EMT affects these structures due to loss of cadherin, causing decreased contacts, loose associations and attainment of migratory properties resulting in anchorage-independent survival. E-cadherin binds to beta-catenin, alpha-catenin and p120-catenin in these junctions, mediates intracellular signalling and establishes a tight integration of intercellular junctions as well as a firm link with the ECM [52]. However, upon the advent of EMT, E-cadherin expression is lost and mesenchymal markers such as N-cadherin and vimentin are expressed [53, 54]. EMT is activated by several redundantly occurring biochemical pathways, but the most proficient one noted so far is the TGF-β signalling pathway (Fig. 2a).

Binding of TGF-β to the tetrameric type II TGF-β receptor leads to phosphorylation of the type-I TGF-β receptor, which phosphorylates the C-termini of a downstream protein complex, SMAD. The receptor activates SMAD2 after which SMAD3 recruits SMAD4. This SMAD complex translocates to the nucleus where it, in conjunction with other transcription factors such as TWIST, SNAIL and ZEB, regulates EMT. SNAIL1 and SNAIL2, also known as SLUG, are the major regulators of EMT during cancer progression [55]. They represses expression of the gene encoding E-cadherin, *CDH-1*, by binding to its E-box through a ZINC finger domain. Upon binding to the E-cadherin gene promoter, SNAIL1 recruits Polycomb repressive complex-2 (PRC-2), which mediates histone modification, mainly methylation and acetylation, thereby silencing the *CDH1* gene. While downregulating the epithelial gene signature, SNAIL upregulates mesenchymal genes such as those encoding Fibronectin and N-cadherin. TWIST, a basic helix-loop-helix transcription factor, is also involved in downregulating E-cadherin expression. HIF1α, under hypoxic conditions, induces TWIST expression and thereby EMT. Another class of transcription factors that mediates EMT is ZEB. Activation of ZEB is often brought about through SNAIL [55]. TWIST interacts with SNAIL which leads to ZEB expression (Fig. 3). ZEB expression has been found to be high in breast and lung cancer. It represses the E-cadherin gene by binding to the carboxyl-terminal binding protein (CTBP) co-repressor [56]. Additionally, EMT may be induced by several other signalling molecules such as PI3K, PDGF, FGF and HGF. EMT is a reversible process. When a cancer cell undergoes EMT and detaches from the tumour mass, it travels across the body to other organs and establishes a secondary tumour by undergoing mesenchymal to epithelial transition (MET). During MET, previous cellular events are reversed in order to establish secondary tumours. EMT mostly facilitates cell invasion. It is a common feature exhibited by fibroblasts and stem cells during wound healing and is commonly observed in glioblastoma and melanoma.

Amoeboid cell invasion has been described as the fastest migration pattern with a velocity of up to 20 mm/min. This type of migration mostly develops after treatment of cancer cells with integrin-blocking antibodies or protease inhibitors. Blocking β1 integrin in cancer cells results in loss of cell-cell adhesion, after which the cells transit from a collective to an amoeboid mode. Collapsin response mediator protein-1 (CRMP1) has been found to attribute to a reduced transition of the epithelial to mesenchymal phenotype and to stabilized F-actin in an *in vitro* prostate cancer model by associating with SNAIL and WAVE1, respectively [57]. Disruptor of telomeric silencing 1 like (DOT1L) interacts with the c-Myc-p300 complex and causes methylation and acetylation of histone H3 at lysine residue 79 (H3K79). This, in turn, leads to the upregulation of SNAIL, ZEB1 and ZEB2, and induces cancer stem cell (CSC) properties in breast cancer cells and metastasis initiation. Gain of epithelial markers and loss of mesenchymal markers has been observed when psammaplin A analog (PsA-3091) was used to inhibit the DOT1L protein [58, 59]. A recent study has suggested that there may be a single switch between EMT and MET. YAP, Wilms Tumour-1-YAP (WT1-YAP) and YAP-TRIO-Merlin were found to cause loose cell adhesion and to downregulate E-cadherin, and to regulate Rho GTPases involved in cytoskeleton arrangement and migration, respectively. At the same time, WT1 is known as a regulator of MET [60]. Currently, there are several ongoing clinical trials (Table 1) evaluating whether EMT and its associated molecular characteristics can be used as a diagnostic biomarker for cancer progression, metastasis and treatment response.

### 2.2 Circulating tumour cells (CTCs)

Cells detached from the primary tumour mass that enter into blood vessels and travel along the body through the circulatory system are called circulating tumour cells (CTCs). They are a hallmark of metastatic progression and may be used for diagnostic purposes [61]. CTCs have been shown to express EPCAM (epithelial cell adhesion molecule), a biomarker of distant metastasis and tumour recurrence [62]. Several

techniques are employed at the clinical level to detect metastasis, including X-ray, radiography, computed tomography scan (CT scan), nuclear imaging including PET and SPECT, magnetic resonance imaging (MRI), lymphadenectomy and lymphangiogram. However, these techniques show accuracy only in detecting local metastases that most commonly present themselves by spreading across nearby lymph nodes. CTCs provide a scope for metastasis diagnosis using a simple non-invasive technique, i.e., isolation from blood. Techniques are emerging to accurately detect cancer by isolating CTCs and circulating tumour DNA (CtDNA) from blood, the so-called "Liquid biopsy" approach, which is readily feasible for downstream 'omics' analyses serving the purpose of understanding cancer biology and clinical interpretation [63].

In the bloodstream, cancer cells are exposed to adverse conditions like oxidative stress, immune attack, shear forces, anoikis, and lack of growth factors and cytokines. Thus, many CTCs are likely to die. However, growing evidence suggests that tumour cells may associate with platelets to withstand varying degrees of oxidative stress and to undergo reversible biochemical modifications to withstand the adverse conditions and to shield themselves from shear forces. The association between CTCs and platelets allows the secretion of platelet granules that provide resistance to apoptosis. Platelets release TGF-β and induce the TGF-β pathway, which helps CTCs to maintain mesenchymal characteristics [64]. CTCs associated with platelets facilitate extravasation by allowing the cells to arrest in the capillary bed. HER2/EGFR/HPSE/NOTCH represent gene signatures identified in CTCs of breast cancer patients that potentially metastasize to the brain [65]. CTCs expressing CD44 and CD47 in breast cancer patients serve as markers of the metastasis-initiating population of CTCs [66]. CTCs may also withstand death signals by producing various anti-apoptotic factors such as BCL-2.

Clusters of CTCs, called circulating tumour micro-emboli (CTM), are assumed to be the harbingers of metastasis. Cell enrichment analysis has provided insight into the genomic and transcriptomic profiles of CTCs, thereby opening up the scope to understand relationships between primary and secondary tumours. CTCs provide clinical utility like target discovery, patient monitoring, drug responses and screening. The immune system plays a dual role in cancer [61]. In the early stages of tumour development, the immune system kills and rejects cancer cells. However, after initiation of the metastatic cascade, the immune system plays an immunosuppressive role. CTCs adopt numerous complex mechanisms to escape from immune attack. They readily recruit macrophages and NK cells by secreting cytokines and chemokines, such as IL-6 and IL-8, and undergo genetic alterations to lower their immunogenicity [67].

