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Epithelial-to-mesenchymal transition (EMT) and cancer metastasis: the status quo of methods and experimental models 2025

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Abstract

Epithelial-to-mesenchymal transition (EMT) is a crucial cellular process for embryogenesis, wound healing, and cancer progression. It involves a shift in cell interactions, leading to the detachment of epithelial cells and activation of gene programs promoting a mesenchymal state. EMT plays a significant role in cancer metastasis triggering tumor initiation and stemness, and activates metastatic cascades resulting in resistance to therapy. Moreover, reversal of EMT contributes to the formation of metastatic lesions. Metastasis still needs to be better understood functionally in its major but complex steps of migration, invasion, intravasation, dissemination, which contributes to the establishment of minimal residual disease (MRD), extravasation, and successful seeding and growth of metastatic lesions at microenvironmentally heterogeneous sites. Therefore, the current review article intends to present, and discuss comprehensively, the *status quo* of experimental models able to investigate EMT and metastasis in vitro and in vivo, for researchers planning to enter the field. We emphasize various methods to understand EMT function and the major steps of metastasis, including diverse migration, invasion and matrix degradation assays, microfluidics, 3D co-culture models, spheroids, organoids, or latest spatial and imaging methods to analyze complex compartments. In vivo models such as the chorionallantoic membrane (CAM) assay, cell line-derived and patient-derived xenografts, syngeneic, genetically modified, and humanized mice, are presented as a promising arsenal of tools to analyze intravasation, site specific metastasis, and treatment response. Furthermore, we give a brief overview on methods detecting dissemination and MRD in carcinomas, highlighting its significance in tracking the course of disease and response to treatment. Enhanced lineage tracking tools, dynamic in vivo imaging, and therapeutically useful in vivo models as powerful preclinical tools may still better reveal functional interdependencies between metastasis and EMT. Future directions are discussed in light of emerging views on the biology, diagnosis, and treatment of EMT and metastasis.

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Background

Epithelial-to-mesenchymal transition (EMT) is a multistep dynamic cellular event in which cells lose their epithelial phenotype and gain mesenchymal traits. A mesenchymal state is characterized by the lack of epithelial markers like E-cadherin, occludin, and cytokeratin, and elevated mesenchymal markers like N-cadherin and vimentin [1]. Essential molecular pathways regulating EMT, like TGF-β and Wnt/β-catenin, induced upon activation of transcription factors (TFs) such as Snail, Slug, Twist, Six1, are widely conserved across species [2–4]. EMT is often regarded as a binary process that transitions between an epithelial polarized phase, and a mesenchymal phase with reduced cell polarity and enhanced migratory capacity [5]. It has been linked to an array of cancer characteristics, which involve tumor initiation, intravasation into vessels, tumor cell migration, cancer cell stemness, metastasis, and therapeutic resistance [1, 6, 7]. Metastasis, a leading cause of cancer morbidity and mortality, is the process in which cancer cells disseminate from the primary tumor site to distant organs [8]. The initiation of metastasis involves cancer cells departing from their primary site, followed by extracellular matrix (ECM) degradation, invading and circulating through the bloodstream, adapting to the environment of a secondary metastatic niche, and evading immune cell destruction [9]. The loss of intercellular adhesion within the tumor leads to the detachment of some tumor cells from the main tumor mass. These detached cells then invade the surrounding ECM, which is termed as invasion, which involves enhanced motility and migration of tumor cells, as well as the degradation of the ECM. Following the invasion step, some tumor cells penetrate blood vessels, degrading their basement membrane, and enter into the bloodstream. This process is known as intravasation. The tumor cells then disseminate systemically, can encounter immune resistance, mechanical stresses of the blood flow, and prime themselves in interaction with systemic molecular factors and specific niches during passaging. Some tumor cells will eventually survive and adapt a process to leave the blood circulation, known as extravasation. Finally, the tumor cells must survive in their new environment and eventually form a secondary tumor at a distant location, as depicted in Fig. 1 [10]. Some specific molecular factors have been described to be highly important, to achieve a number of aspects of these key steps biochemically. For example, urokinase plasminogen activator (uPA), a serine protease, is often elevated in various malignancies including breast, ovarian, lung, colorectal, thyroid, gastric, prostate, and other cancers, as further discussed below. Interaction of uPA and its receptor uPAR leads to the conversion of plasminogen into plasmin, and activates, e.g., matrix-metalloproteases, and concerted actions of these players lead to degrading components of the ECM and further phenomena associated with EMT and metastasis, as further mentioned during our review [11–30]. Circulating tumor cells (CTCs) or disseminated tumor cells (DTCs) can be detected at extremely early stages of cancer disease already, as soon as the primary tumor has access to lymph or blood vessels after intravasation. Thus, their dissemination reflects the transition of a carcinoma into systemic disease [16, 31–55]. It has been shown that some of these cells can give rise to niches of minimal residual disease (MRD) which is able to reside in dormant states after therapy, increasing the risk of later relapse, metastasis, and therapy resistance, which is often associated with metastasis [55–57]. As a result, early detection of MRD has become crucial for monitoring cancer progression and providing preventive treatment. However, due to its multifaceted nature, it is still extremely challenging to treat advanced cancer, especially the metastatic stage. Therefore, research on metastasis and all the aspects associated with its molecular understanding, diagnosis, treatment, and prevention, is still one of the most urgent needs of our time.

An improved understanding of cancer progression and treatment requires precise and physiologically relevant experimental models able to reflect critical aspects of EMT and the steps of the metastatic cascade [58]. Although in vitro models can control the majority of experimental variables and promote quantitative analysis, they reflect a limited portion of the tumor microenvironment, and the current approaches are limited to the investigation of specific steps in the metastatic cascade. Furthermore, it is still challenging to replicate several aspects of metastasis in vitro. In this context, in vivo and animal models are an inevitable extension, offering a more comprehensive approach to investigate metastasis and allow for additional validation of outcomes acquired through in vitro platforms.

Nevertheless, there is an increasing arsenal and potential of methodology able to advance success of current EMT and metastasis research. Moreover, the current developments of personalized oncology urge for an encouragement of young scientists and clinicians to enter the field of basic, translational and clinical research which can contribute to overcoming cancer metastasis. Therefore, in this review, we comprehensively compile and highlight the actual status of experimental models employed to study EMT and different steps of the metastatic cascade in vitro and in vivo, including a brief overview on the status of disseminated tumor cell and MRD detection in solid cancers. The relevance, advantages, and limitations of the respective methods are discussed. For educational transparency, we describe the models and

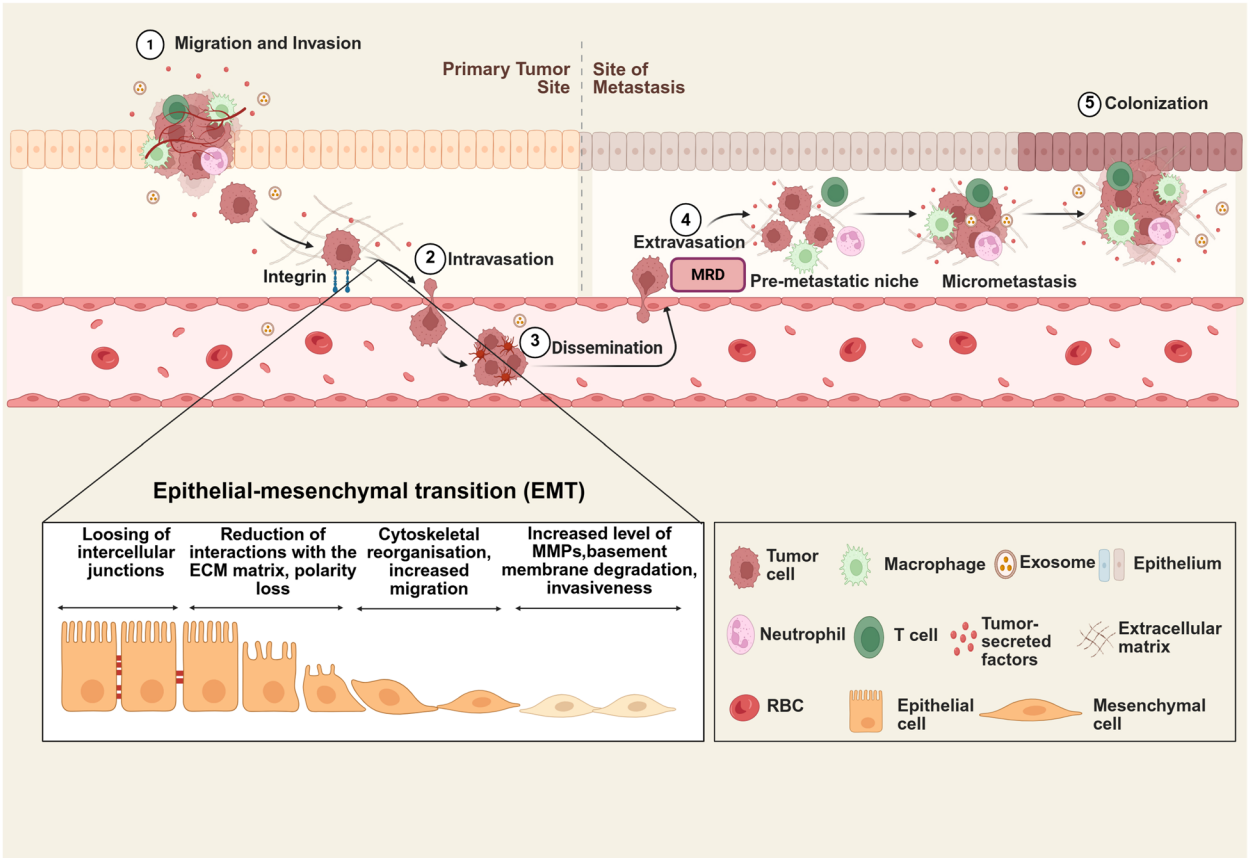

Fig. 1 A schematic illustration depicting epithelial-to-mesenchymal transition (EMT) and the metastatic cascade. The figure shows tumor cells undergoing EMT, characterized by the reduction of epithelial markers and the acquisition of mesenchymal properties, promoting migration and invasion. Cancer cells then enter the vasculature, disseminate as disseminating tumor cells (DTCs), and extravasate to distant sites

technologies available according to the steps of the metastatic cascade in the following chapters.

Classical migration-, invasion-, and ECM degradation assays

The ability of increased migration emerges as a major phenotypic characteristic in EMT, however, especially the ability to invade into surrounding tissue, and thereby actively degrading components of the extracellular matrix (ECM), are considered to be decisive for the first steps of cancer cells within the metastatic cascade. Tumor-associated proteases, their secretion, overexpression, and/or concentration at invasion fronts by cancer cells have been shown by many pioneers at the end of the 1980 s and beginning of the 1990 s to be the essential players in this context [10–24, 31, 32, 59–68]. Especially, the urokinase-receptor (u-PAR) and the urokinase (u-PA)-system have been described decades ago as one of the most important tumor-associated proteolytic systems overexpressed in almost all human "major killer" carcinoma types, being a deleterious and mostly independent prognostic factor when overexpressed in numerous major human cancer entities such as breast, gastrointestinal, ovarian, prostate carcinomas, and others [11–18, 25–30]. Activating plasminogen to plasmin, receptor-bound uPA activates further cascades of, e.g. matrix-metalloproteinases (MMPs), of which MMP-2 and MMP-9 are most relevant to degrade collagen IV and basement membranes of vessels for intravasation. Further MMPs have been shown to degrade other components of the extracellular matrix such as laminin, different types of collagen, or proteoglycans [19–24]. Overexpression of the u-PA system has been shown also to impact chemotherapy response in particular subtypes of breast cancer, and has continuously been suggested as a biomarker for aggressive cancer phenotypes [27, 69–71]. Specifically, u-PAR can interact with particular integrins and fibronectin, thus being able to provide a switch between tumor cell proliferation and dormancy [72]. Therefore, it becomes one of the most likely molecular candidates important for maintaining minimal residual disease in carcinomas and causing re-activation of dormant cells, years after curative primary

tumor therapy, towards disease recurrence. Towards this end, u-PAR was described almost 30 years ago by Allgayer et al. as a molecule to characterize metastatically relevant phenotypes of single disseminated gastrointestinal tumor cells, predicting later metastasis and recurrence in the clinic [16, 31, 73]. They later demonstrated the major tumor suppressor Pdcd4 in colorectal cancer (CRC) as a molecule downregulating u-PAR, and re-activation of an early metastatic capacity by miR-21 which is targeting Pdcd4 [74, 75]. Work by other groups showed that osteopontin regulates cell motility and NFκB-mediated u-PA secretion through PI3-Kinase/Akt signaling pathways in breast cancer cells [76], proposed key u-PAR regulating networks for MRD- and niche preparation, and hypothesized u-PAR, besides factors like CXCR4, as a potential characteristic of metastatically relevant cancer stem cells [77–79]. It is promising that molecules like u-PAR are gaining increasing actual attention again, possibly also as promising diagnostic markers or biomarkers [80]. However, it is a highly concerning general trend that increasing numbers of actual articles, also on this topic, tend to exclusively cite very recent papers or reviews only, but seem to ignore the many pioneers in this field as cited here, who did the major discoveries.

Some of the experimental methods to investigate migration and invasion have entered routine use in many laboratories; some more specific ones might have been forgotten and deserve a regain of attention. Moreover, some highly sophisticated ones have increasingly pushed, and visualized, our detailed understanding of processes such as migration and invasion in different settings, and of phenomena such as invasion fronts [81–83]. Functional assays like wound healing assays (or scratch assays), transwell migration, and (Matrigel) invasion assays provide valuable insights into molecular changes associated with invasive characteristics [84, 85]. In these assays, molecules like u-PA/u-PAR or MMPs (see above), and EMT induction have been shown to significantly increase migration and invasion [86].

