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# Evolution of Metastasis Study Models toward Metastasis-On-A-Chip: The Ultimate Model?

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**For decades, several attempts have been made to obtain a mimetic model for the study of metastasis, the reason of most of deaths caused by cancer, in order to solve the unknown phenomena surrounding this disease. To better understand this cellular dissemination process, more realistic models are needed that are capable of faithfully recreating the entire and essential tumor microenvironment (TME). Thus, new tools known as tumor-on-a-chip and metastasis-on-a-chip have been recently proposed. These tools incorporate microfluidic systems and small culture chambers where TME can be faithfully modeled thanks to 3D bioprinting. In this work, a literature review has been developed about the different phases of metastasis, the remaining unknowns and the use of new models to study this disease. The aim is to provide a global vision of the current panorama and the great potential that these systems have for in vitro translational research on the molecular basis of the pathology. In addition, these models will allow progress toward a personalized medicine, generating chips from patient samples that mimic the original tumor and the metastatic process to perform a precise pharmacological screening by establishing the most appropriate treatment protocol.**

## 1. Introduction

Non-communicable diseases (NCDs) are currently responsible for the majority of deaths worldwide, and cancer is expected to be the leading cause of death and the most significant barrier to increased life expectancy in all countries of the world in the 21st century. The World Health Organization (WHO) estimates for 2018 that cancer was the leading or second leading cause of death before age 70 in 91 of 172 countries, and it is the third or fourth leading cause of death in 22 other countries with ≈18 million new cases and 9.5 million deaths worldwide.[1,2]

Concerning these cancer-related deaths, ≈90% are not associated with primary tumors, but with secondary ones originated by a metastatic process,[3] a dynamic, systemic, and poorly understood process where primary tumor cells travel through the blood and lymphatic vessels to healthy tissues where these malignant cells are implanted to develop a secondary tumor. This process and its formation are inherently inefficient, in the sense that only a low percentage of cells can complete the metastatic process.[4] When successful, the cancer usually becomes incurable.[5] The cells heavily involved in this process are cancer stem cells (CSCs), a class of pluripotent cancer cells that behave analogously to normal stem cells in their ability to differentiate into the spectrum of cell types seen in tumors.[6] Recent studies defends that CSCs are immortal tumor-initiating cells that can self-renew and have pluripotent capacity. Due to these characteristics, CSCs are thought to be the basis for tumor initiation, development, metastasis, and recurrence.[7] In this way, a cancer cell from the primary tumor performs the following steps to produce metastasis: i) locally invades the surrounding tissues; ii) changes phenotype and cell–cell and extracellular matrix-cell (ECM-cell)

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adhesion through the epithelial-mesenchymal transition (EMT), process that confers multiple malignant features associated with the loss of epithelial properties and the acquisition instead of certain mesenchymal characteristics necessary to the metastatic process;[8] iii) enters in the microvasculature of the blood and lymphatic system (intravasation); iv) survives far from its tissue in the bloodstream and lymph; v) translocates through the blood system to the microvasculature of a distant tissue (extravasation); and finally vi) invades the new tissue where it will proliferate and form the secondary tumor (colonization).[5,9]

For years, attempts have been made to generate in vitro cancer study models without achieving a mimetic model, due to the cancer is a very complex and multifactorial disease in which the microenvironment plays an essential role. The conventional 2D in vitro models are not capable of modeling the 3D TME, which is so fundamental for metastatic processes.[10] In this sense, and for a better study, rudimentary 3D models began to be generated, such as the use of cellular spheroids and hydrogels,[11] to later move on to more complex techniques such as bioprinting, capable of organizing cells in a spatially precise manner.[12] Thus, transendothelial migration assays of tumor cells can be performed, using cancer cell spheroids for example, which more accurately model cell–cell and cell-ECM interactions between tumor cells and the surrounding tissue microenvironment, and endothelial cells that act as a barrier.[13] 3D in vitro tumor models present a great advantage over animal models which are not optimal for quick studies and high throughput scenarios, because of the often-long experimental time courses and the difficulty of scaling study sizes, and are not necessarily predictive of outcomes in humans.[8]

To develop 3D tumor models, bioprinting makes possible to create a 3D multicellular construction by adding layer by layer a cell-laden biomaterial called bioink. These bioinks can be biocompatible hydrogels or liquids that solidify through chemical or physical procedures, resulting in hydrogels too.[14] Among all the possibilities, the use of hydrogels such as alginate, gelatin methacryloyl (GelMA), collagen or Poly(ethylene glycol) diacrylate (PEGDA), is the most optimal option to produce multicellular constructs.[15] To control the architecture of these microgels with great precision, multiple types of bioprinting could be chosen.[16] All of them can be used in combination with organ-on-a-chip, which have developed and evolved rapidly over the last decade.