## 2.3 Extravasation

The final stage of metastasis is called extravasation or also referred to as colonization. Metastatic cells begin colonizing

**Table 1** Clinical trials evaluating EMT as a biomarker of cancer progression and metastasis

| Clinical trial number | Study goal | Start date and status |
|---|---|---|
| NCT04021394 | Dynamic Influence of the Epithelial-to-mesenchymal Transition (EMT) on Circulating Tumour Cell (CTC) Generation, Phenotype, and Disease Progression in Prostate Cancer | Started: June 5, 2019, Ongoing |
| NCT04323813 | High Levels of EMT-TFs for the Diagnosis of Colorectal Cancer (CRC) | Started: September 1, 2017, Ongoing |
| NCT04137406 | Role of SIRT1 in the regulation of EMT in Breast Cancer Lymph Nodes Metastasis for Luminal A Subtype | Started: January 1, 2019, Ongoing |
| NCT04323917 | Detection of High Expression Levels of EMT-TFs mRNAs in Patients with Pancreatic Cancer and Their Diagnostic Potential | Started: November 2, 2017, Ongoing |
| NCT03381326 | Biomarkers Study: Circulating Tumour Cells (CTC), Free DNA, Stem Cells, and EMT Related Antigens as Biomarkers of Activity of Cabazitaxel in Castration-resistant Prostate Cancer (CRPC): a Proof of Concept. | Started: December 15, 2014, Ongoing |
| NCT03509779 | EMT, Reactivation of Embryonic Transcription Factors and Alteration of the miR Signaling Network as Prognostic and Predictive Markers in Lung Cancer | Started: October 20, 2014, Ongoing |

at distant sites way before they actually arrive at the desired location. They do so by forming a pre-metastatic niche (PMN). Tumour cells shed vesicles or exosomes that are involved in establishing the PMN. PMNs have been found to be very organ-specific. In case of the liver, for example, tissue inhibitors of metallopeptidase (TIMP1), stromal cell-derived factor-1 (SDF1) and macrophage inhibitory factor (MIF) serve as PMN biomarkers, whereas bone morphogenetic protein (BMP), WNT, fibroblast growth factor (FGF) and insulin-like growth factor (IGF1) serve as bone PMN biomarkers. The PMN recruits CTCs and creates a home for metastatic cells by altering and modifying its microenvironment [68]. The extravasated cells must adapt well to the microenvironment of the newly encountered organ to be able to colonize it. Various molecular factors as well as the biological topography of the secondary organ microenvironment affect local tumour cell growth. Mesenchymal to epithelial transition (MET) is one factor associated with colonization of the second site. Significant effectors of MET include c-Met, Frizzled-7, Frizzled-2 and FGFR2b [69, 70]. Although primary tumours have a certain specificity towards the site of metastasis (organotropism), it is ultimately defined by the primary tumour type, the secondary site microenvironment and the preferred biological interactions between them [71].

## 3 Site specificity of metastasis and its clinical management

The dynamic process of metastasis with its multiple interdependent as well as independent biological mechanisms and organotropic properties demands a distinct treatment strategy for each type of metastasis. So far, metastasis is mainly managed by medicines, without reaching ultimate cure. Tracking metastatic progression at the systemic level is a big hurdle, as major experiments are being carried out in murine models with a relatively short life span. In humans, however, secondary metastatic tumours are encountered even after more than two years. Recent 3D culture systems are equipped to study static primary tumour parameters, but cannot replicate dynamic events leading to distant metastasis. Although *in vivo* models may provide a better scope to investigate these processes, it still needs to be determined which experimental techniques and *in vivo* models accurately reflect the actual clinical situation. There are also several challenges associated with designing clinical trials for metastasis and for deciding the endpoint. Current anti-metastatic drugs mostly target the cell migration and colonization phenomena. Hence, they do not yield a classical clinical outcome like complete response (CR) or partial response (PR). Therefore, stable disease is the commonly used criterion to measure outcome. Most trials dealing with metastasis rely on surrogate endpoints.

A well curated resource called MetMap (metastasis map) launched by the Broad Institute enables real time revelation of organ-specific patterns of metastasis. The investigators injected a pool of barcoded cell lines into immunocompromised mice after which the ability to metastasize was studied by bioluminescence imaging. This method was performed across 500 cell lines across 21 types of solid tumours, Quantification of barcoded breast cancer cells in brain metastases using RNA-sequencing revealed interesting specific signatures such as expression of PI3K and ERBB2, which enables breast cancer cells to metastasize to the brain and other organs for comparative purposes [72]. On a pre-clinical account of metastasis, several compounds have been validated through detailed experimental studies carried out *in vitro* and *in vivo*, but they are yet to be implemented in rationally designed clinical trials and authentication through it in order to be incorporated in standard of care (SOC) therapeutic protocols for metastatic cancer [73]. Clinical reports of breast cancer cohorts suggest that there is no linear relation between tumour size, lymph node status and distant metastases. This essentially evokes a question for the conventional practice of tumour - nearby nodes - metastasis (TNM) staging, that as the tumour enlarges it is more prone to invasion/metastasis [74]. In fact, a tumour may already have metastasized by the time it is diagnosed. The major initiating contributions of the ECM, cytoskeleton modulating factors and angiogenic drivers in the metastatic cascade has placed them in the frontline of potential targets. In addition, there are strategies in practice to directly target CTCs as well. However, since the number of detectable CTCs may be low, the use of kinase and immune checkpoint inhibitors is considered as an efficient strategy in medical oncology. Although several inhibitors have already passed FDA approval to be used in trials for recently discovered targets, so far the outcomes of trials specifically for mitigating metastasis are still hazy. Figure 4 provides information on various tumours and their metastatic sites and highlights the drugs available for the respective metastatic conditions.

### 3.1 Brain metastasis

Common cancers that metastasize to the brain are lung, breast and kidney cancer. Access to the brain through the vascular system is tightly regulated by the blood-brain barrier (BBB). In metastatic colonization to the brain, the BBB is squandered, allowing the cancer cells to interact with the brain parenchyma and with glial cells to colonize it. Associations of transport systems with the BBB mediates selective transport of molecules. Due to this barrier, the application of conventional drugs for e.g. lung cancer and melanoma brain metastases have shown very little pharmacodynamic effects due to their low penetration [75]. $HR^{-}/HER2^{+}$ (HER2-enriched) breast cancer has a relatively high chance of forming brain metastases, which is at least partly due to its molecular features