Wound healing assays (scratch assays)

The migration of cells is one of the most initial steps in EMT and the metastatic cascade, involving cytoskeletal rearrangements within migrating cells. The latter present themselves in different forms, such as amoeboid or mesenchymal migration, driven largely by integrins and proteases [87]. Collective means of migration of cells and associated changes were described by Friedl, Rørth, or te Boekhorst et al. [88–91]. Despite a continuously increasing complexity of knowledge on migration, one of the simplest and oldest in vitro techniques to investigate migration is the wound healing scratch assay [92–95], which involves several key steps. Briefly, the cells are cultured until they form a complete monolayer. A straight"scratch"is made through the cell layer using a standardized tool. Then, the cells are washed and fresh medium is added, sometimes containing test compounds (e.g., therapeutics). Standard microscope images are taken at fixed time points, and the percentage of wound closure is measured by the formula, % Wound closure $=[(\text{Area at } t=0)-(\text{Area at } t=x)]/(\text{Area at } t=0)\times 100$. It is possible to test the impact of molecular modulators and therapeutic compounds on migration of adherent cell lines in this easy-to-perform assay at low costs. Several different protocols and modifications exist, depending on the type of cell lines studied, the research question and budget [84, 96–102], the scratching material and technique ranging from manual scratching to automated tools like lasers or electricity [97, 103–106]. Likewise, analysis of the migration process varies widely, from image-taking with light-field microscopes at defined points of time, image software, measurements of impedance on the migration plates, and automated optical cameras in real-time, up to highly sophisticated real-time live imaging platforms as pioneered by Friedl and others [82, 87, 107–110].

Each version of the protocol has advantages and disadvantages and it would extend beyond the scope of this review to discuss all of them, especially since previous authors have done so [96, 110–112]. However, a major issue of most of the protocols is still a potential false-positive bias in sensitivity towards the proliferation of cells. Different strategies to overcome this bias have been introduced, reaching from thorough rinsing after the scratch, shorter experimental time, or serum starvation to pre-treatment with mitomycin C, the latter having been suggested to be the most secure way to exclude any proliferation bias. Still, of course, the choice of protocol applied needs to depend on the individual research question asked, since an interference of mitomycin C with therapeutic compounds tested needs to be excluded [84, 110, 113, 114].

Transwell migration assays

A more advanced method to study migration, especially when the experiment should involve particular chemoattractants to simulate a particular condition, is the Boyden chamber assay (or transwell migration assay). This method also has become routine as easy-to-perform and cost-efficient. It was initially developed by Boyden et al. to investigate leukocyte chemotaxis [115], then modified to study the migration of other cell types, especially cancer cells [76, 116–122]. Briefly, the Boyden chamber consists of two compartments, one as an insert into the other, filled with cell culture medium of appropriate composition depending on the cell type

studied. The compartments are separated through a microporous filter, through which the cells of interest can migrate. The lower chamber most often contains an additional chemoattractant to stimulate directed migration of the cells through the porous membrane from the upper to the lower chamber [95, 123, 124]. (Cancer) cells of interest, which again can be modified or treated in different ways, are placed into the upper compartment, and migration through the membrane into the lower compartment within a defined period can be measured. This can be achieved by different methods, but generally, the membrane between the two compartments is isolated to allow an evaluation of the number of cells that have migrated to the lower part of the membrane and the lower chamber.

For evaluation, the migrated cells are fixed and stained at the bottom of the membrane (e.g., by crystal violet). They can either be counted manually and visually, using a grid pattern in the microscope, or by overlaying a grid on photos taken from the membrane. Alternatively, the number of migrated cells can be counted by metabolic assays, such as ATP-based measurement using luminescence [25, 74, 80, 85, 109, 125–128]. The most obvious advantages of crystal violet staining are the technical simplicity (simple staining procedure, minimal equipment needed, relatively inexpensive reagents), visualization benefits (permanent staining, samples can be stored, photos for documentation/later analysis), the versatility of the assay (works with most cell types, compatible with different membrane types, can be quantified in multiple ways), reliability (reproducible results, widely accepted). However, there are also technical limitations (time-consuming manual counting, potential counting bias, not suitable for large sample numbers), methodological issues (live from dead cells cannot be distinguished, staining of non-specifically attached cells, risk of cell loss during washing steps) or quantification challenges (such as the comparability between different methods or investigators who count cells).

For ATP-based cell counting, advantages are a much higher sensitivity (detection of fewer than 10 cells), linear measurements over a wide range of cell numbers, measurement of viable cells only, fast performance (quick procedure, no need for cell washing or separation, automated applications available for high-throughput screening), higher validity (no operator bias, quantitative results, highly reproducible), versatility (works well with adherent and suspension cells). Technical limitations of the ATP method are that different cell types in mixed populations are not distinguishable, and that ATP content varies between cell types, their metabolic or cell cycle states, and culture conditions. Moreover, this method requires special reagents that are expensive compared to traditional counting. Finally, this method allows single time point measurement only, due to the necessary cell lysis which might induce ATP degradation bias.

Invasion assays

Invasion is largely a biochemical process whereby tumor cells gain and exert an ability to degrade diverse components of the surrounding extracellular stroma such as collagens, proteoglycans, or laminins. When it comes to intravasation, specific components of the basement membrane of vessels need to be invaded, especially type IV collagens and laminins. Based on the Boyden chamber concept, a major in vitro assay has been developed which is largely known as the Boyden chamber invasion assay, or -Matrigel invasion assay when specifically using Matrigel [81, 85, 96, 116, 117, 123, 125, 129–132]. It can include diverse chemoattractants in the lower chamber suitable for the particular research question. The porous membrane separating the upper and lower chamber is coated with particular extracellular matrix components as barriers, through which the cancer cells of interest need to invade actively towards the lower chamber. Several different components of the ECM have been used, however, the Matrigel invasion assay has been established as the most widely used setting [116]. Matrigel represents a reconstituted basement membrane and its primary biochemical components are laminin, collagen type IV, entactin, and the heparin sulfate proteoglycan perlecan [133]. Briefly, cells of interest are applied onto the Matrigel (or matrix of interest)-coated filter, and incubated for varying times. Depending on the type of tumor cells and the experimental question, the filter is harvested, fixed, and washed, and the cells having arrived successfully at the lower part of the filter can be quantified with different approaches following the same strategy as described in the section for migration assays. Therefore, clear advantages of this assay include a variety of modifications, either of the cells to be investigated (transfections with molecules to be tested, gene editing, knockouts, etc.), the surrounding conditions (buffer composition with different growth factors or chemoattractants), or (therapeutic) compounds added to the system. Since the assay can be done in 48- or 96-well plates, large numbers of different settings can be studied with relatively low numbers of cells and small amounts of components. Thus, the assay is cost-effective, quick, quantifiable, and effectively standardized. Also, in particular settings, it is possible to recover the invaded cells for further investigation [116]. Due to the high technical similarity with the Boyden chamber migration assay as described above, many potential disadvantages are comparable concerning the counting strategies. Moreover, additional caution should be exerted towards proliferation bias: An appropriate time window for the invasion

assays should be selected when tumor cell proliferation is not, or not yet, affected since the results could be biased by different proliferation rates when comparing wildtype and modified cells.

Laminin and ECM-degradation assays

The aforementioned assays give a generally accurate impression of the primary invasive capacity of the cancer cells investigated. However, in particular scientific scenarios, it would be important to show that the degradation of a specific component of the extracellular matrix is brought about by a particular (proteolytic) molecule or molecular system. It seems rather amazing that more specific assays in this regard have either been forgotten and/or not developed further. An example is the laminin degradation assay developed by Schlechte and Boyd [134]. This pioneering method enabled a quantitative measure of specific laminin degradation by the u-PAR/u-PA system [134, 135], also differentiating the ability of receptor-bound versus free u-PA to degrade the specific basement membrane component laminin [134, 135]. Briefly, after seeding of the cultured cancer cells of interest onto radioactive laminin-coated dishes, allowing the cells to attach overnight [11, 134–138], cell surface u-PARs were saturated by incubating the cells with urokinase, and unbound u-PA was removed by washing. The cells were replenished with serum-free medium containing plasminogen (without plasminogen as negative control) to start specific u-PA/u-PAR mediated laminin degradation. The latter was measured at varying time points by withdrawing aliquots of the culture medium and counting for radioactivity, which represented solubilized laminin.

Certainly, the original protocol of this method has the disadvantage of significant exposure to radioactivity for the experimenting persons, however, it might not be difficult to modify such a protocol according to actual standards with non-radioactive, e.g. luminescent or immunological detection using Western blot- or ELISA-based methods [139, 140]. One could also anticipate that similar protocols, as demonstrated by Rangaswami et al., could be developed for the molecule-specific degradation of other important ECM components [141]. The benefit of being able to selectively, and quantitatively, measure specific degradation of a particular ECM component by specific molecules certainly adds precision to investigations on the role of particular molecules in matrix degradation, invasion, intravasation, and other metastasis-associated degradation processes.

Similarly, approaches and protocols already exist for other key components of the extracellular matrix, like collagen degradation, which is crucial for studying matrix remodeling, tissue repair, and particular disease processes. For example, particular assays study heparan sulfate proteoglycan (HSPG) perlecan degradation, which is crucial for understanding ECM remodeling and tissue homeostasis, or entactin (nidogen) degradation, which is important for studying basement membrane remodeling and ECM turnover [142]. An attachment assay to fibronectin was applied in a recent publication, however, it was rather used as an adhesion assay and generally measured attached cells to fibronectin-coated dishes, not as an assay measuring ECM-degradation by particular molecules [143].

Gelatin zymography

Zymography is a widely preferred approach to examine extracellular matrix-degrading enzymes like matrix metalloproteinases (MMPs) and antioxidant enzymes like SOD and catalase in cells and tissue extracts. For the first time, Gross and Lapière reported about MMPs and introduced zymography to detect collagen degradation in tadpole tissue [144]. There are several zymography methods available for specialized applications, including substrate-based (gelatin, casein, collagen, heparin, etc.), reverse, and in situ zymography [145]. The primary objective of gelatin zymography is to identify (activity of) the gelatinases MMP-2 and MMP-9. Its high sensitivity can detect MMP-2 levels as low as 10 pg [146].

In-gel zymography, also known as overlay or indirect zymography, was initially used to separate protease-containing components with a resolving gel, and overlay them on an indicator gel to detect substrate degradation. Plasminogen activators are detected by converting plasminogen to plasmin and disintegrating fibrin films in the indicator gel [147]. Later, a direct zymography technique was developed and improved into a semi-quantitative assay. The resulting approach effectively identifies both latent and active forms of gelatinases, specifically 72 and 64 kDa for MMP-2 and 92–95 and 82 kDa for MMP-9. By using zymography to correlate elevated MMPs with clinicopathological factors, Rocca et al. examined the activity of circulating gelatinases in the sera of breast cancer patients [148]. Kundu and his team have been refining similar protocols for more than 20 years [76, 117, 119, 124].

All assays of this chapter are depicted in Fig. 2.

In vitro models to study cell–cell and cell–matrix interactions in EMT

The focus of the previously described in vitro methods lay on cultured cancer cells. However, in EMT and metastasis, the interaction between cancer cells and their microenvironment is extremely important. Therefore,

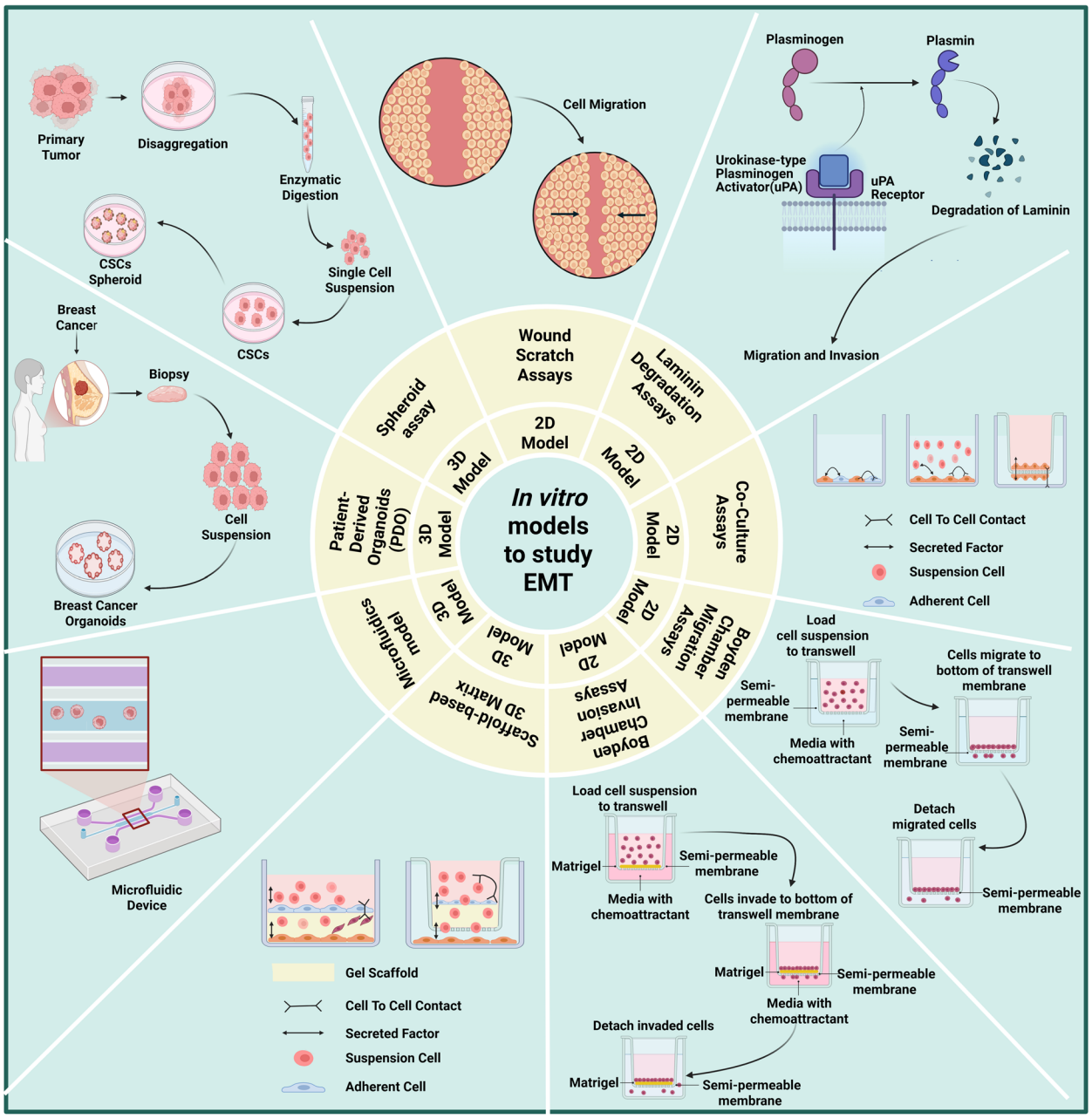

Fig. 2 Depiction of various 2D and 3D in vitro models to study EMT in Cancer. In 2D culture, cells grow under adherent conditions, which confine them to microenvironment. These models are easy to implement, however, they only support to study cell–cell interactions. In contrast, 3D culture models mimic the tumor microenvironment by enabling both cell–cell and cell–matrix interactions within a three-dimensional structure. Moreover, 3D models allow for the co-culture of multiple cell types, effectively mimicking intra-tumor heterogeneity

this chapter summarizes rather straightforward and fast 3D in vitro models to address these interactions.