These organ-on-a-chip technologies use hydrogels or organoids generated from human cells with functional and dynamic microvascular networks, which can recreate highly promising human TME as a new resource for cost-effective and high throughput analysis of cancer drugs.[17] The platforms on which these hydrogels rest are generally made of polydimethylsiloxane (PDMS), which is optically transparent and allows simple and reproducible observation at cellular and subcellular levels, without disturbing the tissue microenvironment. Moreover, the pressure and fluid flow conditions on these platforms are easily controlled, making it possible to recreate the characteristics of the human vascularized tissue microenvironment and to closely observe cellular behavior under precise conditions.[18]

The present work provides a new vision about the still paradigms of metastasis, showing all of them as well as the evolution of the development of new study models to solve the unresolved biological processes of this complex pathology. Special emphasis will be placed on the new tumor-on-a-chip models that involve the use of 3D bioprinting along with these new technologies. In this way, a new perspective will be addressed to understand how future models will be developed, in combination with the current ones, which will allow to finally understand the metastatic processes and to establish a precise pharmacological screening with a small sample of the original tumor to implement a personalized medicine adapted to each patient.

## 2. Biological Steps Related to Metastatic Process

Over 90% of cancer-related deaths are due to metastasis. Once produced, metastasis is generally uncontrollable, and only in very early and localized stages the treatment is effective. The metastatic process is multifactorial involving genetic, epigenetic, and microenvironmental factors in both the primary tumor and the organs that receive the metastatic cells.[19] In addition, in the TME there are a large number of cells that are naturally found in tissues such as T cells, macrophages, dendritic cells, B cells, fibroblasts, or neutrophils (**Figure 1**), accompanying the tumor cells which in turn present different genetic alterations, allowing them to overcome physical limits, spread and colonize a distant organ.[20] During this process, the primary tumor cells must acquire the ability to migrate and establish themselves in distant organs, a process in which there is a continuous evolution and phenotype selection of tumor cells capable of surviving all phases of the process, culminating in a metastatic phenotype.[21] In this way, metastasis is a succession of these individual steps,[22,23] in which metastatic cells are rare clones that appear in a very small proportion in the primary tumor. In animal models, 0.01% or less of the cancer cells that enter the circulation become metastatic cells.[24] The intrinsic genomic instability presents in tumor cells, evident in the chromosomal gains, losses, and rearrangements associated with cancer, increases the frequency of alterations necessary to acquire this metastatic capability. In this way, DNA integrity is compromised by aberrant cell cycle progression, telomeric crisis, inactivation of DNA repair genes, and alteration of epigenetic control mechanisms.[22–24]

Besides, it has been determined that interactions with the TME assist in the selection of a highly aggressive phenotype, with survival capacity and capable of evading the immune response.[25] Subsequently, metastasis will be addressed from the different biological processes that occur, such as origin, EMT process, premetastatic niche formation, or tumor colonization.

### 2.1. Local Invasion and Origin of Cellular Heterogeneity

Local invasion involves the entry of cancer cells of the primary tumor into the stroma associated with the surrounding tumor and then into the adjacent parenchyma of normal tissue. To invade the stroma, tumor cells must first rupture the basement membrane (BM), a specialized ECM that plays a major role in organizing epithelial tissues, in part by separating their epithelial and stromal compartments.[26] In addition to the