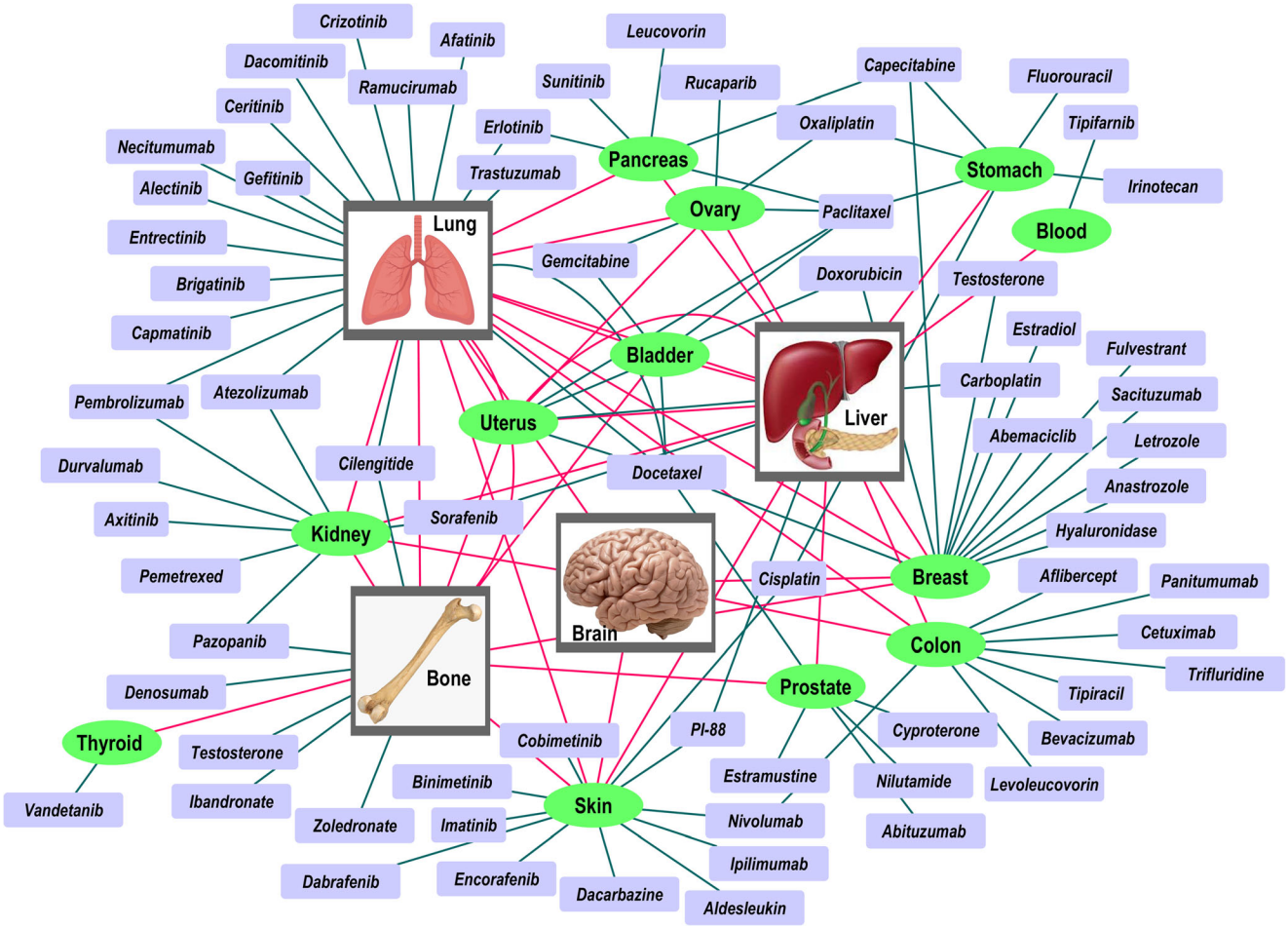


**Fig. 4** Representation of the site-specific metastasis of different types of primary tumours and the drugs available for each of the metastatic cancer types. The images within the grey boxes represent the major metastasis sites. The green nodes represent the primary malignant tumours, and the grey coloured rectangles represent the therapeutic drugs available. Green lines represent drugs available for the particular cancer treatment, and pink lines show the primary tumour's major metastatic sites

including the expression of L1CAM, COX-2, IL-8, CK-5, CXCR4, VEGF and CD44 [71]. Melanoma to brain metastasis is led by activation of STAT3, which dysregulates the expression of bFGF, VEGF and MMP-2 [76]. A number of microRNAs regulating crucial metastasis-associated genes across several lung cancer types to brain metastasis has been found to be dysregulated, such as downregulated miR-768-3p, miR-95-3p and miR-146a, and upregulated miR-378, miR-197 and miRNA-184 [77]. Combination therapy of carmustine with methotrexate or radiation therapy has been found to be effective in managing breast cancer to brain metastasis [78, 79].

A small scale clinical trial on the combination of dacarbazine (BOLD) and intermittent Granulocyte-colony stimulating factor (G-CSF) revealed that a majority of patients reached complete response (CR) for stage 4 melanoma to brain metastasis [80]. The DNA repair enzyme MGMT (O[6]-methylguanine-DNA methyltransferase) causes tumour cell resistance to alkylating agent-induced damage. Temozolomide (TMZ) and lomustine (CCNU) are the two most used alkylating agents to treat glioblastoma and brain metastasis. A phase III study was conducted with the combination of TMZ and CCNU, and TMZ alone in patients with glioblastoma, and a better overall survival rate was observed [81, 82]. Recently, clinical trials have been initiated to study BRAF inhibitors in combination with ipilimumab, and the epidermal growth factor receptor 2 (EGFR2) inhibitor lapatinib for melanoma to brain and breast to brain metastasis [83] (Fig. 4).

### 3.2 Bone metastasis

Tumours that metastasize to bone may provide insight into reciprocal molecular adaptations that occur between cancer cells and the host stroma. The most common cancers that metastasize to the bone are breast and prostate cancer. Metastasis in the bone microenvironment is regulated through osteoclastic or osteoblastic mechanisms. Breast cancer metastasis to bone is induced by hyperactivation of bone-resorbing osteoclasts, which clinically present with painful fractures.

Osteolytic breast cancer cells inhibit osteoblastic pathways like the BMP and Wnt pathways, and overexpress osteoclast-inducing factors such as Parathyroid hormone-related protein (PTHrP), IL-8, IL-11, CTGF and RUNX2. They also secrete a protease that cleaves Receptor activator of nuclear factor kappa-B ligand (RANKL) into an active form, thereby activating osteoclast activity. On the other hand, prostate cancer cells secrete a high amount of osteoprotegerin, a RANKL inhibitor that prevents osteolytic reactions.

Prostate cancer metastasizes to the bone by stimulating osteoblasts, which leads to increased bone density and, eventually, bone marrow displacement [84, 85]. Forkhead box F2 (FOXF2) functions as the major transcription factor for conferring bone colonizing properties to breast cancer cells (osteomimicry) by activating BMP and SMAD1 signalling and expressing bone differentiation-associated genes [86]. Bisphosphonates are the standard of care for bone lesions. Clodronate (clodronate disodium, Bonefos) is a non-nitrogen-containing bisphosphonate used in breast to bone metastasis and blocks bone resorption and osteoclast activity [87]. The administration of another bisphosphonate, pamidronic acid, to breast cancer to bone metastasis patients showed reduced levels of platelet endothelial cell adhesion molecule-1 (PECAM-1) and angiogenesis factors [88].

Administration of ibandronate and zoledronic acid has been the most common practice for skeletal event management in breast cancer, multiple myeloma and prostate cancer patients [89, 90], whereas Samarium Sm 153 Lexidronam (Quadramet) may provide relief from pain associated with bone metastasis from prostate cancer [91]. Denosumab is a monoclonal antibody directed against RANKL that prevents bone resorption and has shown to be effective against bone metastasis [92]. It provides metastasis-free and disease-free survival in cases of prostate cancer and breast cancer to bone metastasis. A small molecule inhibitor of RANKL, AS2676293, has been found to markedly inhibit the metastasis of human breast cancer MDA-MB-231-5a-D-Luc2 cells to the bone and to reduce RANKL-mediated tumour migration of B16F10 melanoma cells to the bone in a mouse model [93] (Fig. 4).

### 3.3 Lung metastasis

Metastasis to lungs is relatively common as the lungs provide a large surface for tumour cell growth and survival. It starts at the small pulmonary arterioles where the cells burst through the endothelial cells and the underlying basement membrane. Metastatic extravasation can be halted for a while by inhibiting the expression of the cytoskeletal anchoring protein Erzinin in osteosarcoma cells that metastasize to lungs [94]. Extraordinary lung metastasis cases detected from renal cell carcinoma after 37 years of nephrectomy have been reported, suggesting an extremely dormant metastatic phase, yet intangible to scientific understanding [95]. A diverse array of gene signatures has been found, like those associated with breast cancer stem cells (BCSCs) that regulate breast cancer metastasis to the lungs, and other factors like interactions between CXCL12 and CXCR4, and interactions among $\alpha 4\beta 1$ integrin and VCAM1 [96]. Also upregulation of miR-374a, miR-18a, miR-115 and miR-9, and downregulation of miR-22, miR-26a, miR-320a and miR-302a along with the expression of metastasis enhancing factors such as secreted protein, acidic, cysteine-rich/osteonectin (SPARC) and colony-stimulating factor 1 (CSF-1) have been associated with breast to lung metastasis [97].