Co-culture assays

Co-culture studies interactions between cells through autocrine and paracrine mechanisms relevant to the disease, its diverse states, or potential therapeutics. Cellular polarity, cell–matrix adhesion, cell–cell and -microenvironment communication, metastatically relevant pathways, drug action, and further issues related to EMT can be studied in co-culture. Co-culture systems are classified into direct and indirect methods. Direct co-culture includes different types of cells communicating in a culture dish, whereas, in the indirect method, two different

types of cells reside in separate chambers, whereby interaction occurs between the cells by secretory molecules. Various technical co-culture systems include microfluidic, Petri dish, or solid support systems, bioreactors, and transwell systems. Co-culture can be performed 2-dimensionally on a Petri plate, or 3-dimensionally in suspension media, based on experimental questions, requirements, and individual budgets. Hay and Greenburg were the first who co-cultured embryonic epithelial cells with an adult anterior lens in a 3-dimensional collagen gel system to study EMT during embryonic development [149]. Co-culturing is still one of the best artificial methods to understand key steps of EMT [150], as illustrated by the following examples:

Montero et al. have demonstrated that MCF-7 cells co-cultured with senescence-conditioned media (SCM) or cytokines IL6 and IL8, promote EMT [151]. In another study, T cells were modified with B7 homology 4 (B7-H4) specific chimeric antigen receptors and co-cultured with Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeled SKBR3 (wild type/knockout) breast cancer cells, suggesting that B7-H4 is important for the cancer cells to escape from cytotoxicity and enhance EMT activity [152]. Xu et al. co-cultured MCF-7 with human adipose-derived mesenchymal stem cells (MSCs) and observed MCF-7 to transform into a prolonged phenotype of EMT, which was regulated by TGF-β1 dependent ZEB/miR-200 signaling [153]. MDA-MB-231 (human breast cancer cells) and pre-osteoblast MC3T3-E1 cells in the Tissue Mimicking Co-culture Device (TMCD) facilitated a cross-talk to promote EMT in MDA-MB-231 cells [154]. The co-culture of CD133+ hematopoietic progenitor cells (HPCs) or CD133- HPCs with MCF-7 or MDA-MB-231 cells enhanced the expression of EMT markers and suppressed E-cadherin in breast cancer cells [155]. Another group demonstrated that co-culturing tumor organoids with inflammatory-like cancer-associated fibroblasts (iCAFs) caused an upregulation of EMT markers [156]. Further, co-culturing HUVEC (Human Umbilical Vein Endothelial Cells) with SiHa (cervical squamous carcinoma) cells led to an induction of EMT and invasion in the latter [157]. Taken together, each of these instances demonstrates the advantages of co-culture methods in examining EMT, which can be conducted relatively quickly and in a cost-efficient manner.

3D matrix models to study EMT

2D models are limited in replicating physiological complexity. For example, the rigidity of 2D surfaces does not accurately represent the dynamic ECM interactions present in breast cancer tissues, which are crucial for proper EMT regulation [158]. Furthermore, 2D cultures lack the spatial gradients of oxygen, nutrients, and signaling molecules that occur in in vivo systems, potentially skewing cellular responses [159]. Studies have demonstrated that cells in 2D culture often exhibit altered gene expression profiles, with higher expression of stress response genes and differences in EMT-related pathways compared to 3D culture [160]. These discrepancies highlight the need to interpret 2D EMT and further functional findings with caution and, where possible, to complement them with 3D or in vivo models.

3D matrix models provide several advantages, including the ability to better mimic the native tissue architecture and microenvironment [161]. Bissell et al. have demonstrated that cells in 3D culture exhibit distinct morphological, phenotypic, and functional properties compared to 2D models [162]. In 3D cultures, cells interact with their surroundings in more closely resembling in vivo physiological conditions [163].

Scaffold-based 3D matrix models

Several types of 3D matrix models have been developed to study EMT, which can be broadly classified as scaffold-based or scaffold-free. Scaffold-based hydrogels, or hydrophilic 3D networks, provide a hydrated and biocompatible environment that promotes cell survival. Their adjustable qualities allow for mechanical strength modifications, but their lack of stability and microstructure control can be limiting [164]. The internal structures of scaffold-based hydrogels are networks of cross-linked polymers that can be shaped through mild gelation conditions with low cytotoxicity [165]. The hydrogel can be supplemented with growth factors (EGF, FGF) or signalling molecules that induce, or inhibit, EMT. Cells are resuspended in the hydrogel solution and then plated into culture dishes. The hydrogel is allowed to polymerize to form a stable gel matrix. After gelation, a suitable medium is added to the wells to support cell growth. Cells are cultured in this 3D environment for a defined period, to allow for EMT to occur. The cells are analyzed through various methods, including immunofluorescence staining for EMT markers (E-cadherin, N-cadherin, or others), gene expression analysis via qPCR, or functional assays like migration and invasion [166].

Scaffold-free bioprinting creates three-dimensional structures by printing cell aggregates directly. This approach provides exact control over cell location, allowing for more complicated drug screening and research designs. However, bioprinting is still in its early stages, with high costs, technological obstacles, and scaling constraints, limiting its widespread adoption. Cell viability can also vary depending on the technology used [167].

Spheroid assays

Spheroid tumor models, which are composed of cells in various metabolic and proliferative states, have been shown to have great potential for the development of novel anticancer therapies [168]. Scaffold-free or anchorage-independent techniques generate spheroids via non-adherent cell-to-cell aggregation; they include Hanging drop plate- and low attachment plate technologies [169]. There are several steps in the development of a spheroid. First, individual cells in the suspension group together to create cell spheroids that are weakly adherent. Initial aggregation is promoted by extracellular matrix fibers, which also include complementary binding of the peripheral cell surface to integrins. E-cadherin then establishes homophilic interactions between peripheral cadherins, promoting strong adherence to the initial cell aggregate. In addition, signal transduction is facilitated by the β-catenin complex [170]. Tescalcin (TESC), an EF-hand protein that binds with calcium, plays an essential role in spheroid cultures of liver cancer stem cells (LCSCs) through its impact on EMT [171]. In colon cancer, EMT may explain the stemness and malignancy of spheroid cells, which have characteristics of colon cancer stem cells (CCSC). The Wnt/β-catenin signaling cascade might play a crucial role in CCSC EMT [172]. Crotoxin (CTX) suppressed the expression of growth factors, chemokines, and mesenchymal markers linked to the EMT process in spheroid (3D) model [173]. Due to the increase of EMT-promoting microRNAs and the inhibition of EMT-inhibitory miRNAs, MSCs in 3D spheroids are being pushed towards EMT [174]. In another paper, scaffold-free and scaffold-based A549 spheroid cell cultures showed higher expression of proteins linked to drug resistance and EMT [175]. Furthermore, 3D models showed how decreased EMMPRIN expression boosted drug resistance, invasiveness, and EMT status while inhibiting proliferation and angiogenesis. The 3D setting also increased cell dormancy and stopped colon cancer (CT26-KD) cells from developing metastatic-like lesions when seeded on basement membrane extract (BME) [176].

Patient-Derived Organoid (PDO) models in vitro

Recent years have seen the widespread use of three-dimensional (3D) cell cultures as preclinical models for studying various diseases, including cancer, in an attempt to bridge the gap between two-dimensional (2D) cell culture and animal models [177]. However, the initial 3D models like tumor spheroids still have limitations, the most significant being the lack of tumor cell interactions with the neighbouring stroma, which restricts their potential as viable preclinical tumor models. The PDO approach has recently emerged as an extremely promising strategy in personalized cancer therapy and translational cancer research, as it preserves the inherent genetic heterogeneity and cellular complexity of the original malignant tissue [178]. Organoid cultures are composed of clusters of organ-specific cells that are developed directly from tumor fragments. Numerous studies have shown that organoids partly reconstruct the original tissue, retaining its structural and functional characteristics [179].

Bates et al. have developed an EMT model of colon cancer to explore the involvement of stromal elements. They have revealed that LIM 1863 organoids transition from a well-differentiated spheroid structure to a migrating monolayer phenotype in response to TGF-β. Furthermore, they discovered that TNF-α, a product of active macrophages, significantly accelerates TGF-β-mediated EMT. Moreover, an autocrine loop of TNF-α that is reliant on ERK activation is established when organoids are exposed to TNF-α. According to this, TNF-α accelerates EMT by a mechanism involving p38 MAPK activation [180].

In 2015, a biobank of several CRC organoids was developed using appropriate colon segments, with an overall success rate of 90% [181]. These organoids were subsequently cryopreserved, with a mean survival rate that exceeded more than 80% after thawing, indicating a significant benefit over PDX models. Thereafter, 18-gauge biopsies of metastases were effectively used to generate CRC PDOs, with a success rate of over 70%. Significantly, these PDOs retained the genetic diversity of their primary site of metastasis [182]. CRC organoids were cultured with niche factors such as TGF-β inhibitor (A83-01), Noggin, epithelial growth factor (EGF), Wnt3A, p38 inhibitor (SB202190) (ENAS), and R-spondin1. The transition from adenoma to carcinoma was accompanied by decreased niche factor needs in CRC organoids, indicating the accumulation of genetic alterations throughout this process [183]. In 2018, a live PDO biobank of over 100 organoid lines of breast cancer from a diverse range of primary and metastatic tumors was established. This particular study examined several primary and metastatic breast tumors and identified 95 breast cancer organoids. PDOs were shown to have characteristics in common with a broad range of breast cancer subtypes that differ greatly in terms of histology, human epidermal growth factor receptor 2 (HER2), and hormone receptor status [184]. 3D organoids have been used to successfully represent neuroendocrine prostate cancer, an aggressive form of this cancer with relatively few pre-clinical models available [185]. Ovarian cancer (OC) can also be modelled using PDO. Kopper et al. developed around 56 organoids that accurately replicated the histological as well as genetic characteristics of the parent tumor, representing all major subtypes of OC

[186]. Similarly, PDOs can be used in the investigation of bladder carcinoma. In 2018, numerous bladder cancer organoids were developed and shown to accurately replicate tumor progression and the therapeutic responses of their parent tumors [187]. A majority of the organoids are grown in a synthetic medium containing specific chemicals as well as growth factors, and depending upon the type of cancer, the growth medium may be supplemented with additional specialised factors. For instance, it has been shown that Neuregulin 1, a ligand for human EGF receptor tyrosine kinases which is associated with carcinogenesis and mammary development, can efficiently develop breast cancer organoids and sustain continual growth for over 20 passages [184]. Individual ligands of fibroblast growth factor (FGF) family play various roles in lung organoid development. For example, FGF7 and FGF10 produced greater organoid branching than FGF2 and FGF9 [188]. Furthermore, a greater concentration of dihydrotestosterone (DHT) is critical for the survivability of certain forms of prostate cancer organoids [189]. However, the possibility of combining organoids with diverse functional assays or in vivo model settings still might not have been explored enough, which limits the potential of PDOs to investigate molecular insights compared to other experimental settings. Figure 2 illustrates various in vitro models utilized to study EMT in cancer.

Invasion fronts and beyond: advanced imaging and spatial resolution as sophisticated tools to understand the landscape of metastatically relevant compartments

In the past decade, a new field of imaging and spatial technology has developed, providing detailed insights into cellular interactions at the subcellular level, particularly relevant to understand metastatic stages. Since early pioneering works on live cell imaging by Ann Chambers and colleagues, fundamental advancements into an understanding of invasion fronts, different types of migration, guidance of invasion by tissue structures, intra- and intercellular changes and molecular dynamics during invasion, etc., have been brilliantly achieved by specialized groups such as Peter Friedl et al. [81, 82, 87, 88, 91, 190–193]. Various imaging methods have emerged, ranging from 2 to 3D and in vitro to in vivo approaches. One notable technique is fluorescent lifetime imaging microscopy (FLIM), initially used for 3D living tissue cultures [190]. Additionally, third harmonic generation (THG) microscopy allows for visualization of cell functions and interactions without tissue staining, thus providing deep-tissue images and detailed structural observations [194]. THG, in contrast to other imaging techniques such as confocal microscopy, does not need tissue staining and enables the acquisition of deep-tissue images, being able to achieve a detailed resolution of structural, cell, and tissue architecture [195]. THG can scan, and reconstruct, the volume of 3D samples, and allows long-term time-lapse imaging [196]. A potential weakness of this label-free technique is limitations in providing molecular information and therefore, it could be considered as complementary to other imaging approaches in the field of advanced fluorescence imaging. Nevertheless, it has strong potential to emerge as a standard tool of infrared multiphoton microscopy in metastasis research and investigate cell–matrix interactions, tissue remodeling, and the turnover of microvesicles such as exosomes, in unlabeled cell- and tissue samples and in small animal models. Clinically, there is a high potential of this technology in oncology, vascular biology, neurooncology, and other fields, to analyze microanatomy, new materials, 3D tissue organization, the positioning of different cell types in a tissue context, or their potential modifications following therapy [194]. Multiphoton fluorescence microscopy (MPFM) is another advancement that enables the study of biological tissues at high resolution and provides valuable insights into tumor dynamics and interactions [197]. Furthermore, super-resolution microscopy has been developed to enhance diagnostic capabilities in cancer research, including the detection of single, metastatic microRNA [197–199]. However, these techniques can be expensive and challenging for non-experts to perform, limiting their routine use in clinical settings.

In studying exciting compartments and tumor cell areas like invasion fronts, the need for different spatial transcriptomics and spatial resolution–omics is important to discuss. A comprehensive review of all technologies available in this field would require a separate analysis, as recent reports already offer a good overview [200]. Thus, this current review provides only a summary of it. Current spatial transcriptomics platforms can be sequencing-based, imaging-based, probe-based, or image-guided, to provide spatially resolved, single-cell RNA sequencing. Chen et al. and Stahl and colleagues, have highlighted the advancement of spatial transcriptomics in studying tumor heterogeneity in details. It adds an important spatial context to gene expression data, allowing researchers to map gene expression patterns while retaining information about the location of cells in tissue samples. This technique offers new insights into tumor heterogeneity and interactions within the microenvironment [200–202].