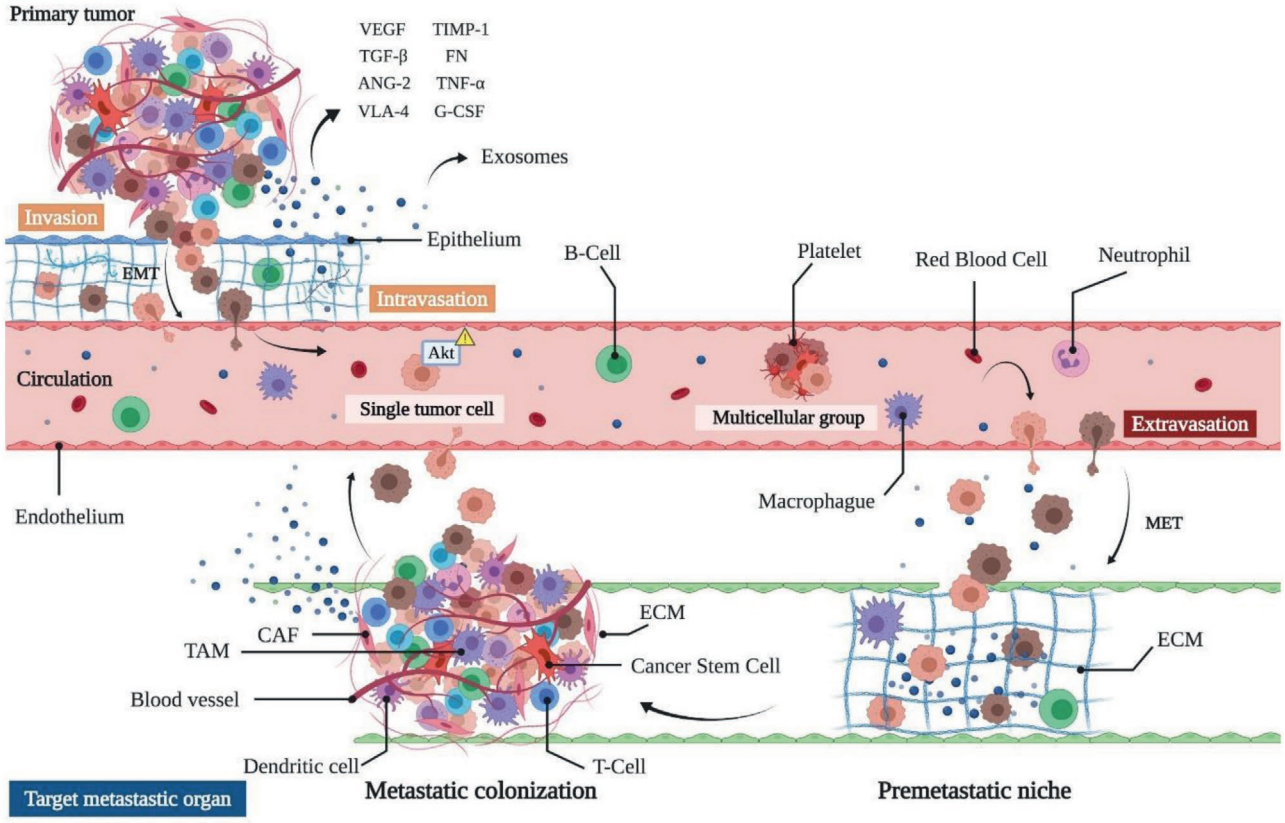


**Figure 1.** Graphic representation of the metastatic process. The primary tumor produces local invasion through the epithelium of the tumor organ, managing to intravasate the blood and lymphatic vessels to initiate the metastatic process. Tumor cells, individually or in groups together with the presence of platelets, start their journey through the bloodstream and lymph, where their survival due to different molecular processes, among which the continuous activation of Akt stands out. Once the target organ is reached, already prepared thanks to TDSF and the exosomes released primary tumor cells forming the premetastatic niche, colonization occurs to trigger another metastatic process.

structural roles played by the BM, the components of this ECM contain a reservoir of growth factor, such as tumor-derived colony-stimulating factor 1 (CSF-1) and macrophage-derived epidermal growth factor (EGF).[27–29] These factors are secreted by tumor-associated macrophages (TAMs) along with cancer-associated fibroblasts (CAFs),[30] and facilitate tumor cell invasion through a paracrine signaling loop.[31] These cells are also the major source of proteases, such as cysteine cathepsins[32,33] and metalloproteinases,[30] which are capable of breaking down the proteins in the ECM. Specifically, TAMs facilitate angiogenesis and the breakdown and remodeling of the ECM, as well as promoting tumor cell motility. Recent studies reveal that direct communication between macrophages and tumor cells leads to the invasion and exit of tumor cells into the blood vessels (intravasation).[28] Thus, different tumor-derived signals seem to recruit a subset of monocytes that express a marker not normally found in this type of cell and which is restricted to endothelial cells, tyrosine-protein kinase receptor Tie2, and which are responsible for neoangiogenesis. Signals for this macrophage recruitment include, among others, hypoxia, which induces hypoxia-inducible factors transcription factors in these cells. The main targets of this transcription factors include genes for many angiogenic factors, such as vascular endothelial growth factor (VEGF), whose action improves angiogenesis in these avascular areas.[20] On the other hand, CAFs, as an abundant and active population of stromal cells in EMT, function as the signaling center and the remodeling machine to help create a desmoplastic tumor niche.[30] CAFs are fibroblasts found in the stroma of human cancers, but they differ from normal fibroblasts in their increased production of collagen and ECM proteins[34,35] and in their increased secretion of pro-angiogenic factors, such as fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor A (VEGFA) promoting tumor proliferation.[36]

In addition to these cells, the BM also plays a crucial role in signal transduction events within the carcinoma through pathways initiated by integrin-mediated adhesions of the cellular matrix. There proteins are the main receptors of cell adhesion. Through multifaceted functions such as signaling molecules, mechanotransducers and key components of the cell migration machinery, such proteins are involved in most steps of cancer progression, from the development of a primary tumor to metastasis, so that the alterations they cause are reflected in cell polarity, proliferation, invasion, and survival of the cells.[37,38]

Therefore, the invasion is a well-determined and well-known process, although questions still arise. There are different cell lineages that have an intrinsic capacity for migration. Would

this capacity help certain cell lineages in the invasion process, as well as in the subsequent metastatic progression after the acquisition of the tumor genotype and phenotype? The answer to this question could, in part, explain the high aggressiveness and high metastatic capacity of certain types of tumors. Furthermore, this could be solved by developing more realistic models that would allow the faithful recreation of the human TME, as it happens with decellularized matrix hydrogels, which will be discussed later.