The genes observed to be affected in lung metastasis are distinct from those observed in bone metastasis. This underscores the distinct architecture, as well as genomic and metabolic profiles of the secondary site mediating organ specificity and demanding new therapeutic targets [98]. Alectinib and Ceritinib are the most widely used drugs for ALK-positive metastatic non-small cell lung cancer (NSCLC) and its metastasis to the brain [99]. Atezolizumab, a PD-L1 inhibitor combined with kinase and angiogenesis inhibitors, is currently being studied in several clinical trials for brain or CNS metastasis and first-line therapy treatment of metastatic non-squamous NSCLC and cisplatin-ineligible urothelial carcinoma [100, 101]. Experimental results suggest that Atezolizumab exerts its inhibitory effects on metastasis by downregulating cell invasion and EMT-associated genes CXCR4, METTL7A, ICAM3, LPPR3, S100A8, SUSD3, HVCN1, MMP9, MMP19, GLIPR1L2, N4BP2L1, CDHR2, IL11RA, CSRP2, FOXQ1, KLF8, FGF11, CLDN1 and CLDN24, and upregulating the KISS1 metastasis suppressor gene in MDA-MB-231 cells [102].

Clinical trial evidence shows that the PD-1 inhibitors Nivolumab and Pembrolizumab have become important players in the treatment of metastatic lung and renal cell carcinomas and melanomas. They enable the inhibition of these cells to from a tumour microenvironment and help to maintain immune system-mediated cancer cell killing. The emergence of the anti-angiogenic drugs as being anti-metastatic resulted in the development of several vascular endothelial growth factor (VEGF) inhibitors. Bevacizumab, as an example, has potentially minimized the problem of metastasis in clinical settings. It has shown beneficial results for non-squamous NSCLC with brain metastasis as concluded after multiple clinical trials. Bevacizumab in combination with Paclitaxel/Fluorouracil/Leucovorin/Oxaliplatin has shown efficient anti-metastatic activity in breast and colorectal cancer cases [103] (Fig. 4).

### 3.4 Liver metastasis

Supplied by both systemic and portal circulation, the liver is the metastatic destiny of most gastrointestinal cancers. The

discontinuous lining of the endothelial cells in the liver makes it highly porous, allowing entrapment of CTCs. Other primary tumours like breast, melanoma and lung cancer metastasize to the liver through systemic vasculature. In colorectal cancer, increased serum levels of HSP60 and IGFBP-2 are indicative of liver metastasis [104]. Increased LOX mRNA and LOX nuclear localization in CRC tumour tissues are associated with liver metastasis. The tyrosine-phosphatases PRL-3 and PIAS2, a protein inhibitor of activated STAT2, are significant biomarkers for CRC to liver metastasis. Claudin-2 is associated with breast to liver metastasis by inducing c-Met (specific tyrosine kinase receptor), allowing matrix and adhesion remodelling. IL-6, IL-8 and TNFA are specific drivers of prostate to liver metastasis [105].

Floxuridine (FUDR), targeting thymidylate synthase, is the most commonly administered drug in the case of gastrointestinal metastasis to the liver [106]. Also, new strategies are being developed for treating metastatic conditions. In particular TGF-β has attained interest due to its known role in cancer metastasis. Next to its involvement in EMT and cell proliferation, TGF-β is involved in mediating cell dormancy and autophagy, biological processes that are closely associated with tumour progression and metastasis. In clinical trials, the small molecule inhibitor of TGF-β type I receptor (ALK5) Galunisertib, combined with Gemcitabine, has shown potential anti-tumour/anti-metastatic effects in pancreatic cancer [107]. An improved anti-tumour activity was seen in a phase I clinical trial that was carried out in patients with advanced solid tumours with M7824 (MSB0011359C), a bifunctional fusion protein composed of a mAb directed against programmed death-ligand 1 (PD-L1) fused to TGF-β [107, 108]. The study showed better efficacy and safety than administration of PD-L1or TGF-β inhibitors alone in advanced solid tumours. Palbociclib is a recently approved CDK4/CDK6 inhibitor employed to treat HR-positive, HER2-negative advanced or metastatic breast cancer (Fig. 4). CDK4 and CDK6 play prime roles in the transition of G1 to S phase during cell division. Palbociclib arrests cells in the G1 phase of the cell cycle and, thereby, prohibits their entry into the S phase. It inhibits phosphorylation of CDK4/6 kinase by ATP, causing hypo-phosphorylation of pRb thereby suppressing activation of several transcription factors required for cell cycle progression. It has recently been implemented in clinical trials for cancers with metastatic potential to brain and bone [109]. A drug targeting the perinuclear compartment called Metarrestin is currently under investigation and shows potency to suppress metastasis in a pancreatic cancer xenograft model [110, 111].

## 4 Targeting metastasis: insights from pre-clinical investigations

The composite protein-protein interaction networks associated with the various metastasis-associated genes illustrate their complex modes of action and the difficulty to identify suitable targets (Fig. 5). Efforts that are put in pre-clinical metastasis studies do not reciprocate in the clinical end, resulting in a gap between pre-clinical output and clinical implementation. The emerging approach of systems biology in understanding metastasis using integrated data across different levels of tumour biology holds promise in interpreting the multidimensionality of the metastatic cascade by touching upon its genetic and physiological conditions from the single cell up to the clinical state. The quest for metastasis targets and therapeutics may be fulfilled by an integrated network of computational and experimental models scaled up to a clinical understanding. In the context of drug discovery, several target complexes and their inhibitors are available, but a holistic understanding is still a requisite. Several proteins known to be involved in metastasis interact with each other, but how exactly their dynamics, interactions and functionalities change after drug administration and how this affects the metastatic cascade warrants further understanding [112].

EMT is one characteristic associated with metastasis and several transcription factors are involved in carrying out metastasis-related EMT. Dysregulated EMT-inducing transcription factors may form a unique class of drug targets that mediate the epithelial to mesenchymal switch. TWIST, an EMT-inducing transcription factor, appears to be a potential target in reversing EMT and, hence, metastasis. Harmine, a harmala alkaloid, has been found to inhibit multiple properties of TWIST1 and to potentially show marked anti-tumour activity in oncogene-driven NSCLC, including EGFR, KRAS and MET mutant NSCLC [113]. Another important transcription factor driving EMT is SNAIL. CoIII-Ebox has been found to proficiently inhibit SNAIL activity in SKBR3 and MCF7 breast cancer cells by inhibiting the decrease in epithelial markers such as E-cadherin and cytokeratin and by inhibiting the increase in mesenchymal markers such as Fibronectin and MMP-9. It could also effectively delay a gain in invasive phenotype [114]. ZEB1, yet another transcription factor, may upon inhibition reverse paclitaxel resistance in ovarian carcinoma cells and reduce their metastatic properties [115].