It has been widely applied in cancer research, such as characterizing various phenotypes of tumor-associated macrophages (TAMs) and forming new hypotheses on how they interact with their surroundings [203, 204]. Spatial transcriptomics has provided deeper insights into gene expression in different tumor regions and tracked

how cancer cell populations evolve and spread [205–207]. It also helps identify regions with specific gene expression patterns associated with treatment resistance [208]. Additionally, it allows precise identification and characterization of immune cell populations within the tumor microenvironment and enhances our understanding of stromal interactions [209–211]. The advancements in this field are remarkable, enabling hypothesis generation about cell and molecular interactions at both single-cell and subcellular levels. Current technologies boast increased speed, sensitivity, coverage, multiplexing abilities, and the flexibility to apply to various sample types, including paraffin-embedded and living tissues. Beyond spatial transcriptomics, other platforms have been developed, including spatial genomics, proteomics, epigenomics, and metabolomics, along with combinations and single-cell sequencing [200, 202, 207, 211–215]. These developments aim to analyze larger sample areas in 3D and observe time-dependent changes in molecular landscapes. Ongoing enhancements focus on improving spatial resolution, higher multiplexing capabilities, and the spatial analysis of living tissues, alongside new bioinformatic platforms to address emerging challenges [200, 216–222].

There is still a need for independent validation of hypotheses from spatial–omics platforms through classical experimental models in vitro and in vivo. Developing novel experimental models for verification at the 3D multicellular level may also be necessary. If achieved, these platforms can significantly improve our understanding of critical compartments, such as invasion fronts and metastasis in specific microenvironments.

Intravasation and beyond—the chorionallantoic membrane model (CAM model) as an in vivo model to differentiate specific steps of metastasis

Intravasation is the most essential step to transform cancer into a systemic disease. It is amazing, however, that hardly any models are able to study this step specifically. The CAM model of the bred chicken embryo is a unique in vivo model in which different steps of the metastatic cascade can be studied. Briefly, primary tumor growth on the upper chorionallantoic membrane (CAM), invasion into the upper CAM, intravasation into the systemic circulation of the egg, angiogenesis, and establishment of distant metastasis in organs of the nascent chicken embryo can be specifically studied and differentiated. Regarding angiogenesis, the development of tumor-associated vessels can be studied in the primary tumor and quantified [123, 124, 132, 223–225]. The ability of this model to, quantitatively, measure the crucial step of intravasation in a highly sensitive manner sets it apart from other in vivo models, including the majority of mouse models. For example, the protocol that the Allgayer group established with a TaqMan-based human ALU-PCR in 2004 has proven to be very powerful [226]. For a graphical overview on the CAM-model, please see Fig. 3.

The first discoveries to use bred chicken eggs experimentally were made by Rous and Murphy, who implanted tumors from various species onto the CAM, demonstrating that these could be maintained and continuously passaged from egg to egg [227]. In further studies, the ability of some of these tumor grafts to metastasize to chicken organs became apparent [75]. Over time, the most utilized experimental technique for effective tumor grafting evolved as the removal of an eggshell square, with the junction point of major blood vessels on the visible CAM serving as the ideal implantation site. Gradually, the ideal host age for transplanting xenograft tissues to the CAM was shown to be 9 or 10 days of incubation, since earlier timing was associated with less developed CAMs but later time points limited the length of time for experimental observation. Furthermore, as the nascent embryos progressively mature, the model loses its natural immunodeficiency [228–230], the initial detection of B- and T-cells starting at days 11 and 12, but full immunocompetence being seen with days 18 and later only [223, 231]. Over time, the CAM model has been refined to investigate different aspects of cancer cell invasion, intravasation, angiogenesis, and metastasis of many (human) cancer cell lines, whereby the unique strength of the model is a specific ability to delineate intravasation from other steps of the metastatic cascade in vivo [75, 226, 230]. During this development, the highly pioneering work of Liliana Ossowski and colleagues, Bender et al., Weiss & Andres, cannot be emphasized enough [232–234]. Ossowski and Reich discovered that tumor growth and metastasis are sensitive to embryonic age, determined day 10 as optimal for the inoculation of cancer cells/tumors onto the upper CAM, and gave proof that sequential passaging of tumor cells on the CAM maintained their aggressive phenotype and metastatic potential [234, 235]. Later, their work in the CAM model led to the discovery that the invasive potential of tumor cells correlated with tumor-associated proteolysis brought about by the interaction between uPA and its receptor u-PAR. The same model prompted later essential discoveries that the u-PAR can induce dormancy in carcinoma cells, providing a switch between dormancy and proliferation, depending on the (microenvironmental and intracellular) molecular context [72, 235–240].

These first CAM-models used radiotracers and semi-quantitative evaluation of invaded and intravasated cells. Gradually, model development took advantage of the

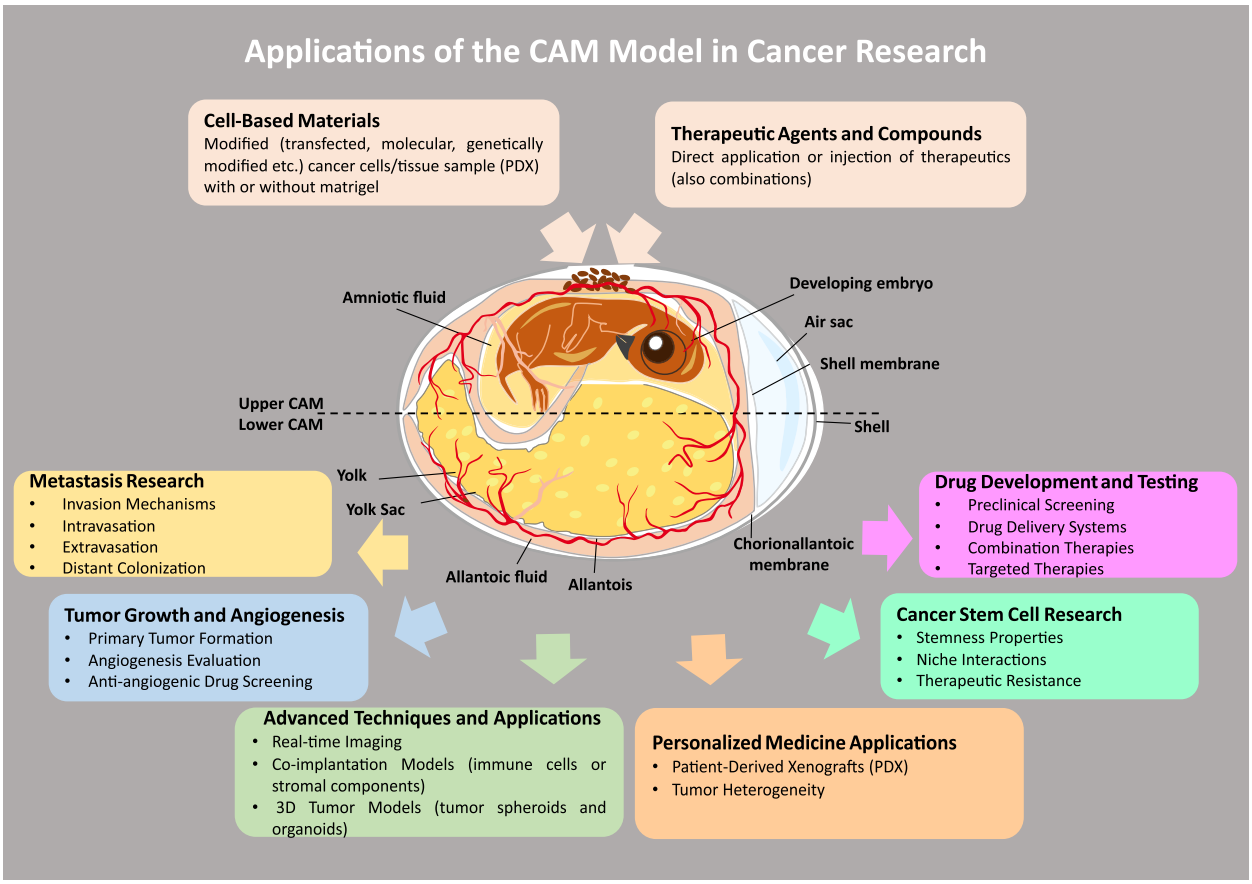

Fig. 3 Schematic representation of the chorionallantoic membrane (CAM) model of the bred chicken egg and possible applications for cancer research. Schematic overview of the developing egg. Possible material to be used for the CAM model and potential applications (materials or compounds) are shown at the top level of the graph. Major compartments of the egg model are shown, including the lower and upper CAMs which are physiologically defined with respect to the window cut for placing the cells/material to be investigated (dotted line). Many implications of the model for cancer research are demonstrated in the lower part of the figure. Especially, after inoculation of the cells or tissues onto the CAM, invasion into the upper CAM can be measured, as well as tumor growth and angiogenesis. The lower CAM can be harvested specifically to evaluate the cells that have intravasated into blood vessels. PDX models are also possible. The model is excellent for rapid in vivo drug testing, amongst all of the other applications shown

highly species-specific presence of eukaryotic *ALU* family sequences, or short interspersed repeated DNA elements (SINES). These sequences are also distributed throughout the human genome, and therefore, highly species-specific *ALU*-PCR protocols for the quantitative detection of intravasated tumor cells into the CAM and specific organs of the nascent chicken embryo enabled a quantitative, highly sensitive detection of single human cells on the chicken embryo background [226, 241].

This modern quantitative protocol briefly works as follows: A square carefully cut into the top shell of a bred chicken egg exposes a window of the upper CAM. Human cell-line-derived tumor cells (which can be manipulated experimentally in many different ways, e.g. transfected, the genome modified or edited by site-directed mutagenesis, CRISPR/Cas editing, etc.) are seeded onto this upper CAM part around day 9 of breeding [226]. Tumor cells invading the CAM can be harvested 2 days later to give a specific quantitative measure of local invasion. Furthermore, only the tumor cells able to intravasate will enter the amniotic vessels and, on day 11, can be measured at high specificity and quantified with a human ALU-specific TaqMan-PCR, using cells isolated from the lower CAM which has been harvested before according to, e.g., ref. [226]. Allowing the model to incubate further, one can also measure metastasized cells into the lungs and livers of the nascent immature chicken embryo [226]. It is also possible to study the impact of therapeutic compounds on different steps of the metastatic cascade in this model, within much shorter time frames than needed for mouse and other in vivo models, applying the compounds either onto the upper CAM after tumor cell inoculation, or, more challenging, by microinjection into amniotic vessels [74, 75, 80, 126–128, 242].

Thus, the CAM model offers a number of advantages. First, as already mentioned, it is uniquely able to specifically measure intravasation in vivo in a highly sensitive and quantifiable manner. In the protocol introduced by the Allgayer group detecting ALU-YB8 subfamily sequences, genomic DNA of human tumor cells are detected within the lower CAM (intravasation) or internal organs of the chicken embryo (organ-specific metastasis) at a sensitivity of 10 invaded/colonizing cells [226, 243]. Compared to especially mouse models, it enables a less challenging analysis of the individual steps of a complex physiological process such as metastasis in vivo. In parallel, it ideally fulfils the objective of saving and sparing animals that otherwise would be required for classical in vivo models, e.g. mice [243]. In addition, the usage of fertilized eggs instead of animals can be done at much lower costs, since neither the purchase of SPF-eggs nor the incubators or care result in high costs or need for specific personnel. Finally, the CAM model has a significant advantage regarding experimental time, since an animal model investigating metastasis, e.g. in mice, usually lasts 4–10 weeks. In contrast, questions related to intravasation can be answered after 11 days in the CAM-model, or after 17 days for metastasis.

Recent exciting modifications of the CAM model pushed its application as a patient-derived xenograft (PDX) model, as reviewed by DeBord and colleagues [225]. Advantages of a CAM-PDX model as compared to rodents include a high take rate (successful engraftment of the transplanted tissue), and good possibilities to study angiogenesis around the grafted tumor. However, secondary hyper-angiogenetic responses due to inflammation have been observed as a potential limiting factor. Still, PDX-grafts on the CAM reflected particular subtypes of primary tumors regarding their metastatic potential in terms of higher viability of the latter, potentially allowing predictions of particularly aggressive phenotypes in a patient. The CAM also allowed successful engraftment of rather problematic tumor entities such as musculoskeletal or nasopharyngeal ones [225, 244–246]. Easy accessibility for the testing of single or combined chemotherapeutic drugs, classical therapeutics, photodynamic therapies and, certainly, molecularly targeted drugs, is a clear further advantage [225]. The CAM-PDX turned out to be a good model for testing drug combinations, although the question of comparability of the doses in the model with the therapeutic doses applied to the patient, bioavailability, etc., might still be issues. Here, translational-experimental studies at the CAM-PDX paralleling clinical trials on particular drugs or their combinations should be done, including analyses in retrospect of whether individual clinical outcomes matched predictions by the CAM model [225]. A limitation of CAM-PDX models in drug testing might be the original strength of a short experimental time window, when studying chronic development of drug resistance or long-term toxicology. Here, sequential re-transplantations of grafts into fresh-bred eggs and CAMs might be a solution, although the issue of potential molecular and stromal changes in the graft by repeated passaging on the chicken background needs to be addressed. Other still existing limitations of CAM-PDX settings include mixed results on the detectability of disseminated or (micro-) metastasized cells from the graft in the chicken egg, thus potentially limiting options to study intravasation and metastasis functionally as compared to cell-line-based settings. Further open questions on the degree of representability of the grafts down to the molecular and genomic level as compared to the original patient tumor, especially after repeated passaging, or the influence of the (gradually chicken-derived) stromal component on the graft, need to be addressed in future testings. However, the latter issues are at least as challenging in PDX models in rodents. Therefore, besides still existing questions, the CAM model might be a highly interesting in vivo model in the development of smart PDX concepts, such as PDX-based clinical trials [225].

Dissemination and Minimal Residual Disease (MRD) in cancer

One of the major obstacles to the complete eradication of carcinomas is MRD, which can be caused by the rather early step of systemic dissemination in the metastatic cascade [16, 31–45, 55]. Advances in cancer treatment have significantly reduced cancer-associated mortality and morbidity [247]. However, many patients with disseminated epithelial malignancies still cannot achieve complete curation of their disease, or, at the very least, successful transformation into a controllable chronic illness that is compatible with life. Residual cancer cells might persist locally, in distant organs as DTCs, or in the circulation as CTCs, in spite of certain individuals exhibiting high sensitivity to treatment [248]. These residual cells are able to develop MRD, which can lead to therapy failure and tumor recurrence [249]. Frequently investigated tumor-derived analytes within the peripheral blood for the assessment of a potential MRD are circulating tumor DNA (ctDNA), which detects somatic DNA alterations, and CTCs, which detect cellular residual disease [250]. MRD is well known in hematological malignancies, and initial treatment is effective for many of these tumors [251]. In carcinomas, single cytokeratin-positive cells were discovered in the bone marrow and blood of cancer patients, providing the first implications

for MRD in solid tumors. These cells, previously termed as micrometastatic cells, are now defined as single DTCs as opposed to the official pathology term of micrometastasis [252]. MRD might exist for years or decades after surgery before metastasis develops [16, 31–36, 46, 253]. Tumor dormancy is the term used to describe this prolonged latency period [72, 193, 239, 254–259].