### 2.2. Physical Translocation and Spread of the Cancer Cells: EMT Process

Cancer cells must acquire the ability to migrate and invade with the aim to separate from the primary tumor and begin the process of metastasis. These skills allow CSCs, to degrade and move through the ECM of the surrounding tissue into the blood and lymph vessels, pathways to distant secondary sites thanks to its higher tumor initiating capacity.[39] A central question, not yet addressed, is whether this acquisition of malignant traits, which involves physical translocation and spread of the cancer cells, occurs as an almost inevitable consequence of primary tumor progression or as an accidental and therefore arbitrary product of the tumor.[40] A widely accepted, but as yet unproven model of primary tumor formation, postulates that cancer cells acquire a sequence of genetic and epigenetic alterations, each of which confers one or another phenotypic form for each cell type. Thus, only one of these alterations can trigger a clonal expansion of the cells that have acquired it, leading to a succession of clonal expansions that resemble a pattern of Darwinian evolution, essential for cell migration.[9,41]

A key event in promoting tumor cell migration and invasion is the EMT.[42] In the last 3 decades, developmental biologists have defined the existence of this cellular biological program, which plays a critical role in early embryonic morphogenesis.[43] This EMT program also plays a fundamental role in tumor processes, conferring on epithelial cells, both normal and neoplastic, properties that are critical for invasion and metastatic spread, considerably increasing mobility, invasiveness and the ability to degrade the components of the ECM.[44] EMT is a group of cellular biological programs that share common characteristics but differ in certain critical details, depending on the site of the tissue, the degree of malignancy and the contextual signals experienced by the individual neoplastic cells. These complex programs are orchestrated and coordinated by a series of master transcription factors that induce EMT (EMT-TFs), including Snail, Slug, Twist, and Zeb.[45,46]

Although it is clear that EMT process is involved in metastatic events in cancer, its involvement in other events may also be highly relevant to tumor progression:[43] i) it causes resistance to cell death and senescence, through the expression of Twist Family BHLH Transcription Factor 1 and 2 (Twist1 and Twist2), which prevent cells from experiencing oncogene induced senescence by inhibiting p16/ink4a and p21/cip;[47] (ii) it provides resistance to chemotherapy and immunotherapy, thanks to the transcription factor Snail, which confers resistance to paclitaxel, adriamycin, to radiation by antagonizing p53-mediated apoptosis[48] and dendritic cell-based immunotherapy;[49] iii) it promotes immune survival and immunosuppression, by inducing immune tolerance and phenotypic changes through immunoedition (tumor selection process where cancer cells overcome immune restrictions during the cancer's dormant period);[50,51] and iv) it provides stem cell characteristics, which induces the change from E-cadherin to N-cadherin, developing less cell adhesion, as well as the expression of Snail, vimentin, and metalloproteases,[43] which favor cell polarization and remodeling.

As it can be seen, the role of the EMT phenomenon is important, since it helps the cell dissemination thanks to several complex molecular processes. However, as we have commented before, the intrinsic characteristics of the cells could be sufficient for the cell migration, depending on the cell lineage, which would put in doubt the total need of this process. Furthermore, the loss of cell adhesion generated by this process highly compromises certain theories of cell migration that occur during metastasis, as we will see below. Molecular genetic studies in models that specifically simulate human TME may reveal how this EMT occurs, and how the TME influences in the entire metastatic process.

### 2.3. Circulation of Tumor Cells in Search of a Target Organ

Individual invasive carcinoma cells and invasive cohorts arising from primary tumors may invade the blood and lymphatic vasculature of adjacent normal tissues, or the neovasculature that has been assembled within the tumors themselves by the process of angiogenesis. The resulting intravasation provides access to a pathway for circulating tumor cells (CTCs) to travel to distant sites, where they can seed new metastatic colonies.[52] These travelers can move as single cells or as multicellular groups (Figure 1) with platelets,[53] that can persist in the circulation to the small-caliber microvessels of distant tissues (often with luminal diameters as small as 8 μm). The resulting physical entrapment would seem to ensure that the vast majority of intravascular CTCs remain in the general circulation for only seconds or minutes after their initial entry into the vasculature. Although most CTCs can be rapidly eliminated, it has recently been reported that even groups of CTCs are capable of maneuvering through capillary-sized vessels, making them like a single-cell chain held together by adhesive interactions.[8,54]

Animal models suggest that less than 0.01% of CTCs survive and are capable of forming metastases, and for this reason they need molecular mechanisms that allow them to survive away from the primary tumor.[55] Activation of protein kinase B (AKT) signaling plays an essential role in the survival of metastatic tumor cells at multiple anatomical sites. In circulation, the tropomyosin receptor kinase B (TrkB) (also called NTRK2) inhibits tumor cell anoikis, a programmed cell death induced by the detachment of cells from the ECM,[56] by activating the phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K)-AKT54 pathways,[57] whereas in the bone marrow, the SRC kinase mediates AKT signaling in response to bone-specific factors.[58] In pulmonary metastasis, macrophage binding to tumor cells via vascular cell adhesion molecule 1 (VCAM1) and integrin b4 triggers AKT56 signaling for prosurvival.[59] It should be noted that AKT phosphorylation in Ser473 can be detected in

BM in lung cancer patients, and in vitro functional analysis supports an important role of AKT1 and AKT3 in proliferation and survival, thus highlighting the importance of this enzyme and suggesting a blocking strategy for future antimetastatic treatments.[60]

Therefore, many genes and molecular events are involved in cell survival. However, not only the intrinsic processes to the tumor cell would play a fundamental role. External factors could also contribute to the future of the metastatic cell. What influence do the circulation patterns and the areas through which the metastatic cell travels have? Not all cells travel through the same circulatory pattern or interact with the same organs, and perhaps this interaction could determine the survival of the migrating cells as well as the organ they will subsequently colonize. The development of more realistic models that comprise complex and functional vascular networks will allow a more in-depth study of tumor cells survival in the bloodstream.