Drug repurposing revealed an EMT inhibitory effect by downregulating miR-21 expression when MCF7 breast cancer cells were treated with propofol [116]. Also, many potential MEK inhibitors have been investigated. One of the most widely studied is selumetinib (AZD6244; ARRY-142,886; AstraZeneca), a highly selective allosteric inhibitor of MEK1/2. Upon administering selumetinib to TNBC cells in a xenograft mouse model, EMT was reversed, as indicated by a reduced mesenchymal phenotype with stem cell-like characteristics and a $CD44^{+}/CD24^{-}$ expression pattern, to an epithelial phenotype and reduced lung metastasis [117]. In another study, combinatorial treatment with a Src inhibitor (AZD0530) and a MEK1/2 inhibitor (AZD6244) in a breast cancer model induced apoptosis in a large fraction of the dormant cells and delayed metastatic outgrowth [118]. Recently,

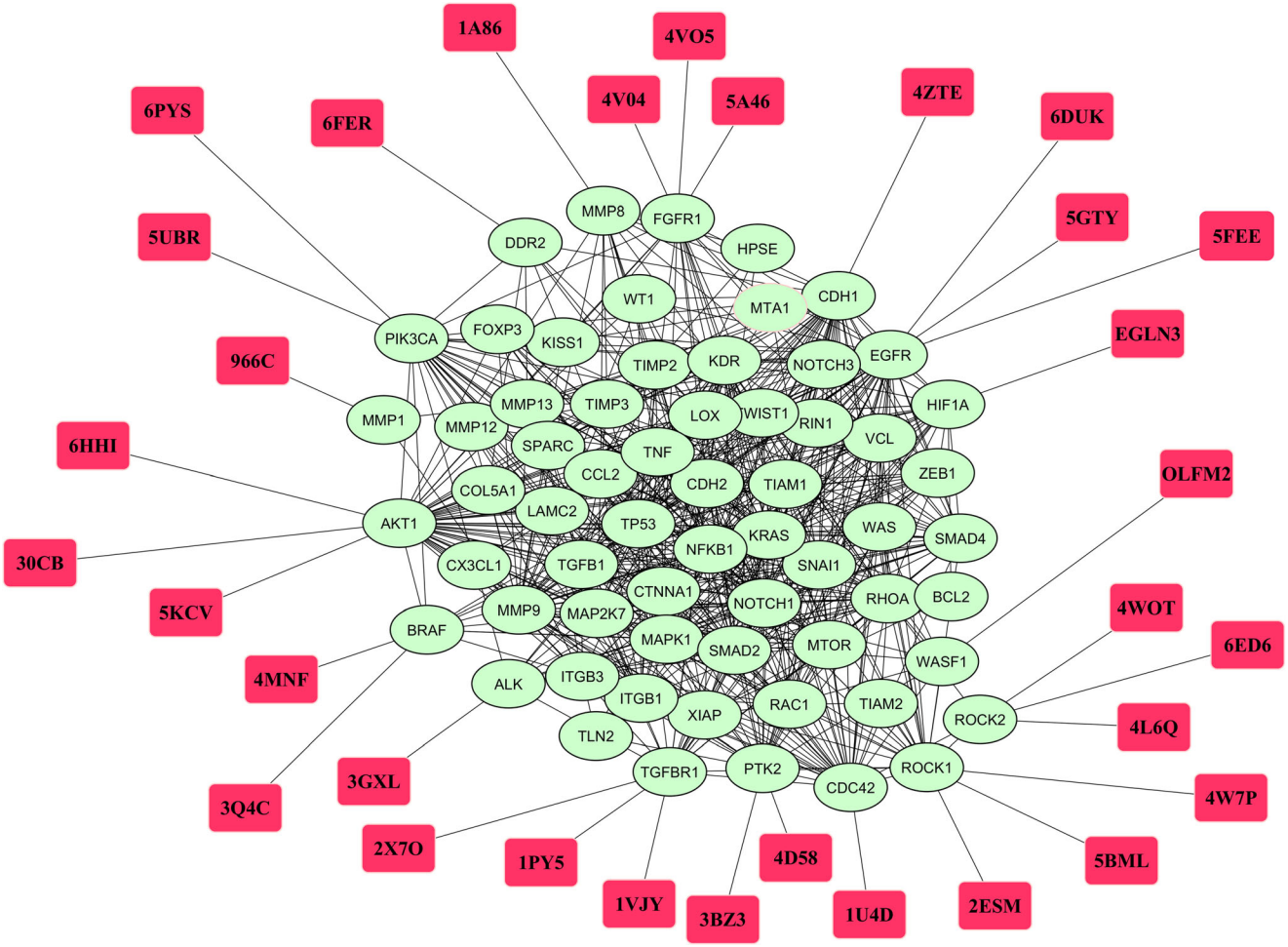


**Fig. 5** Protein-protein interaction network among metastasis-associated factors and inhibitors marked with their respective protein data bank IDs

it has become evident that miRNAs may play important roles in regulating cancer progression and metastasis. In renal cell carcinoma, miR-193b has been found to inhibit growth and metastasis by decreasing insulin-like growth factor 1 receptor (IGF1R) expression, providing new insight on targeting metastasis [119].

Multiple efforts on developing small molecules and antibodies as inhibitors of TGF-β have been flourishing recently and studied well across various cancer cell lines. All of it supports the very idea of reduced cell proliferation and invasion, indicating that TGF-β may serve as one of the potential targets of metastasis and supporting the reason that it is involved in mediating several downstream reactions of cell motility and invasion. LY2109761, a small molecule kinase inhibitor selective for TGF-β RI/II (TGF-β receptors I/II), has been found to suppress the metastasis of pancreatic cancer to the liver and other abdominal sites in a murine model [120]. A highly metastatic type of human breast cancer, MDA-MB-435, has shown reduced metastasis to lung and bone when treated with [3-(pyridine-2yl)-4-(4-quinonyl)]-1 H pyrazole, an ATP-competitive inhibitor of the TGF-β receptor I kinase [121]. SD-208, another TGF-β inhibitor, reduced primary tumour growth as well as metastasis in syngeneic R3T or 4T1 mammary carcinoma-bearing mice [122]. An anti-TGF-β antibody has also been shown to be effective and to prevent bone loss and bone metastasis of breast cancer in mice [123]. Novel ALK5 inhibitors, EW-7203 and EW-7195, have been found to inhibit TGF-β induced Smad signalling, EMT and breast tumour metastasis to the lung in another mouse model [124, 125]. TGF-β inhibition has shown impressive anti-metastatic effects across a multitude of cancer types. It underscores extending TGF-β as a valid target in clinical trials and in monitoring metastatic profiles. Several natural products such as Biemamides A-E, a group of marine natural products, and ZL170 have also been found to inhibit the TGF-β pathway, and several plant-based compounds have come a long way to treat cancer and manage metastasis by modulating EMT [126, 127]. A subtle metabolic regulation is observed in EMT and some metabolism inhibiting drugs have shown EMT inhibiting features. For example Simvastatin, targeting

HMG-CoA, is FDA approved for hypolipidemia and is currently under clinical trial for brain metastasis and small cell lung cancer [128].

Also, major emphasis has been put on designing potential anti-metastatic agents by inhibiting the action of chemokines. It has been discovered that the stromal cell-derived factor 1 (SDF-1)-CXCR4 axis plays a critical role in determining the metastatic destination of tumour cells. AMD3100, a small molecule inhibitor has been found to effectively block the SDF-1/CXCR4 axis and to significantly slow down lung to brain metastasis [129]. It also blocks EMT and liver and lung metastasis in prostate cancer [130]. SB225002, a pharmacological inhibitor of chemokine (C-X-C motif) receptor 2 (CXCR2) in combination with sorafenib when introduced in an in vivo model of ovarian cancer exhibited stabilization of tumour progression [131].