Evidence that disseminated tumor cells exist in patients with carcinomas has been pioneered by groups across Europe including Germany around the beginning of the 1990 s [33, 34]. Towards this end, disseminated tumor cells were detected with classical immunocytochemical methods based on human epithelial-cell cytokeratin antigens (most often CK 18, but also CK 19) in the bone marrow and blood of the patients. These cells were evidenced in patients with even very early stages of different carcinomas like breast, lung, gastric, colorectal, or prostate cancer [33, 35–38, 41, 46, 48–54]. Establishing immunocytochemical protocols for double-staining, some groups succeeded in differentiating distinct molecular phenotypes amongst DTCs associated with a rather critical prognostic fate of patients, recurrence, and/or metastasis even years after curative resection and primary tumor therapy, or to represent a phenotypic advantage in the dynamic development of MRD over time [16, 31, 32, 37, 39, 41]. Within the high heterogeneity of disseminated tumor cells found in human cancer patients, molecules such as c-erbB-2 and the urokinase receptor u-PAR were proposed as representing metastatically capable phenotypes. The former is an established oncogene and target for therapy, while the latter is one of the essential biochemical drivers and focus of tumor-associated proteolysis in many steps of the metastatic cascade, and represents a switch between tumor cell proliferation and dormancy (see chapter "invasion"). Since these pioneering times, certainly, completely novel technologies have been developed, and the field of liquid biopsies to diagnose, and phenotype, single DTCs has been pushed tremendously by specialized groups, e.g., Pantel et al., Alix-Panabieres et al., Kalthoff et al., just to name a few. We refer the reader to reviews by groups in this field who gave an excellent overview on the current technological status [16, 31, 35–37, 40, 42–44, 55, 59, 60, 62, 64, 72, 73, 77, 240, 260–266].

To give a brief overview, ctDNA is a form of cell-free DNA that malignant tumors release into the circulatory system and serves as a novel diagnostic approach to detect cancer by liquid biopsy [267]. Moreover, liquid biopsy assays have revolutionized MRD detection in patients who lack any radiological or clinical indications of metastasis or residual tumor cells that remain after initial therapy [268].The first evidence that a cell culture can be generated by some DTCs in carcinoma patients has been given by the Pantel group [269]. Technologically, the field of liquid biopsies tackles the detection, and in part characterization, of DTCs by several high throughput technologies such as single-cell analysis at the genome, epigenome, transcriptome or proteome level, along with next-generation sequencing to understand their molecular alterations and heterogeneity [270–273].

For efficient CTC enrichment, positive selection uses cell surface proteins such as epithelial cell adhesion molecule (EPCAM), cytokeratins, or HER2, whereas negative selection uses antibodies against CD45, CD146, or CD34 to eliminate non-malignant cells [274]. However, it remains a matter of critique if most of the markers used for CTC selection are entirely tumor cell specific. The Epithelial Immuno SPOT (EPISPOT) assay and its variant, EPIDROP (the EPISPOT in a drop), are functional assays to detect viable CTCs [275, 276]. EPISPOT detects CTCs using the fluorescence of epithelial proteins released by viable CTCs in short-term cultures [275]. EPIDROP detects single-cell CTCs in fluid microdroplets, distinguishing between viable and apoptotic cells, and detecting EPCAM+ or EPCAM- cells. This approach can also detect EMT in malignancies such as breast, colorectal, and prostate cancer [277–279]. It is currently being improved for quicker and more effective use in clinical settings. Sementsov et al. developed a mass spectrometry-based method for detecting mutations from single CTCs that does not require complicated bioinformatics techniques or whole gene amplification (WGA). The method was tested at CTCs of patients with metastatic melanoma and the mutations compared with those detected in ctDNA and tumor tissue. A greater number of mutations, including those linked to melanoma driver genes, resistance to therapy, or metastasis, were discovered in CTCs than in ctDNA, however, ctDNA analysis had better concordance with tumor tissue [280].

Animal models to reflect CTCs, a disseminated disease stage of carcinomas, and MRD have been challenging. To investigate cell signalling and the tumor microenvironment during MRD and recurrence, Janghorban et al. have developed a Wnt/iFGFR^{GFP} (iFGFR^{GFP}) model. To reveal the alterations in the microenvironment, they have used immunophenotyping and single-cell RNA sequencing (scRNA-seq) to create four stages of tumorigenesis: primary, MRD (dormant), long-term dormant (long-term DT), and recurrent. They established that the gene expression profiles of Ki67 −MRD cells resemble those of growth-arrested cells, using dormancy markers such as Cdkn1b, Gas6, Zfp281, and Nr2f1 [281]. Preventing relapse by targeting MRD has emerged as a potential salvage strategy of targeted therapies [282]. Rambow et al. created PDXs (MEL006, MEL007, and MEL015) from BRAFV600E/K mutant melanoma patients, showing that

MRD exhibits both cellular as well as geographical heterogeneity [283]. They also discovered that this strategy may be more challenging than initially anticipated, based on bulk analysis and in vitro culture investigations [284, 285]. Functional studies in PDX models, developed using CTCs, may reveal characteristics of these cells necessary for the DTCs to proliferate and for homing to secondary sites to develop overt metastases [286, 287]. Using a HER2-driven mouse breast cancer model, Hosseini et al. indicated that early disseminated cancer cells account for at least 80% of metastases [288].

Dormancy in DTCs is considered to be a crucial event in the development of MRD that can become reactivated later, and is an inactive state that is regulated by both the tumor microenvironment and cellular mechanisms [289]. It is accompanied by stromal remodeling, involving endothelial cells and fibroblasts, as well as immune cell recruitment, including myeloid and lymphoid cells, the majority of which are immunosuppressive or nonfunctional and cytotoxic [284]. Excellent reviews addressing dormant cells, and potential overlaps to the characteristics of stemness, have been given recently by Barkan, Chambers, and further pioneers in this field [259, 290–293]. Specific model advancements such as those done by Barkan et al. to specifically study stemness and dormancy are promising [256, 257, 290, 294–296]. Pioneer and ongoing work to understand, model, and target dormant cancer cells has been, and is being, continuously done by key groups such as Aguirre-Ghiso et al. and others [254, 255, 297–300]. Still, as already pointed out above, the ultimate characterization of the fraction of disseminated tumor cells that would render a clear definition of the "metastasis-initiating cell" remains to be put forward [78]. To do so, larger interactive consortia bringing down hypothesis generation from a systematic characterization of metastatic lesions towards a "micrometastasome" might be needed to arrive at a clinically applicable, phenotypically clear diagnosis of metastatically critical, single disseminated tumor cells within an individual patient [45, 301–307]. Still, the suggestions of early pioneers in this field on particular, metastatically relevant markers on such cells, such as c-erbB-2 or u-PAR have held their biological promise in our opinion as to their role in tumor progression, metastasis, dynamics between proliferation and dormancy, or else, and might be revisited [45, 78]. The development of suitable models to study dormant cells mechanistically is a highly exciting actual field of development, in the context of advancing our understanding of tumor cell dissemination and the dynamics of DTCs in their interface between intravasation, dissemination, settlement in particular compartments of the body, and requirements for metastatic outgrowth of these cells.

Microfluidic models to study extravasation and more

Cell extravasation is a precisely controlled process in which cells escape the bloodstream, traverse the endothelium, and eventually migrate into tissues. Microfluidic devices are efficient tools for multiplex PCR, electrophoresis and hybridisation to be executed on chips, also in the context of extravasation. Concentration gradients, cell–cell analysis, and ECM component analysis can be captured by microfluidics, which then provide a greater picture of the TME [308]. The technology offers further variations like isolating CTCs, cell phenotyping, studying shear stress, cancer drug screening using droplet microfluidics, mimicking TME on-a-chip, analyzing angiogenesis, and organ-on-a-chip options [309]. Moreover, novel human-on-a-chip models offer 3D culture systems that mimic various organs of the body simultaneously [310]."Tissue chips"with a single channel lined by cells from a single tissue type, or more intricate organ chips that combine two or more tissue types that can be interfaced directly across a porous ECM-coated membrane, or separated by an ECM gel that fills one or more microchannels, can be made using microfluidic devices [311]. To recreate tissue–tissue interfaces that are essential for reconstituting organ-level structures and functions, one of the most intricate organ chips has distinct parenchymal and vascular microchannels divided by a porous ECM-coated membrane, with organ-specific epithelial cells on one side and stromal cells (or immune cells) on the other. Cells cultured on either side of these membranes form their basement membranes, which cover many pores, allowing cells to stretch and make direct contact with the extracellular matrix of the surrounding tissue [312]. These devices make it feasible to replicate intricate physiological microenvironments by incorporating a variety of cellular populations into a 3D microenvironment [313]. Thus, microfluidic models can incorporate crucial features of the extravasation process, such as blood vessel geometry and a 3D environment, into a cell culture apparatus. Finally, these models improve insights into cell motility controlled by chemical gradients, which aids prospective treatment development [314]. Through the use of microfluidic models, it has been shown that TNF-α increases vascular permeability and alters endothelial intercellular adhesion, which aids in understanding how inflammatory stimuli affect cell extravasation [315]. Song et al. preconditioned breast cancer cells with varying metastatic potential in low-oxygen settings before putting them into a microfluidic device. This experiment demonstrated that cells exposed to hypoxia had a higher rate of extravasation than those under normoxia conditions [316].

In another study, Humayun et al. utilized an organotypic microfluidic model of human vasculature to explore

how multiple secreted factors, resulting from cancer-vascular interactions, contribute to cancer cell extravasation [317]. The model consists of a tubular endothelial vessel created from induced pluripotent stem cell-derived endothelial cells, within a collagen-fibrinogen matrix, with breast cancer cells injected and cultured along the vessel lumen. This system identified cancer-vascular crosstalk, specifically for invasive breast cancer cells, which elevated their IL-6, IL-8, and MMP-3 secretion. These results highlight the potential of microfluidic organotypic models to reveal the mechanisms by which cancer-vascular interactions promote extravasation, as well as to serve as a platform for assessing therapeutic drugs that can prevent extravasation in cancer metastasis [317].

In vivo animal metastasis models

Traditionally, metastasis research in vivo has relied on models generated in mice or rats. Transplantable models fall into two categories: syngeneic and xenograft. For metastasis in vivo models, different routes of, even heterotopic, tumor cell administration can reproducibly mimic different sites of metastasis formation. It is possible to analyze whether particular tumor models or subpopulations are capable of forming/establishing metastasis at distant sites, including preferences for a specific metastatic site, representing their matching"soil"(liver, lung, bone marrow, brain, etc., the "soil", however, coming from the host animal). This can be correlated to specific molecular signatures that are driving these metastatic properties. In addition, such metastasis models allow for the quantification of the metastatic potential of tumors by measuring the number and size of metastases (by specific qPCR, immunohistochemistry, bioluminescence of reporter gene expression models) formed at distant sites, as well as the time required for metastasis formation in vivo.

Cell Line-Derived Xenografts (CDX)

Drug development and cancer research have benefited greatly from the decades-long usage of conventional cancer cell lines as in vitro and in vivo tools [132, 150, 318, 319]. These models exhibit consistent tumor growth among animals and are easy to establish. Creating a CDX involves injecting animals with specific cell lines that have been grown in vitro. Given the benefits of high reproducibility, short turn-around time, wide availability, and cost-effectivity, CDX models are commonly used in drug discovery, pharmacokinetics (PK), and pharmacodynamics (PD) research [320, 321].

Besides heterotopic concepts, some mouse models are orthotopic, the cell lines of interest implanted into the specific organ of their origin. Zhu et al. developed an orthotopic model by injecting human bladder cancer T24-tumorigenic cells into a mouse bladder. The sublines T24-parental and T24-t-lung were obtained by excising the original tumor and lung metastases and growing them on tissue culture dishes with G418 [322]. By simulating the metastasis of real bladder cancer, the sublines T24-P and T24-L were utilized to examine the function of ZEB1 interaction with HIF-1α and EMT in the invasion and metastasis of bladder cancer cells [322, 323].

Intrasplenic transplantation of CRCs can induce liver metastasis. In one example of the Stein group, the efficacy of intervention strategies and novel drug combinations was confirmed. SCID beige mice were xenotransplanted intrasplenically with stable luciferase overexpressing HCT116 cells, to investigate combinatorial MACC1/S100 A4 inhibition against metastasis at human-equivalent doses [324]. Bioluminescence monitoring revealed that negative-control mice acquired prevalent liver metastases over time. The quantity of human satellite DNA in the livers was used to measure metastasis development. Single doses of niclosamide, atorvastatin, or fluvastatin significantly reduced liver metastases by 50%, 41%, and 40%, respectively, whereas combinations achieved up to 95% reduction [325]. These results have strong translational potential for personalized anti-metastatic treatment.

Syngeneic mouse models

Syngeneic transplantable models often involve mouse or rat cancer cell lines, or tissues that cause tumors in inbred animals with the same genetic background [326–328]. Previously, syngeneic cell lines were produced from carcinogen-induced or spontaneous malignancies in mice or rats. Syngeneic models provide the advantage of using the same species for the transplanted tissues, tumor microenvironment, and host [329]. This is especially interesting for studying the interaction between the tumor and the host during metastasis. However, these model systems lack key properties found in actual malignancies. They are typically produced from homozygously inbred mice and lack the genetic complexity of human malignancies. Species-specific variations in oncogenesis, such as carcinogenic xenobiotic metabolism, may result in different mutations or other molecular changes as compared to human patients [329]. Thus, validating findings and conclusions from models is crucial for determining their applicability to human cancers.

Moreover, syngeneic mouse models allow researchers to analyze the evolution of tumor and immune cells in vivo, as well as immune cell interactions. Syngeneic mouse models involve inoculating murine tumor cell lines into immunocompetent animals from the same strain. Many protocols focus on 4T1 and B16F10 models in this context [117, 319, 330]. They are particularly

relevant for evaluating immunotherapy for tumor metastasis [331].