### 2.4. Premetastatic Niche: A Guide to CTCs

A key step in metastasis is the entry of the CTCs into secondary sites or distant from the primary organ, in which the tumor appeared to become disseminated tumor cells for subsequent metastasis; however, this step is critically affected by the local microenvironment of the target organ, which determines whether colonization of the tumor cells can occur. However, primary tumors can "prepare" the local microenvironment of distant organs for colonization by tumor cells even before their arrival (Figure 1).[61] Tumor cells, either at the primary sites or in the circulation, may release soluble factors or extracellular microvesicles[62–64] to convert the incipient metastatic sites into compatible "premetastatic niches" (PMNs).[55,65]

The premetastatic niche can be defined as a supportive and receptive tissue microenvironment that undergoes a series of molecular and cellular changes to form the fertile "soil" for metastasis that is ready for the colonization of tumor cell, the "seeds". Thus, it supports the settlement of the tumor in the distant organ and promotes tumor metastasis.[66] The primary tumor-derived components, tumor-mobilized bone marrow-derived cells (BMDC) and the local stromal microenvironment of the host organ are the three main factors crucial to the formation of the PMN. Many molecular and cellular components have been identified that contribute to the formation of the PMN in different tumor models allowing the recruitment of different cell types for the establishment of the TME (**Tables 1** and **2**). These niche-promoting molecular components, in addition to being secreted by tumor cells, can also be produced by myeloid cells and stromal cells, working together with the cellular components to initiate, polarize, and establish a PMN in future metastatic organs.[61]

Soluble molecules secreted by the primary tumor play critical roles in preparing distant sites for de novo PMN, thus promoting metastasis and even determining metastatic organotropism. These tumor-derived primary molecular components include tumor-derived secreted factors (TDSF), extracellular vesicles (EV), and other molecular components.[61] Several TDSF have been shown to promote the formation of PMNs by mobilizing and recruiting myeloid cells directly from the bone marrow into the premetastatic niche.[62] For example, vascular endothelial growth factor (VEGF)-A and placental growth factor (PlGF), derived from a primary tumor, mobilize and recruit VEGFR1+VLA-4+ BMDCs to rich premetastatic sites in the lung (Table 1).[66] TDSF, such as tumor necrosis factor $\alpha$ (TNF-$\alpha$) and transforming growth factor $\beta$ (TGF-$\beta$), together with VEGF-A, induce the expression of S100A8 and S100A9 in the lung to develop PMNs (Table 1).[70] Moreover, hypoxia induces the expression of some factors, such as granulocyte colony-stimulating factor (G-CSF), which can initiate and regulate pre-metastatic niche formation.[74]

Tumor-derived EVs potentially travel far from their original site to act as potential mediators by educating the target organ to generate the pre-metastatic niche. Thus, EVs can be grouped into three broad categories: exosomes (30–100 nm in diameter), microvesicles (100–1000 nm in diameter), and a recently identified population of cancer-derived EVs called "'large oncosomes'" (1–10 mm in diameter).[75] The first to be mentioned, exosomes, are nano-sized vesicles (30–150 nm in diameter) that are secreted by most cells. They are enclosed by a lipid bilayer and carry various biomolecules, including proteins, glycans, lipids, metabolites, RNA, and DNA (Table 2).[76] Exosomes released from cancer cells contain a number of proteins (Table 2) including oncogenic proteins, integrins, and signaling molecules; some are shared between different cell types, while others are uniquely packaged, reflecting the cell of origin.

Exosomes can also contain various RNA types (Table 2). Most researches to this effect showed that microRNAs (miRNAs) and non-coding RNAs were the predominant RNA species transported by exosomes; however, messenger RNA (mRNA), ribosomal RNA (rRNA), and transfer RNA (tRNA) were also reported.[80] Exosomal RNA-mediated communication greatly influences the education of all cells that conforms the TME, not only in the target organs of metastasis, but also in the primary tumor, playing a key role and, therefore, presenting a high potential as biomarkers of cancer.[85,86] Even CSCs can secrete these exosomes; however, it is not yet well known but it has been seen how this exosomal release is able to induce the EMT process in adjacent cells[87] or even induce immunosuppression.[88]

With all this, it could be thought that different types of tumors have a metastatic predisposition for another specific type of organ. The characteristics of organotropism may be innately related to the PMN since certain types of cancer have a predisposition to metastasize to specific organs with a selective microenvironment. The primary tumor prepares organ-specific sites for metastasis by secreting TDSF to change adhesion molecules and ECM components in these secondary organs.[61]

Due to the great complexity of this sequential multistep process, new questions are constantly emerging. Previously, the possible influence of circulation patterns on the survival of metastatic cells has been discussed. With this, it would also be logical to think that these patterns could influence both the formation of the premetastatic niche and future extravasation. Future studies and the development of models simulating the connection between organs will bring to light the behaviors that surely contribute to the metastatic process.