Based on the redundant nature of the various signalling pathways converging towards initiating the metastatic cascade, it has been suggested that target selection should be made on the most downstream effectors that cannot be bypassed to swamp the trouble of developing drug resistance [10]. In this context, cells migrate through the development of actin-rich extensions, invadopodia and lamellipodia, which is mainly enabled by cytoskeletal component remodelling, actomyosin contractility and actin polymerization. The major signalling molecule that activates these phenomena is the Rho-associated protein kinase (ROCK). Therefore, several inhibitors have been passed through various experimental models to check their impact on decreasing metastasis and cell invasion. Fasudil is one of the ROCK inhibitors that has shown marked reductions in breast cancer cell migration and invasion properties. It also reduces the contractility and invasion potential of cancer stem cells, those with metastatic potential [132, 133]. Another ROCK inhibitor (Y27632) also showed a reduced bone metastasis of breast cancer in a mouse model [134, 135]. Netarsudil, Ripasudil and Fostamitinib are among the already FDA approved ROCK inhibitors, but they are yet to be validated in the context of metastasis. Silencing of the gene encoding Cofilin induces invasion in carcinoma cells. Decreasing levels of PRPF4B, UD31 and BPTF cause down-regulation of genes involved in focal adhesion and ECM-interaction pathways. PRPF4B has been found to be an essential component for TNBC metastasis formation *in vivo*, turning it in a candidate for drug development [136]. FAK is activated in several types of cancer and involved in activating an intracellular adaptor protein called Paxillin for cytoskeletal rearrangement during metastasis. Several of its inhibitors have been designed as pyrimidine-based (NVP-TAE-226, PF-573,228, PF-562,271 and GSK2256098) or pyridine-based (VS-6063, VS-4718, and VS-5095) inhibitors and checked across several cancers in pre-clinical models [137, 138]. The crystal structure of FAK revealed that it can bind to methanesulfonamide diaminopyrimidine inhibitor, and bis-anilino pyrimidine inhibitor (PDB ID: 3BZ3 and 4D58), providing a potential blueprint to trace key inhibitory residues and their functional impacts in metastasis.

A vast number of inhibitors has been developed against MMPs by focusing on extracellular matrix (ECM) disintegration during the initiation phase of metastasis. MMP-2, -9 and -14 also enable extravasation by degrading blood capillary basal lamina [9]. An effective decrease in MMP activity and metastasis in the lung was observed when SD-7300, an oral inhibitor of MMP-2, -9 and -13, was administered in an aggressive mammary carcinoma 4T1 mouse model [139]. Liposome-based delivery of a MMP inhibitor, marimastat, to the tumour microenvironment of breast cancer showed a reduction in metastatic lung nodules and in angiogenesis [140]. Another MMP inhibitor, SB-3CT, has been found to selectively inhibit prostate cancer to bone metastasis [141]. WRG-28, an extracellular small molecule inhibitor of DDR, inhibits the receptor-ligand interaction in an allosteric manner. It does so by disrupting DDR receptor clustering. A potential reduction in breast cancer cell invasion and migration to the lung was observed. However, it did not inhibit the growth of the primary tumour and, hence, adding it as adjuvant therapy in clinical practice may serve as a potential anti-metastatic regimen [142]. An anti-fibrosis drug called silibinin induced decreased LOX mRNA levels and inhibition of NSCLC cell migration [143]. LOX2, another member of the LOX family, is known for its role in solid tumour progression. PXS-S1A has been found to show dual inhibition of LOX2 and LOX, whereas PXS-S2A acts as a highly selective LOX2 inhibitor. These compounds show reduced cell migration and invasion when administered to the MDA-MB-231 triple-negative human breast cancer model. They also show decreased angiogenesis and CAF activation [144]. Administration of the LOX inhibitor beta-aminopropionitrile (BAPN) to MDA-MB-231-Luc2 injected mice showed that the activity of LOX is required during the phase of metastasis initiation, as treatment started the day before or at the same day of tumour cell injection elicited a more prominent decrease in metastasis foci [145]. Integrins are attractive targets due to their role in mediation of cell migration and angiogenesis. Cilengitide, a small molecule inhibitor of integrins $\alpha v\beta 3$, $\alpha v\beta 5$ and $\alpha 5\beta 1$ has been under the clinical investigation for sarcoma, glioma, melanoma and lung cancer [146–149] and has been found to reduce breast cancer metastasis in *in vivo* mouse models.

Next to the advent of small molecule inhibitors, the therapeutic strategy for metastasis has recently turned into the direction of generating antibodies directed against metastasis-associated proteins. Several antibodies have already been tested in preclinical models and have shown potential anti-metastatic properties (Table 2). It has also turned to targeting small RNA molecules as they have emerged as core components acting as oncogenes, tumour suppressors and metastatic switchers. In a breast cancer model in mice, miR-96/miR-182

**Table 2** Antibodies in pre-clinical stages with anti-metastatic effects

| Antibody | Target | Anti-metastatic effect |
|---|---|---|
| 38 M and 64R mAb | LYVE-1 | Suppresses the migration of HEK293F cells *in vitro* by inhibiting lymph-angiogenesis [154] |
| Anti-AXL antibody 20G7-D9 | AXL | Inhibits EMT, tumour growth and metastasis in TNBC cell xenograft and Patient-derived xenograft (PDX) [155] |
| Tocilizumab (anti-IL-6 receptor antibody) | IL-6 and CCL5 signalling | Reduces tumour growth and thoracic metastasis of TNBC [156] |
| Anti-MACRO mAb | Pattern recognition scavenger receptor (MACRO) a subtype of TAM | Inhibits metastasis in breast cancer, colon cancer and melanoma models [157] |
| 211−12 and 211−14 | A disintegrin and metalloproteinase 28 (ADAM28) | Anti-metastatic effect seen in PC-9 cells [158] |
| chLpMab-7 | Podoplanin (hPDPN) | Suppress metastasis of hPDPN expressing tumours via ADCC and CDC in LN319 (glioblastoma cell line) and PC-10 (lung cancer cell line) [159] |
| 6B12 antibody | S100A4 Protein | Reduces attraction of T cells to the pre-metastatic niche in CSML100 cell line [160]. |
| Anti-TGF-β antibody | TGF-β | Reduces breast metastasis to bone [161] |

has been found to downregulate the level of paladin, thereby reducing migration and invasion properties. This microRNA-based therapy was successfully established by supplementing a hydrogel embedded gold-nanoparticle-based delivery system that provided specific and sustained microRNA release into the breast cancer mouse model, abated the metastatic properties and caused tumour shrinkage. MicroRNA-based therapies have been combined with chemotherapeutic drugs such as cisplatin, and proven to have anti-metastatic potential [150]. Expression knockdown of miR-21 suppressed the growth, invasion and metastatic properties of breast cancer cells [151], whereas a miR-655-3p mimic, when administered with oxaliplatin in a colorectal cancer model, reduced the occurrence of liver metastases [152]. Administration of an anti-miR-214, R97/R98, to melanoma and breast cancer cells elicited reduced levels of trans-endothelial migration [153].