The 4T1 model is a spontaneous breast cancer model that closely resembles triple-negative cancer (TNBC) [332]. It involves injecting mouse 4T1 cancer cells orthotopically into the breast fat pad. Three weeks following injection, primary tumors are removed and lungs are analyzed to assess metastasis. The B16F10 model uses an intravenous injection of the mouse metastatic melanoma cell line, B16F10, which forms tiny tumors in the lung. This model is fast and dark tumor foci allow for easy identification. Metastatic load can be assessed without further analysis. Both the 4T1 and the B16F10 models are commonly used in the development of novel immunotherapies [333]. To assess Neratinib, a potent pan-tyrosine kinase inhibitor, Nagpal et al. developed a novel syngeneic model of spontaneous HER2-positive breast cancer metastasis (TBCP-1) with a particular ability to metastasize to the brain, besides other locations, in immunocompetent mice. It helped to reveal a novel mode of action for Neratinib and its distinct ability to promote ferroptosis [334]. The original NT2.5-lung metastasis (LM) cell line was serially passaged to generate a novel subline known as NT2.5-LM, which was reported by Baugh et al. as an orthotopic, immunotolerant model of HER2+breast cancer that promotes development of spontaneous metastases. Their results distinguished NT2.5-LM as an improved proliferative and metastatic breast cancer model [335].

Genetically engineered mouse models

In the late 1980 s, the first transgenic mouse cancer models were developed by genetically modifying mice to express dominant oncogenes. These"oncomice"enabled researchers to investigate inherited traits required for cancer development and to find potential targets for novel therapies. Later, more precise genetic modifications were achieved with the development of knockout and knock-in mice [336]. They are also referred to as germline genetically engineered mouse models (GEMMs), which include both knock-in and knockout mouse models. There are various approaches to generating transgenic mice by inserting DNA into their genomes.

To investigate the fundamental role of Twist1 in EMT, growth, and metastasis of breast cancer, Xu et al. established mouse models with oncogene-driven mammary tumors that either expressed wild-type (WT) Twist1 or had a tumor cell-specific knockout of Twist1 (Twist1^{TKO}). In advanced Twist1^{TKO} tumor cells, the knockout of Twist1 significantly decreased the expression of the EMT-inducing transcription factors such as Snail, Slug, Zeb2, and both basal and mesenchymal markers, while maintaining the expression of luminal markers. Mice with advanced wild-type tumors showed significant lung metastasis as opposed to mice with advanced Twist1^{TKO} tumors. Their study revealed that Twist1 is essential for the activation of other EMT-inducing transcription factors and these factors collectively cause partial EMT, basal-like tumor growth, intravasation, and metastasis [337]. In another example, transgenic animal models gave in vivo proof that MACC1-induced tumor progression in CRC acts, at least in part, via the newly discovered MACC1/Nanog/Oct4 axis [338].

Recently, even more sophisticated special mouse models have been developed that can delineate particular genetic contributions to metastasis. Towards this end, Welch et al. introduced mitochondrial-nuclear exchange (MNX) mice, demonstrating that mitochondrial DNA (mtDNA) polymorphisms can contribute to modulating metastasis. mtDNA can do so by, for example, changing epigenetics, the generation of reactive oxygen species (ROS), or altering microenvironmental interactions [339, 340]. Such developments are highly promising in terms of an increasing arsenal of specific in vivo models for EMT and metastasis research.

Patient Derived Xenograft (PDX) models

PDX models have revolutionized cancer research and certainly bear high promise, also in metastasis research. For the very first time in 1969, Rygaard and Povlsen implanted colon cancer tissue fragments into nude mice which established the first PDX as a robust in vivo model [341]. In 2006, Hidalgo's group established a pancreatic PDX platform for drug screening and biomarker discovery, which was one of the pioneers of its kind [342]. Another study established a CRC PDX model and characterized HER2 inhibitors for patients with cetuximab resistance [343]. In the meantime, Gao et al. have established 1000 PDX models to predict drug response in clinical trials [344]. These models showcase a myriad of advantages. It retains the genomic landscape of patients across the subtypes, stages, and varied treatment regimens that the patient has undergone. Further, these human-originated models are an ideal choice for studying various situations of tumor heterogeneity, metastasis, interactions with the (immune-) microenvironment, mechanism of drug resistance, and for conducting preclinical trials. Still, however, it needs to be kept in mind that there might be contaminations with animal-derived stromal cells.

In brief, tissue samples from primary or metastatic solid tumors are collected via surgery or biopsy. Tumors are implanted as tissue pieces or single-cell suspensions, alone or in combination with matrigel, human fibroblasts, or mesenchymal stem cells. The most common site of implantation in mice is the dorsal region by subcutaneous

implantation, but orthotopic implantation may be possible [345]. Butti et al., for the first time in India, established a PDX model based on a hormone-resistant breast cancer patient who was hormone receptor-positive and HER2/Neu negative and had been treated with endocrine hormone therapy. The tumors were excised and subsequent generations of PDXs were eventually generated by implanting a part of the tumor tissue into the right mammary fat pad orthotopically in new female NOD/SCID mice [346]. Wang et al. developed PDX models from CRC liver metastases, demonstrating that these models replicate the characteristics of the original tumors. While the models were mostly consistent with the parental tumors, some alterations have been identified only in the metastases, suggesting their participation in the onset of distant metastasis, underscoring observations at human CRC metastases that were shown to bear, in part, specific alterations that were not shared with the corresponding primary tumors [303, 304, 347]. Cho et al. developed PDX models from primary CRC tumor tissue as well as metastatic areas to test their responsiveness to different treatments. They demonstrated that PDX models originating from metastases had varied responses to targeted therapy, possibly due to subclonal mutations acquired during tumor metastasis [348].

A HER2-positive tumor xenograft model originating from gastric cancer was developed by Shi et al. Trastuzumab was continuously administered to simulate the drug resistance process in human gastric cancer. The model was found to exhibit an evident increase in p-HER4 and HER4 expression, followed by enhanced YAP1 expression. Assessment of tumor samples from trastuzumab-resistant mice further demonstrated EMT, with significantly higher levels of HER4, p-HER4, YAP1, and Vimentin than mice without trastuzumab resistance [349]. Pillai et al. propagated, and molecularly analysed, human DTCs using PDX to assess the clinical relevance of a mouse model for identifying potential prognostic and predictive biomarkers of these cells. Notably, human-specific expression of genes such as SNAI1, FOXC2, GSC, STAM2, and KRT19, originating from DTCs, was detected in the bone marrow of all xenograft mice that developed metastatic tumors. However, no human-specific gene expression was observed in the bone marrow of xenograft lines without evident distant metastases or in non-transplanted control mice [350]. Powell et al. created paired isogenic PDX models of TNBC for longitudinal investigation, differing only in p53 status, to investigate how p53 deficiency influences late-stage tumor growth and metastasis. Mouse mammary glands were implanted with patient-derived isogenic human tumor lines that differed primarily in their p53 status. Bioluminescence imaging was used to track tumor growth and metastasis, and flow cytometry was used to measure the number of CTCs. They found that p53 deletion accelerated late-stage tumor progression by enhancing proliferation and decreasing apoptosis, resulting in increased tumor growth and cell shedding [351].

PDX-derived organoid (PDXO) models

Since mouse PDX models are expensive and time-consuming, researchers are now focusing on PDXOs, which might increasingly become important in EMT-research. To obtain patient-derived xenograft cultures (PDXC), a tumor graft from animals with PDXs is isolated, sliced into small pieces, and dissociated into individual cells, identical to the approach used to create primary cell cultures from tumor biopsies. These cells are subsequently seeded into a culture dish. To establish patient-derived organoid cultures, mild tumor dissociation is conducted followed by differential centrifugation to enrich organoids while depleting individual cells [352]. Guillen et al. developed long-term organoid cultures established from PDXs, and to minimize changes in tumor biology, conditions were optimized to support the sustained growth of the PDXOs with minimal medium supplements. Various conditions were tested, assessing the growth of PDXOs by measuring the area occupied by living cells, intracellular ATP content, and organoid morphology. HCI-002 was utilised as a test model, and it was reported that the Rho kinase inhibitor, Y-27632 effectively supported the growth of HCI-002 PDXOs over 15 days, while other common supplements did not increase this effect and some even decreased viability. Similar results were observed when culturing other TNBC PDXOs under optimized conditions, confirming that Y-27632 was the critical supplement [352]. They obtained 40 PDXO lines from 47 attempts. These lines have been cultured for over 200 days and each PDXO line exhibits variable morphology and behaviour. Sengal et al. developed and examined endometrial cancer (EC) PDXOs that closely resemble the morphological and molecular characteristics of advanced ECs and their corresponding PDX models. They discovered valid preclinical models exhibiting distinct FGFR2c oncogenic splice isoform expression patterns. Furthermore, they showed that PDXOs with high FGFR2c expression depend on endogenous FGF2 for autocrine activation. The findings suggested that the FGFR2c splice variant is a prognostic biomarker in EC, paving the way for a Phase II therapeutic trial combining an FGFR inhibitor with immune checkpoint inhibitors in metastatic patients [353]. In another study on CDK4/6 inhibitors, Navarro-Yepes et al. developed various in vitro models of palbociclib-resistant (PR) and abemaciclib-resistant (AR) cell lines. Furthermore, they generated in vivo PDX and ex vivo PDX-derived organoids

from patients who progressed on CDK4/6 inhibitors. Their findings suggest that individuals with HR-positive/HER2-negative metastatic breast cancer who are palbociclib-resistant could remain sensitive to abemaciclib [354]. Taken together, these models will be of notable value for studying patient-specific crosstalks between different patient-derived cells, as it is important in EMT and metastasis research, and to identify patient-optimized individual therapies. Since they are re-translated from animal-based human PDXs into culture, however, they might have their limitations when it comes to studying metastasis in vivo, and in terms of potential cross-contamination with the respective animal-derived stroma.

Humanized mouse models

The disparities between murine and human biology, along with human-specific characteristics of certain drugs and diseases, have led to the adoption of animal models that accurately reflect the human biological system for disease modelling and preclinical evaluation [355]. The humanized PDX model is a cutting-edge conventional in vivo mouse model, exhibiting tumor fidelity and phenotypic consistency comparable to tumor organoids, resulting in similar results across multiple applications. They enable the assessment of metastatic growth in interaction with the local milieu and immune cells at the injection site, and the potential bias from non-humanized mouse (or in general animal) cells is reduced. Furthermore, they promote an assessment of metastasis and dissemination [356, 357]. Bankert et al. reported a simple and reliable method for effectively engrafting tumor and stroma into NOD-scid IL2Rγnull (NSG) mice by intraperitoneally (i.p.) injecting tumor cell aggregates obtained from fresh ovarian tumor biopsy tissues [358]. Several intraperitoneal xenograft models have been observed before, but none have recapitulated the metastatic patterns observed in patients or established the co-engraftment of the non-malignant tumor stroma [359, 360]. The study introduced human ovarian tumor xenografts, demonstrating metastasis patterns comparable to those observed in ovarian cancer patients [358]. To determine whether humanizing tumor-immune interactions will enhance characterization of metastatic prostate cancer and the effects of androgen receptor-targeted and immunotherapies, Kostlan et al. developed prostate cancer mouse xenograft models using huNOG and huNOG-EXL mice with an intact human immunological system. In addition to exhibiting precise responses to standard-of-care hormonal therapies, the results unveiled the first human prostate cancer model with a fully intact human immune system, replicating an immunosuppressive microenvironment responsive to checkpoint inhibition [361]. In a particular study, morcellized human bone, co-implanted into the flank of NOD/SCID mice along with MDA-MB-231 cells or patient primary metastatic breast tumor tissues, developed osteolytic tumors. Four out of ten patient tumor specimens and the cell line both showed metastases from the original implantation site to a secondary cancer-free human bone implant. There was an increase in the number of osteoclasts and osteolytic lesions. The presence of metastatic lesions in host soft tissues, but not in femurs, suggested that the bone tropism of human breast cancers is species-specific, amongst other possible explanations related to the technique as mentioned below [362, 363]. Qiao et al. postulated that bevacizumab and pembrolizumab can prevent tumor growth by triggering activated T-cell infiltration to prevent metastasis and recurrence. To perform preclinical immunotherapy research, they created a human PBMC (Hu-PBMC) in addition to a neoadjuvant mouse model. In this model, combination therapy created a synergistic therapeutic response in neoadjuvant and advanced tumor applications, implicating clinical implementation [364]. A summary of in vivo mouse models employed to investigate cancer metastasis are depicted in Fig. 4.

Site-specific metastasis models

Understanding the mechanisms of site-specific metastasis to particular organs and compartments requires sophisticated, mostly in vivo models that faithfully recapitulate the metastatic cascade and the distinct microenvironments of target organs. Miarka et al. highlighted the pivotal role of PDXs and syngeneic mouse models in modeling brain metastases, emphasizing their utility in exploring the intricate interactions between DTCs and the central nervous system (CNS). These models incorporated advanced techniques such as orthotopic injections into the brain or carotid artery, and longitudinal monitoring using magnetic resonance (MRI) and bioluminescence imaging (BLI) [365]. Additionally, Boire et al. examined the molecular and cellular interactions that promote brain colonization using genetically engineered mouse models. They highlighted how adaptive mechanisms, like immune regulation and astrocyte-mediated support, allowed tumor cells to proliferate in the brain's extremely selective environment [366].