**Table 1.** Molecular and cellular components promoting pre-metastatic niche formation.

| Source | Molecule | Niche-promoting cells/cell target | Mechanisms | Primary tumor | Target | Refs. |
|---|---|---|---|---|---|---|
| Tumor-derived | VEGF and PlGF | VEGFR1+ HPCs | Recruits VEGFR1+ BMDCs | Lewis lung carcinoma and melanoma | Lung | [66] |
| | TIMP-1 | Neutrophils | Drives cancer cell homing to the liver | Colon cancer and breast carcinoma | Liver | [67] |
| | CXCR4 | SDF1-expressing cells | Recruitment of SDF1-expressing cells | Breast cancer | Lymph nodes, bone marrow, lung and liver | [68] |
| | FN | DPP4-expressing endothelial cells | Recruitment of DPP4-expressing endothelial cells | Breast cancer | Lung | [69] |
| | TGF-$\beta$ | CD11b+/Mac1+ myeloid cells; CD11b+/Gr-1+ myeloid cells | S100A8/S100A9, SAA3 expression to develop an inflammatory PMN; remodels lung parenchyma for PMN formation | Lewis lung carcinoma and melanoma | Lung | [70] |
| | CD15 | Endothelial cell | Prepare the PMN | Non-small-cell lung cancer | Brain | [71] |
| Stroma-derived | ANG-2 | CCR2+Tie2- macrophages | Recruits macrophages and induces inflammatory, angiogenic response of endothelial cells | Breast carcinoma and Lewis lung carcinoma | Undefined | [72] |
| | miR-19a | IBA1-expressing myeloid cells | Silences PTEN increasing CCL2 to recruit myeloid cells into the PMN | Breast carcinoma and melanoma | Brain | [73] |
| | G-CSF | Ly6G+/Ly6C+ granulocytes | Mobilizes MDSCs niche and promotes metastatic cancer cell seeding | Breast carcinoma; Lewis lung carcinoma and melanoma | Lung | [74] |
| | TNF-$\alpha$ | CD11b+/Mac1+ myeloid cells | Induces lung expression of S100A8/S100A9, SAA3 and remodels lung parenchyma for PMN formation | Lewis lung carcinoma and melanoma | Lung | [70] |
| BMDC-derived | Id3 | VEGFR1+ HPCs | Facilitates the mobilization of VEGFR1+ cells to the PMN | Lewis lung carcinoma and melanoma | Lung | [66] |
| | VLA-4 | VEGFR1+ HPCs | Allows adhesion of the BMDCs in the pre-metastatic to provide a permissive PMN | Lewis lung carcinoma and melanoma | Lung | [66] |

VEGF, vascular endothelial growth factor; VEGFR-1, vascular endothelial growth factor receptor 1; HPC, hematopoietic progenitor cell; PIGF, placental growth factor; TIMP-1, TIMP metallopeptidase inhibitor 1; CXCR-4, C-X-C chemokine receptor type 4; SDF-1, stromal cell-derived factor 1; FN, fibronectin; DPP4, dipeptidyl peptidase 4 (DPP4); TGF-$\beta$, transforming growth factor; CD11b, cluster of differentiation 11b; Mac1, macrophage-1 antigen; Gr1, granulocyte receptor 1; S100A8/S100A9, S100 calcium-binding protein A8/A9; SAA3, serum amyloid A 3; CD15, cluster of differentiation 15; ANG-2, angiopoietin-2; CCR2, C-C chemokine receptor type 2; Tie-2, tyrosine-protein kinase receptor for angiopoietins 2; miR-19a, microribonucleic acid 19a; IBA1, ionized calcium-binding adapter molecule 1; PTEN, phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase; CCL2, C-C motif chemokine ligand 2; G-CSF, granulocyte colony-stimulating factor;Ly6G, lymphocyte antigen 6 complex locus G6D; Ly6C, lymphocyte antigen 6 complex locus C6D; TNF-$\alpha$, tumor necrosis factor $\alpha$; Id3, DNA-binding protein inhibitor; VLA-4, very late antigen-4.

### 2.5. Colonization and Adaptation of CTCs in the Target Organ

For CTCs to be successful in colonizing a new target organ, an extravasation process is necessary, where the reversal of the EMT process plays a very important role. In this way, the mesenchymal-epithelial transition (MET) process occurs acquiring tumor cells again an epithelial phenotype, which is a more sessile and proliferative phenotype.[89]

In addition, some cancers have a characteristic tendency to metastasize to certain organs.[90–92] For example, breast cancer extends to the bones, lungs, brain, and liver or distant metastases of prostate cancer occur more prominently in bone (**Table 3**). According to Paget's hypothesis of "seed" versus "soil", compatibilities between the cancer cells (the seed) and certain distant sites (the soil) have been perceived to influence our view of the metastatic process for a long time.[24] However, although it is well known that there are preferences on the part of the primary tumor toward the target organs, much remains to be known about these associations. This complexity could be judged, perhaps simplistically, by a list of the most common tumors and their known metastatic tropisms.[83] However, these and other primary tumors can also form metastases at alternative tissue sites. In each case, the tissue microenvironment of a primary tumor is likely to differ markedly from the secondary site of spread, requiring significant adaptive movements by the newly arrived cancer cells. The details of these adaptive programs would appear to be dictated by the microenvironment of the starting point (the primary tumor) and the microenvironment of the landing site (the parenchymal tissue on which a metastasis is found).[7]