## 5 Precision medicine as solution for metastasis treatment?

The design of targeted approaches has many shortcomings for diseases like metastasis. How to understand the dynamic and simultaneously occurring processes of cancer cells leading to metastasis? How to recruit a large pool of diverse biological data and identify common metastasis-gene associations for developing therapies? How can it reveal the state, stage and specificity of metastasis and/or facilitate its diagnosis? Precision medicine is based on unfolding the raw components of metastatic conditions in tumours. It basically assesses the variability in genes, transcripts, metabolites, non-coding counterparts, disease biomarkers, epigenomic marks, environmental factors and lifestyle. It requires a holistic way of multilevel data integration to understand each component and its influence or autonomy on the other. These criteria, if holistically translated into clinical components, may provide a scope for a better evaluation of patient cohorts with respect to disease susceptibility, treatment response and prognosis.

The stages before actual metastasis can be classified into a pre-metastatic niche phase, a microvascular phase, a pre-angiogenic phase, an angiogenic phase and a growth phase. Each of these phases show a unique genotypic profile. Therapeutic exploration of pan-cancer studies of each of these phases and of CTCs may offer serious clues for a precision medicine approach for metastasis treatment. Some preliminary studies have already found such clues. It has been found, for example, that the dopamine agonist apomorphine can restrict brain metastasis formation from lung cancer by targeting the pre-metastasis-associated genes KIF16B, SEPW1 and TESK2 [162, 163]. However, insights at the genome-wide level may offer more large-scale hints. In this light, we discuss breast cancer and the progress that

has been made in understanding metastasis. Breast tumours are extremely heterogeneous. The intra-tumoral heterogeneity is reflected by the variability of genetic mutations and mutations induced by chemotherapies. On the other side, various types of cells and molecular subtypes occurring in the tumour microenvironment and during tumour progression and metastasis reflect the inter-tumoral heterogeneity [164]. Due to this heterogeneity different patients respond differently to the same treatment. Implementation of molecular profiling and genomic subtyping in triple negative breast cancer (TNBC) clinical trials was used to evaluate the efficacy of targets under an umbrella trial and can be considered a potential future trial design for precision medicine [165]. Breast cancer has been classified into several types, including ER positive, triple negative and HER2 positive. The commonly prevailing treatment modalities include chemotherapy, hormone therapy, immunotherapy and nanoparticle therapies. Genomic data acquisition has evolved from single gene mutation studies to genome wide association studies (GWAS) and next generation sequencing (NGS) studies. These high throughput technologies and emerging big data analysis tools are pushing us towards the clinical setting of tailored precision/personalized medicine and slowly leaving the paradigm of "one model fits all".

GWAS started accumulating big data through collaborative projects across different countries and ethnic groups. The Breast Cancer Association Consortium (BCAC) and the Asia Breast Cancer Consortium were built to assess associations of common genetic variations with breast cancer. GWAS still poses a challenge in uncovering rare variants and to this problem NGS has enabled sequencing millions of DNA fragments. Many of the studies consistently found BRCA1 and BRCA2 mutations to confer a moderate to high risk of breast cancer, whereas rare mutations are found in TP53, ATM, RAD51C, MSH2, MSH6, PMS2, MRE11A, RAD50, NBS1, CDH1, BARD1, NBN, BRIP1, MUTYH, MLH1 and CDKN2A. Several other frequently mutated genes in breast cancer are PIK3CA, TP53, GATA3, PTEN, AKT1, CDH1, ARID1B, CASP8, BRCA1, RB1, MLL3, MAP3K1, MAP3K13, NCOR1, SMARCD1, CDKN1B, TBX3, RUNX1, CBFB, AFF2, PIK3R1, PTPN22, PTPRD, NF1, SF3B1 and CCND3. Copy number alterations in the PIK3CA, ERBB2, TP53, MAP2K4, MLL3, CDKN2A, PTEN and RB1 genes have also been observed [166]. Parallel and comparative analyses across tumour genetic, phenotypic, metabolic, epigenetic and transcriptomic profiles may offer insights into the genetic signatures determining site specificity of metastasis to certain organs, stages of metastasis, mutational and methylation profiles, non-coding RNA mutations and disease associations, novel signalling pathways and networks, drug-disease-gene networks, novel biomarkers, targets and, ultimately, treatment decisions.

### 5.1 Insights from pan-cancer metastasis studies

Recent pan-cancer studies have enabled a deeper, comprehensive and integrative analysis of primary tumour gene and metastatic tumour gene architectures across several cancer types. It facilitates the identification of cancer drivers and mutation hotspots and infers the importance of all types of variants including large-scale genomic rearrangements (via fusions and copy number alterations) which are commonly found in many key oncogenes. In case of lung adenocarcinoma, a comparative whole exome sequencing of both primary and metastatic tumour tissues of stage IV from four patients with chest wall metastasis was performed. It turned out that with the enormous heterogeneity within individual samples, metastatic events occurred simultaneously with those of primary tumour progression. After a global comparison across primary and metastatic samples, TAS2R31 and UMODL1 were identified as genes that may drive metastatic lung adenocarcinoma [167]. Pan-cancer analysis of the numerous complex processes in the tumour microenvironment regulated by matrisome genes revealed mutational landscapes of several genes across several clinical samples of different cancer types. This throws light onto novel cancer therapeutic aspect for targeting the cancer ECM matrisome. It turns out that copy number alteration and mutations of matrisome genes are more frequent than those in the rest of the genome and that the mutated genes have a greater functional impact on ECM proteins such as collagens and proteoglycans. The mutational burdens of matrisome genes like COL6A1 and MUC5B are associated with overall survival. Among the most mutated matrisome genes across 14 cancer types are MUC16, FLG and HMCN1 [168].

The benefit of pan-cancer studies is that they provide an overall detailed insight into the commonalities and differences, which in case of understanding metastasis from a therapeutic perspective, is of utmost importance. Two recent studies on whole genome pan-cancer analysis of solid tumours and pan-cancer analysis across the cancer genome atlas (TCGA) has shown the mutational loads on each tumour type due to single nucleotide variants, multiple nucleotide variants, indels, structural variants, copy number alterations and mutational hotspots. This kind of information may be crucial in assessing the stages of propagation of metastasis. For example, a significantly mutated gene from metastatic breast cancer, ZFPM1, a zinc finger transcription factor without previous links to cancer, was identified this way. Similarly, FHIT and DMD were found to be deleted across 4 to 5 % of the samples and reported to cause localized genomic instability. In the latter study, different molecular signatures for metastasis across different cancer types were observed suggesting distinct molecular pathways leading to metastasis in different cancer types. In contrast, other studies revealed common metastasis causing genes across different cancer types. Also, significantly

different expression patterns of DNA methylation genes, miRNAs and protein coding genes were observed in primary versus metastatic samples. For example, AGER, LRP2 and PIGR were found to be highly expressed in metastatic samples of esophageal carcinoma (ESCA), sarcoma (SARC) and skin cutaneous melanoma (SKCM) compared to their respective primary tumour samples. This information derived from large datasets highlights molecular patterns of metastatic progression guided by common genes versus distinct genes and can be correlated with the pathological stages of metastasis and quantified to delineate the time of progression from the primary tumour state [169, 170].

The pan-cancer studies have also been expanded towards investigation of protein expression data across cancer types acquired from the The Cancer Proteome Atlas (TCPA) and led to the identification of differentially expressed proteins in different cancer types. These studies have clinical potential through the identification of patients for treatment monitoring, cancer stage determination and personalised therapy prescription. Pan-cancer studies of whole proteomes of primary and metastatic samples across different cancer types hold promise for identifying potential biomarkers. As yet, however, the prime limitation is the availability of enough protein expression data [171].