Meanwhile, research on the molecular drivers of metastasis, as demonstrated by Valiente et al., provides critical insights into the regulatory pathways influencing metastatic tropism. By leveraging genetically engineered mouse models and systemic tail vein injection assays, this work elucidated the role of the SRRM4-REST-REST4 axis in enhancing metastatic potential, particularly in lung and liver colonization. Immunohistochemical analyses and gene expression profiling in these models reveal how alternative splicing events contribute to tumor

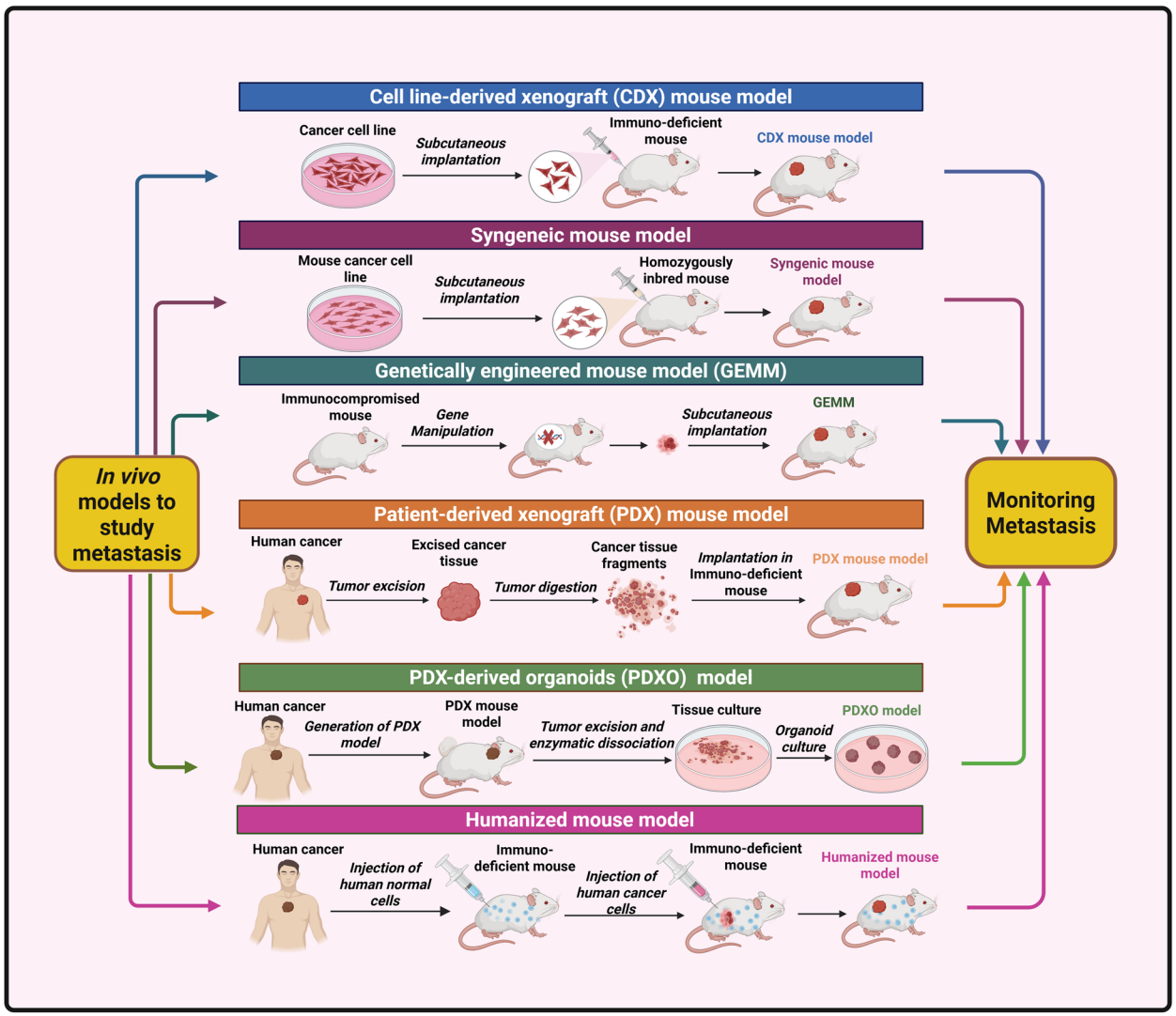

Fig. 4 Schematic illustration of experimental mouse models to study metastasis. Syngeneic mouse models involve spontaneous development of tumors or transplantation of tumors within the same strain. Genetically engineered mouse models have targeted gene mutations which enable tumor development to be monitored from the initial stages. Immunocompromised mice are used for xenotransplantation of human tissues or cells. In the CDX model, a tumor is developed by injecting cells from established tumor cell lines into mice, whereas in the PDX model, the tumor is derived from human tumor tissue, mimicking the human tumor microenvironment. These tumors can be expanded within the same strain, facilitating metastatic studies. In humanized mouse models, the mice are inoculated with human cells (e.g., immune or stromal cells) to study tumor interactions with human normal cells. The PDXO model involves implanting human tumor tissue into mice to create 3D organoid cultures, which mimic the human tumor environment

progression and site-specific dissemination. Valiente et al. also promoted an exciting development of organotropic cell lines, resulting in a panel of brain-targeted cell lines as an experimental resource [367].

In a microfluidic model, cells from breast, bladder, and ovarian malignancies had different affinities for a bone-mimicking environment. Bladder cancer cells had the highest rates of extravasation and migration distance, while ovarian cancer cells had the lowest [368]. Colorectal peritoneal metastasis-derived PDX models and organoids, together with pan-omics, have been developed and employed by the Stein group. For example, a site-specific in vivo metastasis model was successfully applied to evaluate response towards conventional and novel targeted compounds [369].

Contreras-Zárate et al. have developed a PDX-model to investigate breast cancer brain metastases by implanting human metastatic tumor tissues into the brains of immunocompromised mice, to provide a biologically relevant specific platform for this site-specific issue [370]. A

Table 1 Experimental models to study the site-specific metastasis in various cancers

Cancer type	Metastatic site	Models	Reference
Breast cancer	Bone, Lungs, Liver, Brain, Lymph Node	Cell line-derived xenograft model, Syngeneic mouse model, Patient-derived xenograft model, 3D co-culture model	[385–390]
Lung cancer	Brain, Bone	Microfluidic chip model, Patient-derived xenograft model, Cell line-derived xenograft model	[391, 392]
Prostate cancer	Bones, Lung, Kidney	3D scaffold model, Migration Assay, Patient-derived xenograft model, Syngeneic mouse model	[393–396]
Colorectal cancer	Liver, Lung, Lymph Nodes, Peritoneum	Cell line-derived xenograft model, Genetically engineered mouse model, Patient-derived xenograft model, Syngeneic mouse model	[397–401]
Renal cancer	Liver, Lung	Patient-derived xenograft model, Genetically engineered mouse model	[402–405]
Melanoma	Lungs, Brain	Cell line-derived xenograft model, Syngeneic mouse model	[406, 407]
Pancreatic cancer	Liver	Transwell assay, Cell line-derived xenograft model, Patient-derived xenograft model	[408, 409]
Esophageal squamous cell carcinoma	Lung, Lymph Node	Cell line-derived xenograft model	[410]
Hepatocellular carcinoma	Lung	Cell line-derived xenograft model, gelatine zymography, Wound scratch assay, Invasion assay	[411, 412]
Uterine cervix squamous cell carcinoma	Lymph Node	Patient-derived xenograft model, Transwell invasion assay, Wound scratch assay	[413, 414]
Oral cancer	Lymph Node	Transwell migration assay, Syngeneic mouse model	[415, 416]

Overview of various cancer types, prevalent metastatic sites, and experimental models for studying site-specific metastasis, including in vitro and in vivo assays. The table can provide a quick reference to identify available experimental models for metastatic research, giving some reference papers as examples

significant advantage of PDX models lies in their ability to retain the molecular heterogeneity of metastatic lesions [371]. This was evident in a study by Furet et al., where breast cancer brain metastases retained their expression profiles even after multiple passages [372].

Wang et al. demonstrated the utility of brain organoid models in capturing the interactions between metastatic breast cancer cells and the neural microenvironment [373]. In other experiments, cancer cells were co-cultured with organoid systems mimicking the blood–brain barrier (BBB), revealing specific molecular pathways, such S100B upregulation, that mediate metastatic colonization in the brain [374]. Organoid platforms are crucial for identifying therapeutic targets for brain metastasis, and their limitations, including missing systemic factors, can now be addressed through vascularization and immune cell integration. For example, the incorporation of endothelial cells has been shown to replicate BBB functionality more accurately, as demonstrated in recent studies [375]. These innovations are pushing the boundaries of organoid technology, offering increasingly sophisticated models to unravel the complexities of brain metastases.

To mimic liver metastases in pancreatic cancer, researchers use intrasplenic injection methods, whereby, e.g., pancreatic cancer cells are injected into the spleen and subsequently migrate to the liver via the portal vein [376]. Zhang et al. highlighted how such models enabled the identification of metastasis-specific gene signatures, in response to the overexpression of angiopoietin-like 4 (ANGPTL4) [377]. Advances in 3D hepatic organoids have further refined our understanding of tumor-liver cell interactions, especially in drug metabolism and immune evasion [378]. Innovations in lung-on-a-chip technology are enhancing our ability to study metastatic colonization and drug response in the lung microenvironment [379].

Ghanta et al. identified that bone metastasis models for lung cancer often involve intratibial injections, which replicate osteoblastic lesions observed in patients [380]. Prostate cancer shows a strong predilection for the bone, often resulting in osteoblastic lesions. Intratibial and intracardiac injection models have been extensively used to study this process [381–383]. Humanized bone models, where human bone tissue is implanted into mice, offer a unique platform to study human-specific bone metastasis pathways. These models have been pivotal in identifying therapeutic targets such as RANKL inhibitors [384]. The most commonly reported site-specific metastatic models are depicted in Table 1.

Certainly, in the field of site-specific metastasis models, there is still an increasing need to develop more. Besides mouse- or animal-based models, promising potential can

be seen in the in vivo CAM model to a limited extent as discussed above, and still in sophisticated in vitro settings such as with microfluidics. Crippa et al. designed a microfluidic device that enables the independent introduction of human vascularized breast cancer metastatic seeds into a bone-mimicking micro-environment comprised of osteo-differentiated mesenchymal stromal cells and endothelial cells (ECs). The ECs arranged themselves into microvascular networks that connected the bone-mimicking environment to the metastatic seed. This model could provide significant insights into the critical functions of neutrophils in bone metastases, potentially paving the way for the development of novel anti-metastatic treatments that combine traditional chemotherapies with novel immunotherapies [417]. The exciting developments in advanced imaging bear high promise in increasing our prospective abilities to mimic particular metastatic compartments, also in live settings.

Discussion

In this review article, we have tried to comprehensively describe and discuss the techniques and methods available to contribute to our current understanding of EMT and the diverse steps of the metastatic cascade. Each of the aforementioned models have their own advantages and disadvantages, which are listed comprehensively in Table 2.

Certainly, there are still limitations. For example, the wound healing assay is a simple technique to evaluate EMT- and migration-associated parameters like wound area and width, but it is time-consuming and requires accurate quantification [418]. The Boyden chamber cell migration technique, although highly reliable, still faces challenges with quantification [419]. Zymography provides minimum insight into cellular localization and may be impacted by interference from other proteases, potentially lacking alignment with cellular conditions [420]. The microfluidic model is a powerful tool to keep the population of cells in its artificial habitat to study interesting complex aspects of metastasis but requires reprogramming of the niche to study extravasation in a disease model. Its construction needs a fine diameter of microvessels, efficient permeability, eventually a pathological sample and a particular therapeutic in appropriate physiological concentration, the former needing to survive appropriately in culture. If any aspect of the complex experimental setting fails, the accuracy of the results is questionable. Towards this end, cells derived from a patient -iPSC could be a better option to predict treatment for individual diseases [421]. Co-culture models have the potential for studying EMT but have drawbacks like size variation, nutrient supply issues, and cell surface damage when an agitator is used for culturing. The tracking of individual cell types and exchanging media during the spheroid hanging drop method are other issues [170, 422, 423]. PDMCs, including PDOs and PDXs, are useful for drug development but still have limitations like variable correlations with clinical responses [424–426]. CDX is a good model for testing the efficacy of compounds but may not reflect the heterogeneity or natural biological behaviour in situ of patient tumors. This can be due to the selective availability of components for experimentation, inappropriate vascularity, or the host-animal derived microenvironment which may cause epigenetic or other changes leading to a huge difference between the xenograft and the original tumor [427]. Attractive models like the CAM assay are still cell-line or sample-dependent regarding their successful applicability towards particular samples either in general or regarding experimental measures of particular metastatic steps. In some instances, when observations regarding long-term toxicity or side effects of compounds should be studied, its original strength of rendering fast in vivo results can turn into a disadvantage. It also requires experience and, thus might be challenging to some groups due to seemingly complex study requirements [428]. Still, it is one of the only models able to differentiate the important step of intravasation in metastasis. Syngeneic mouse models involve transplanting tumors to the same species. This, to some aspects, strength as compared to heterologous in vivo models faces other challenges like low heterogeneity and inefficient tumor microenvironment. Genetically engineered mice have breeding challenges, and patient-derived xenografts show low implantation rates and limitations due to murine stroma. Humanized patient-derived xenografts have limited immune system reconstitution and short duration, leading to inappropriate immune reconstruction [331]. Further strengths and weaknesses of the particular techniques and models have been discussed in the respective chapters and in Table 2. Therefore, although current models to study EMT and metastasis still might not be fully able to reflect EMT and metastasis as it happens in cancer patients, the various assays discussed here still provide valuable, to some parts encouragingly comprehensive, insights into specific aspects. A graphical summary of the metastatic cascade and the advanced techniques discussed in this review is demonstrated in Fig. 5.