The genomic profile of metastasis has been successfully used to predict the sites of origin of primary tumors. These patterns of gene expression suggest that cancer cells within primary tumors acquire patterns that allow their subsequent colonization in preference to specific organs. These findings

**Table 2.** Exosome cargo and its role in cell–cell communication.

| Macromolecule | Molecule | Cell of origin | Mechanisms | Refs. |
|---|---|---|---|---|
| Protein | EGFR | Gastric cancer | Increased localization and proliferation of metastatic cells | [77] |
| | Podocalyxin | Non-small cell lung cancer | Modulated integrin trafficking in fibroblasts, increased tumor cell migration and invasion | [78] |
| | cMET | Melanoma | Promoted a pro-metastatic phenotype and mobilization of BMDCs to PMNs | [62] |
| | MIF | Pancreatic cancer | Promoted a pro-metastatic phenotype and mobilization of CTC to liver | [79] |
| | Integrins $\alpha6\beta4$, $\alpha6\beta1$, $\alpha v\beta5$ | Breast cancer | Determined organotropism of metastasis | [80] |
| RNA | miR-939 | Breast cancer | Downregulated VE-cadherin, increased HUVEC permeability | [81] |
| | miR-221 | Cervical squamous cell carcinoma | Promoted migration and lymphangiogenesis | [82] |
| | miR-105 | Breast cancer | Promoted a pro-metastatic phenotype and mobilization of CTC to brain | [82] |
| | MMP1 mRNA | Ovarian cancer | Interaction with peritoneal mesothelium barrier, promoted metastasis | [84] |

EGFR, epidermal growth factor receptor; cMet, hepatocyte growth factor receptor; MIF, macrophage migration inhibitory factor; VE-cadherin, vascular endothelial cadherin; HUVEC, human umbilical vein endothelial cell; MMP1 mRNA, matrix metalloproteinase-1 mRNA.

are supported by recent studies showing that genetically distinct cells subpopulations present in primary tumors are responsible for the metastasis. The result of successful colonization is a rapid expansion of metastasis that can now serve as a new focus for the spread of new secondary metastasis showers (Figure 1). It is important for the tumor that many of the cancer cells that are dispatched from this newly successful metastasis can be reversed with a functional colonization program that allows them to colonize a limited subset of sites throughout the body or multiple different tissue types. Finally, the multitude of secondary metastases derived from the first one soon overshadow the initiating metastasis that generated them.[99]

To complete the metastatic process, extravasation has to occur correctly. This event is similar to the invasion that initially occurs, although it is not known exactly if the same genes are involved. It would even be important to know if those different tumors that are predisposed to colonize the same organ use the same genes. Furthermore, during the process of infiltration and colonization, there is a latency period. What happens during this period? How do the cells survive until they manage to colonize the target organ? All this could be solved, again, with models that represent complex vascular networks and, especially in this case, with the synthesis of more sophisticated organoids that allow the deep study of extravasation.

**Table 3.** Incidence of metastasis in different organs after the autopsy (%).

| Cancer organ | Lung | Bone | Brain | Liver | Refs. |
|---|---|---|---|---|---|
| Lung | – | 34 | 39 | 21 | [93] |
| Breast | 71 | 71 | 22 | 62 | [94] |
| Prostate | 45.7 | 90.1 | 1.6 | 25 | [95] |
| Melanoma | 71.3 | 48.6 | 54.6 | 58.3 | [96] |
| Liver | 44 | 8 | 1 | – | [97] |
| Colon | 32 | 8 | 5 | 70 | [98] |

## 3. Do We Know Everything about Metastasis?

Nowadays, a great number of basic and deep concepts about metastasis are known and allow us to develop new techniques to fight it. However, there are still many questions to be solved: do cells migrate individually or collectively? Currently, it is believed that it can happen both ways; if they migrate collectively, how do they do it if they have lost most of the cell–cell interactions to escape from the primary tumor thanks to the EMT program? So how necessary is this EMT process, and is it really essential for cell spread? Although EMT is widely accepted as an important mode of dissemination of cancer cells, its precise role in the behavior of the primary tumor and its essentiality remains unresolved. For example, the invasion of primary tumor cells usually involves the collective migration of large cohorts of cells into adjacent tissues rather than the spread of individual carcinoma cells, although both models are under debate and it is even believed that they could occur at the same time.[55] The organization of these cohorts seems to conflict with the behavior of cells that have passed through an EMT and have lost the cohesive cell–cell interactions. Therefore, these cohorts raise the question of whether EMT programs are essential for the eventual dissemination of carcinoma cells, as explained above, or instead represent only one of several alternative biological cell programs that allow dissemination to occur.[55,100]

Also, another question is whether the TME plays a fundamental role in tumor development. According to different studies, the answer would seem to be a resounding no.[25,36,40] Yet, would it be possible to fully develop the tumor without the participation of this microenvironment? Why does the TME seem to help the tumor when it should protect the individual and slow down the growth of the tumor? We should not forget that only 0.01% of the cells that manage to escape from the tumor to colonize another organ could survive in the bloodstream. How is such an inefficient process able to be so efficient that it accounts for 90% of cancer deaths? How is the communication between the primary tumor and the rest of the organs?