A newly evolving field in precision medicine is radio-genomics. Through this approach, voluminous data are extracted from digital images and, together with the amalgamation of genomic, transcriptomic, epigenomic and metabolomic data integration, it allows to quantitatively stratify a patient's disease condition. It has the potential to generate image biomarkers and to correlate them with the underlying genotypic and phenotypic state of the tumour and its metastases [172]. With respect to treatment, it may help to stratify patient groups to be radiosensitive or radio-resistant based on correlations of gene signatures and radiosensitivity indexes. A pan-cancer genomic signature revealed 10 gene signatures associated with intrinsic radiosensitivity (AR, cJun, STAT1, PKC, RelA, cABL, SUMO1, CDK1, HDAC1 and IRF1). These 10 genes are associated with cell cycle progression, DNA damage response, histone deacetylation, proliferation and

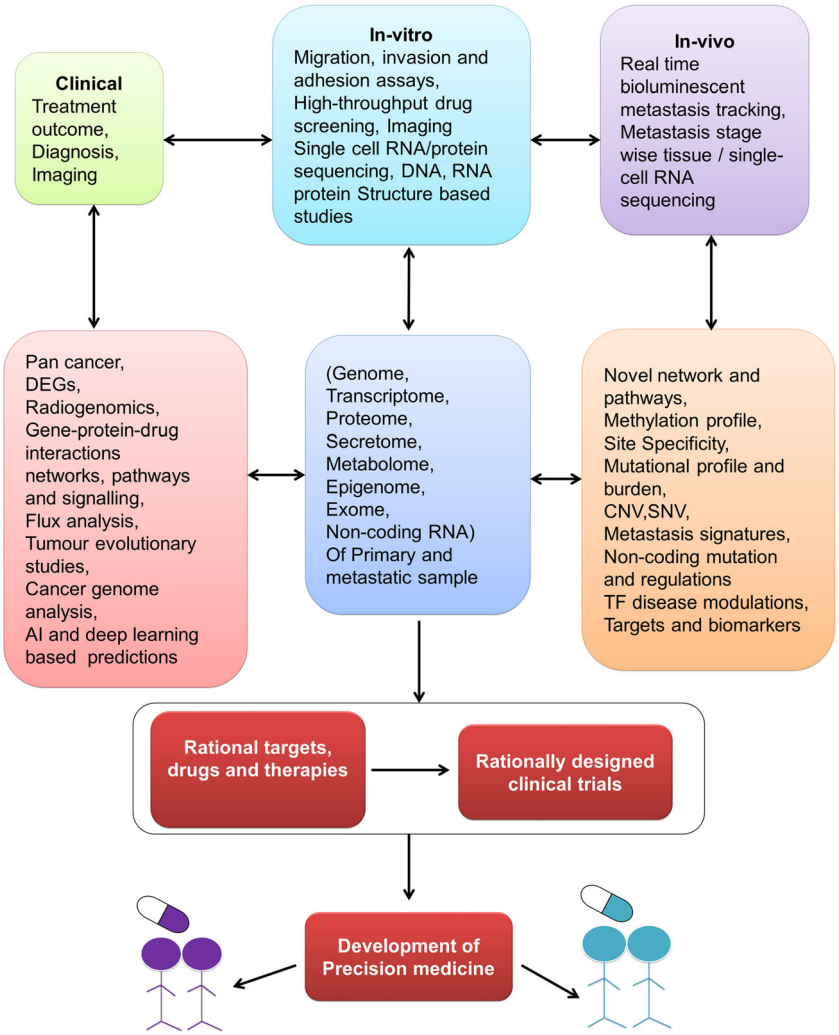


**Fig. 6** Multilevel data integration for comprehensively understanding metastasis and developing precision medicine

apoptosis. These results were used to create a radiosensitivity index (RSI), whereby a lower RSI correlates with a higher radiosensitivity [173, 174].

## 6 Conclusions and perspectives

Despite fundamental and translational research in the field of metastasis, it has been difficult to combat. Fundamental scientific evidence suggests that metastatic cells use native migration mechanisms such as chemokine-mediated signalling, growth factor-mediated signalling and well-studied mechanisms of organogenesis such as EMT, to attain the properties of migration, invasion, survival and growth. There are also molecular mechanisms underlying site-specific distant metastasis as, for example, RANKL-mediated bone metastasis. It is worthy to note that several levels of biochemical changes, molecular changes, metabolic changes and physiological factors may drive this process, signifying its demand to be understood in a holistic manner. The dynamic nature of the metastatic cascade is the reason why developing precision medicine for this phenomenon remains a predicament. Metastases originate from a primary tumour but may pass through several years of latency (dormant state), micro-metastasis or macro-metastasis before actual colonization and secondary tumour formation occurs. This phenomenon is a major drawback in replicating metastasis in *in vitro* or *in vivo* models. Additionally, animal models generally have a shorter lifespan compared to humans. Some patients may, however, show metastasis at early stages of tumour detection.

Various genes, proteins and signalling pathways related to different aspects of metastasis have been widely studied. Some of them have been discussed in this review. This information facilitates the selection of metastasis-specific targets, but in a reductionist way, which only helps to halt or reduce the extent of metastasis. However, a more holistic approach entailing integration of several layers of metastatic data versus primary tumour data across different cancer types may aid, not only in identifying novel targets or biomarkers, but also in understanding the complete tumour and metastatic landscapes across different cancer types including the commonalities and differences between them. Such an integrated approaches may also help in identifying molecular markers determining site specificity and metastatic stage, as well as mutational landscapes and hotspots which can be implemented in the clinical stratification of patients according to genotypic, transcriptomic and epigenetic profiles and classifying them for precision treatment. It can also be used for treatment monitoring.

Profiling of migration, adhesion and invasion in enhanced experimental models *in vivo* and *in vitro* that represent real metastatic scenarios like xenograft models, genetically engineered models, 3D organoids or microfluidic-on-chip models can aid in understanding the basic biochemistry of metastatic transformation and its characteristics. Some important revelations of these techniques have been discussed above in relation to molecular pathways and targets during the metastasis cascade. Integration of such information with 3D imaging and real time metastasis tracing using bioluminescence may provide a basic metastatic framework. However, there are limitations to these models as they still fail to represent the actual metastatic scenario in humans. Single cell RNA-sequencing and protein expression analyses of primary and metastatic lesions across different cancer types can shed light on tumour heterogeneity and interactome profiles, including their role in various molecular and functional aspects. Incorporation of mega-metastasis integrative analyses of genome, proteome, transcriptome, exome, secretome, epigenome, metabolome, non-coding RNA and drug-ligand interaction data using deep learning across several primary and metastatic tumour types by employing pan-cancer studies, GWAS, differentially expressed gene (DEG) studies, expression quantitative trait locus (eQTL) studies, computer-assisted drug development (CADD) studies and their integration with imaging (radio-genomics), protein and drug structural data (structural bioinformatics), gene, protein and non-coding RNA interaction networks as well as drug-related pathways may aid in the identification of global metastatic gene expression signatures and mutational profiles which will help in finding metastatic biomarkers, targets and therapies (Fig. 6). In-depth profiling of RNA-sequencing and pan-cancer data from every step of the metastatic cascade including the tumour secretome, microbiome as well as the microenvironment of primary and secondary tumours, CTCs and studying ctDNA characteristics may be helpful. Essentially, polytherapy and adjuvant therapies can be employed, but only until scientific enrichment helps to explore the several layers involved in metastasis and its main causes. As yet, it is a highly complex phenomenon that demands drug cocktails. Precision medicine can help us to pick the unambiguous combinations for patient-tailored approaches.

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## Declarations

**Ethics approval** Not applicable.

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