Conclusion and future directions

EMT, or epithelial-to-mesenchymal transition, is an important biological process that can have beneficial and harmful effects on organisms. It plays a crucial role during embryonic development and tissue repair, but its activation in tumor growth leads to negative consequences such as increased mobility, stem cell-like characteristics,

Table 2 Advantages and disadvantages of in vitro and in vivo models to study EMT and metastasis

Type of model	Step of metastasis	Advantages	Disadvantages
In vitro			
Wound Healing Assay	Migration	• Easy to perform • Rapid • Affordable	• Cellular stress and edge retraction • Limited information on invasion • Time consuming
Transwell Migration Assay	Migration	• Easy to perform • Cost-efficient • Uniform multi-well system • Diverse conditions, multiple cell types, or varying assay settings may be included in single plate • Commercially accessible • High response to chemoattractant	• Time consuming • Potential counting bias • Not suitable for large samples • Live/dead cells cannot be distinguished • Risk of cell loss during washing step • Quantification is challenging • Restricted duration performance
Invasion Assay	Invasion	• Large number of settings can be performed with low number of cells and reagents to invest • Cost effective • Quick • Quantifiable	• Difficult counting strategies • Biasness towards proliferation
Gelatin Zymography	ECM degradation	• Easy to use and affordable • High accuracy in identifying MMP activity • Ability to distinguish between active and pro-enzyme forms • Assessment of MMP through qualitative analysis	• Semi-quantitative approach • Minimum insight into cellular localization and may be impacted by interference from other proteases, potentially lacking alignment with cellular conditions
Co-Culture	Invasion, Intravasation	• Physiological relevance • Simple to set up • Easy to control cell concentration • Enables simultaneously study the effects of drug on multiple cell types	• Challenging data interpretation due to the complex interactions among different cell types • One cell type could outgrow or outcompete another (size variation, and nutrient supply issue) • Cell surface damage (when agitator is used for culturing) • Conventional 2D co-cultures might not accurately mimic the 3D architecture of tissues
Scaffold-Based 3D Matrix	Intravasation, Extravasation and Colonization	• Offers more physiologically relevant setting that closely mimics the intricate ECM of a tumor • Drug screening and assessment • Allows for more effective exploration of cell-to-cell interactions, migration, invasion, and the impact of the tumor microenvironment	• Technical complexity in handling • Challenges in choosing scaffold materials that closely replicate the native ECM • Challenges in scaling up for large-scale applications
Spheroid Culture	Invasion, Migration, Intravasation	• Mimics in vivo conditions • Low cost, high effectiveness • Preserves intrinsic phenotypic properties and maintains physical interactions that closely resemble behavior in 3D native tissue	• Variable diameter and size, intense work • The diffusion gradient is affected by the size of factors (such as oxygen, nutrients), and self-disassembly is influenced by the rate of factor generation and consumption
Patient-Derived Organoids Models	Migration, Invasion, Dissemination, Metastasis	• Closely replicates the genetic and phenotypic diversity of a patient's tumor • Enables personalized drug testing and provides a better understanding of tumor heterogeneity	• Intricacy of the culture potential variations among organoids • Constraints in mimicking the entire metastatic cascade within a single in vitro system

Table 2 (continued)

Type of model	Step of metastasis	Advantages	Disadvantages
Microfluidics Model	EMT, Invasion, Intravasation, Extravasation, and Colonization	• Precise control over the microenvironment • Efficient mass transfer provided by fluid flow • The ability to integrate with numerous actuators and sensor systems	• Challenging to standardize and scale up • Require additional pumps, connectors, tubing, and valves to function
In vivo			
Chorioallantoic Membrane Model	Invasion, Intravasation, Angiogenesis, Extravasation, and Metastasis into specific organs of the embryo	• Unique model to study intravasation specifically • Assess different metastatic steps, and response to therapeutics, in vivo in short time • Extremely vascularized environment • Immuno-compromised environment • High survival of cells in the CAM microcirculation following arrest for subsequent extravasation • Ease of access • Affordable	• Cell-line and sample-dependent • (Eventually: Nonspecific inflammatory responses) • Difficult to assess the key role of the immune system • Assay duration is limited to 18 days • Reduced availability of chicken-specific antibodies
Cell Line-Derived Xenograft	Migration, Invasion, Dissemination, Dormancy, Metastasis	• Replicate the tumor microenvironment • The establishment period is short	• Inability to replicate heterogeneity • The original cell properties cannot be maintained • Poor predictive value
Syngeneic Mouse Models	Migration, Invasion, Dissemination, Dormancy, Metastasis	• Fully functional immune system • Enable the evaluation of the tumor microenvironment • Genetically identical but still exhibit some level of genetic diversity	• May not accurately reflect the diversity and complexity of human malignancies • May not precisely mimic human immunological responses • Low heterogeneity and inefficient tumor microenvironment
Genetically Engineered Mouse Models	Migration, Invasion, Dissemination, Dormancy, Metastasis	• Immunocompetent host • Fixed genetic background • Tumors develop in the tissue of origin, typically due to clinically relevant mutations	• Breeding challenges • Limited metastatic spread • Inability to replicate heterogeneity of tumor microenvironment • Poor predictive value • Lengthy establishment process
Patient-Derived Xenograft Models	Migration, Invasion, Dissemination, Dormancy, Metastasis	• Replicates tumor microenvironment • Maintains genetic and histologic characteristics • Higher predictive value	• Possible loss of tumor heterogeneity following transplantation • Difficulties in replicating intricate immunological responses • The time and cost of developing these models
PDX-Derived Organoids	Migration, Invasion, Dissemination, Dormancy, Metastasis	• Affordable and quicker • Maintains tumor characteristics • Allows for frequent sampling from patients • Potentially predict the patient's response to therapy	• More expensive than two-dimensional culture • Time-consuming • Difficulty in assessing early EMT events • Expensive and resource-intensive • Low implantation rates

Table 2 (continued)

Type of model	Step of metastasis	Advantages	Disadvantages
Humanized Mouse Models	Migration, Invasion, Dissemination, Dormancy, Metastasis	• Mimics the human immune response in mice • Robust platform for evaluating immunotherapies and other anti-metastatic treatments	• Extended mouse humanization processes • Expensive and technically challenging • Interactions between human cells and the mouse microenvironment can lead to artifacts • Shorter lifespan of mice can restrict long-term metastasis studies

Summary of the benefits and drawbacks of several in vitro and in vivo models that are employed in the study on EMT and metastasis. The table includes in vitro and in vivo experimental models discussed in the review. Each model's advantages and disadvantages are highlighted to help researchers determine the best model for their purposes

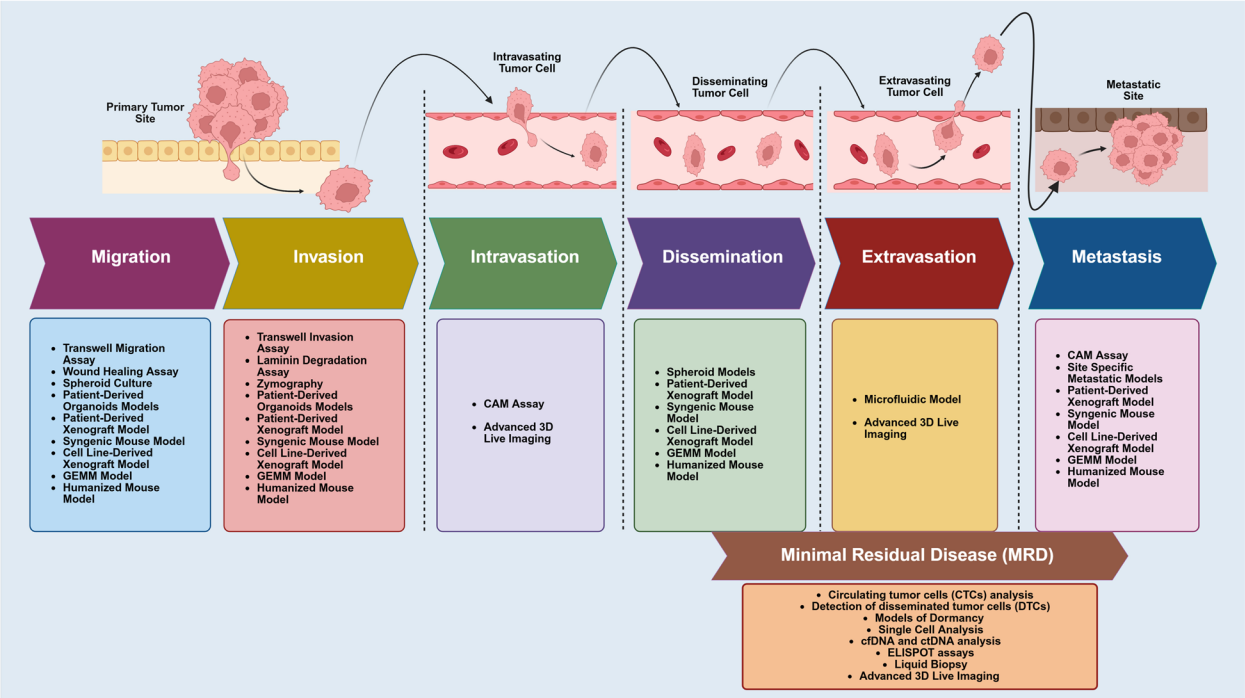

Fig. 5 Comprehensive overview of the metastatic cascade and techniques that can be applied at each stage. Schematic overview of key in vitro and in vitro applications and animal models to investigate individual steps of the cascade of metastasis

contributions to diverse steps of the metastatic cascade, and resistance to therapy in various tissues. Different cancer types display varying molecular expression patterns and biological behavior in the context of EMT, indicating that EMT operates on a spectrum rather than as a strict binary process. Tumor cells often present a partial EMT phenotype with mixed expressions of epithelial and mesenchymal markers, highlighting that tracking a single gene expression only may not fully capture the complexity of EMT [429]. While EMT facilitates certain processes necessary for tumor infiltration, it is not sufficient on its own for metastatic colonization, which also requires the reverse process, MET, to restore the epithelial state in distant organs, besides of course many further factors, some of them mentioned in this review and others [10]. The role of EMT-related molecules varies across tissues and cancer types, which makes it essential to understand precisely how EMT, and other essential molecules, function in individual tumors for effective treatment strategies.

Cancer stem cells (CSCs) are influenced by EMT, placing tumor cells into an intermediate state that enhances their resistance to chemotherapy and ability to regenerate tumors, which can result in relapse. Mechanisms for this resistance include increased drug expulsion and evasion of death signals. EMT also fosters an immunosuppressive environment by increasing the presence of immunosuppressive cells around tumors, with specific factors like SNAIL and ZEB1 driving this accumulation, which can affect the efficacy of immunotherapy [430]. The interactions between EMT and immune cells create a cycle that promotes cancer progression, suggesting that combining immunotherapy with immune checkpoint inhibitors might be effective. Additional efforts are focused on preventing the expulsion of anticancer drugs from cancer cells, particularly by investigating ABC transporter inhibitors [431]. Changes in the epigenetic architecture of tumor cells are thought to contribute to drug resistance, which could be reversed to overcome it. Integrating multi-omics and single-cell sequencing technologies into clinical trials can provide valuable insights into treatment responses and metastasis mechanisms. Shared signaling pathways between cancer metastasis and drug resistance offer novel opportunities for anticancer treatments. Still, of course, such –omics attempts remain at the descriptive level and thus, having a powerful arsenal of methodology at hand that can investigate essential aspects of EMT and metastasis at the functional level is crucial for our future progress in the understanding, diagnosis, therapy, and prevention of these critical aspects of cancer disease.

In this review, we have addressed several techniques to comprehend EMT and the different steps of the metastatic cascade, which in combination can still be powerful

despite some limitations as we have discussed. Personalized cancer management aims to tailor interventions and minimize drug resistance, and we have discussed the respective models in this context as well. For some of them, issues like the high number of required drug combinations with increasing individualization of therapy might pose a challenge. Further technical advances are anticipated to still improve experimental settings. For example, microfluidic devices will be automated soon to improve routine predictability of personalized treatment outcomes; in addition, they can mimic additional factors like mechanical forces as exerted in physiological conditions. Artificial intelligence and nanobots promise to enhance drug screenings and precise treatment delivery. The use of extracellular vesicles (EVs), particularly exosomes, for drug delivery is gaining attention due to their low toxicity, high compatibility, and potential for targeted delivery. Advancements through intensified research of different variations of PDX and PDXO models, involving transplanting patient-derived tumor cells and organoid generation, can further aid drug development and individualized treatment recommendations as preclinical study settings which could run in parallel to clinical trials. Further advancements in imaging platforms will facilitate the visualization of intricate cellular interactions at the molecular level, more and more in live-cell mode, thereby still better understanding the landscape of metastatically relevant compartments. Improving, and increasing the availability of, models resembling MRD, dormancy, and site-specific metastasis, intensifying our collective efforts to delineate the essential molecular and biological characteristics of metastatically relevant cells, and harmonizing them with ongoing extensive -omics-based, single-cell sequencing, and spatial-omics approaches, has a huge potential to improve the existing diagnostic and biomarker development in the clinical setting. Integrating all of these approaches will have an exciting potential, not only for identifying novel diagnostic biomarkers and therapeutic targets, but also for generating more specific anti-metastatic therapies. It should ideally help us to get "ahead of the wave", into a future situation when we can prevent metastases before we observe them in a patient.

Abbreviations

ANGPTL4	Angiopoietin-like 4
AR	Abemaciclib-resistant
B7-H4	B7 homology 4
BBB	Blood–brain Barrier
BLI	Bioluminescence imaging
BME	Basement membrane extract
CAM	Chorioallantoic membrane
CCSC	Colon cancer stem cell
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CNS	Central nervous system
CRC	Colorectal Cancer
CSCs	Cancer stem cells
CTCs	Circulating tumor cells
ctDNA	Circulating tumor DNA
CTX	Crotoxin
DHT	Dihydrotestosterone
DTC	Disseminated tumor cells
EC	Endothelial cells
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
EPCAM	Epithelial cell adhesion molecule
EPIDROP	EPISPOT in a drop
EPISPOT	Epithelial Immuno SPOT
FGF	Fibroblast growth factor
FLIM	Fluorescent lifetime imaging microscopy
GEMMs	Germline genetically engineered mouse models
HER2	Human epidermal growth factor receptor 2
HSPG	Heparan sulfate proteoglycan
PBMC	Peripheral blood mononuclear cell
I.P.	Intraperitoneal
iCAFs	Inflammatory-like Cancer-associated fibroblasts
LCSCs	Liver cancer stem cells
MET	Mesenchymal-to-epithelial transition
MMPs	Matrix metalloproteinases
MNX	Mitochondrial-nuclear exchange
MPFM	Multiphoton fluorescence microscopy
MRD	Minimal residual disease
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
mtDNA	Mitochondrial DNA
NFκB	Nuclear Factor κB
NSG mice	NOD-scid IL2Rγnull mice
OC	Ovarian cancer
PD	Pharmacodynamics
PDMCs	Patient-derived models of cancer
PDO	Patient-derived organoid
PDX	Patient-derived Xenograft
PDXO	PDX-derived organoid
PK	Pharmacokinetics
PR	Palbociclib-resistant
ROS	Reactive oxygen species
SCM	Senescence-conditioned media
scRNA-seq	Single-cell RNA sequencing
SINES	Short interspersed repeated DNA elements
TAMs	Tumor-associated macrophages
TESC	Tescalcin
TGF-β	Transforming growth factor-beta
THG microscopy	Third harmonic generation (THG) microscopy
TMCD	Tissue Mimicking Co-culture Device
TNBC	Triple Negative Breast Cancer
u-PA	Urokinase type plasminogen activator
u-PAR	U-PA receptor
ZEB1	Zinc-finger E-box-binding homeobox 1

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Authors' contributions

HA identified the review topic and defined the structure of the review. HA and GCK did the main part of the writing. SM, BM, BS, SS, SK, KK, VKP, DM, NSP, JHL, HA and GCK participated in the writing of the review and the design of the figures. All of the authors have reviewed and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare no competing interests.

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