There are a large number of molecules that serve as a communication pathway between both,[86,101] and even CSCs are capable of releasing exosomes, but do they play a more important role in this regard? Why does the above-mentioned organotropism exist? And, in another sense, why is there so much heterogeneity in the patterns between individuals? Different studies have revealed that the degree of intratumoral heterogeneity (ITH) in the same patient can be very variable, from 0 to more than 8000 coding mutations that are heterogeneous within primary tumors or between primary and metastatic or recurrence sites.[102] Will we be able to find any kind of pattern among patients despite this very high intratumoral variability?

The answer to all these questions will be the key to get this percentage of dead people to decrease, obtaining an effective treatment for this falsely inefficient process with new models, which will help us to understand it. Thus, there are different study models (models in mice, 3D bioprinting models or lab-on-chip researches) that have helped to understand all the processes that have been previously discussed and that have logically served to fight against this disease. However, in order to discover all the pieces of this biological puzzle and unravel the controversies that exist around it, it is necessary to move toward new and more accurate research models. The approach to reality with new models will surely allow answering all these questions, faithfully imitating the TME and, therefore, obtaining more positive, truthful, and objective results.

## 4. Study Models for Metastasis Research

2D in vitro tumor models have made possible to understand the genetic and epigenetic alterations that can initiate or contribute to the proliferation of cancer cells and other tumor phenotypes.[103] However, as discussed throughout this review, the impact of the TME is significantly important[104] and it is not present in 2D conventional cultures, providing an oversimplified view of tumor biology.[105] To understand the mechanisms underlying these complex tumor-stroma interactions, as well as their impact on tumor phenotypes, it has become clear that better in vitro multicellular models and better animal models are needed.[104] For this reason, in this section we provide an overview of the main metastatic cancer mouse models in use today, as well as the evolution of these models into new and improved forms of research such as tumor-on-a-chip, which implement 3D bioprinting technology. With all this, the advantages and disadvantages of metastasis study models will be observed, reflecting the need for the continuous implementation of new models for the investigation of this pathological process.

### 4.1. Experimental Models on Mice: A Limited Model

#### *4.1.1. Spontaneous and Induced Metastasis Models*

There is a wide variety of mouse metastasis models, including spontaneous metastasis models and induced metastasis models, including in turn allograft and xenograft models. Spontaneous metastasis models allow the study of the cells spread from a primary tumor to secondary sites in animals, that have received ectopic (out of the normal place) or orthotopic (within the normal place) injection of cancerous cells or tissue.[106] However, this model presents serious limitation. Tumor cell lines implanted subcutaneously in mice generally tend to grow rapidly and, therefore, do not mimic the doubling times of most human cancers, which are usually much slower. It is also unclear whether subcutaneously implanted ectopic tumors, which remain a standard methodology, will respond to therapy in the same way if cultured in an orthotopic organ.[107] On the other hand, experimental metastasis models are used to evaluate the ability of cancer cells to extravasate and grow in particular organs after intravascular injection (**Figure 2**), whether it is the lateral tail vein, intraportal and intracardiac, which in turn defines the site of colonization.[108] Nonetheless, this model does not reflect the initiation of the metastatic cascade and is limited to the study of the biological processes that occur once the tumor cells are in the bloodstream. Despite this, these models have been fundamental in the current knowledge of the interactions that CTCs have with secondary organs and their colonization.[106]

The cellular origin of both models is therefore a very important characteristic that must be taken into account. Allograft models are generated by transplanting cancer cells and mouse-derived tumors into mice, that is, between equal species. The use of genetically identical single models, thus preventing graft-versus-host reactions, allows investigation of the immune system in cancer progression and identification of new therapeutic opportunities.[120] Unlike allograft models, xenografts involve human cells, which must be introduced into immunocompromised or immunodeficient mice to prevent host rejection, being this the main advantage; however, the main drawback is the lack of a strong immune system in the host that, as it has been describe above, plays a fundamental role in the metastatic processes.[103] Hence, the use of xenograft mouse models in metastatic studies has been limited to highly metastatic tumors, such as MDA-MB-231 triple negative breast cancer cells, KM12 colon carcinoma cells and WM239A melanoma cells, which overcome the problem of limited metastatic potential.[58,121]

In addition, there are other drawbacks related with the use of mice models. For xenografts, the extraction of cancer cells involves their subsequent in vitro culture, which generally loses the characteristics of the original tumor, not reflecting the phenotypic and genetic heterogeneity of human cancers.[122] In an attempt to solve this problem, patient derived xenografts (PDX) have been developed. To avoid the selection pressures that occur in vitro, PDX are generated from resected tumors and implanted directly in immunocompromised mice by orthotopic or subcutaneous transplantation. While several studies have shown that PDX reflects human cancer diversity by recapitulating the histology and metastatic characteristics of the original tumor,[123–125] other studies show that the grafting rate is low and that the site and frequency of PDX metastasis may vary from that seen in the patient. Furthermore, again the lack of an intact immune system represents an important limitation, which is essential for tumor drift, and the presence of a mouse stromal makes PDX not an ideal model to study the role of the TME in human disease progression.[126,127]

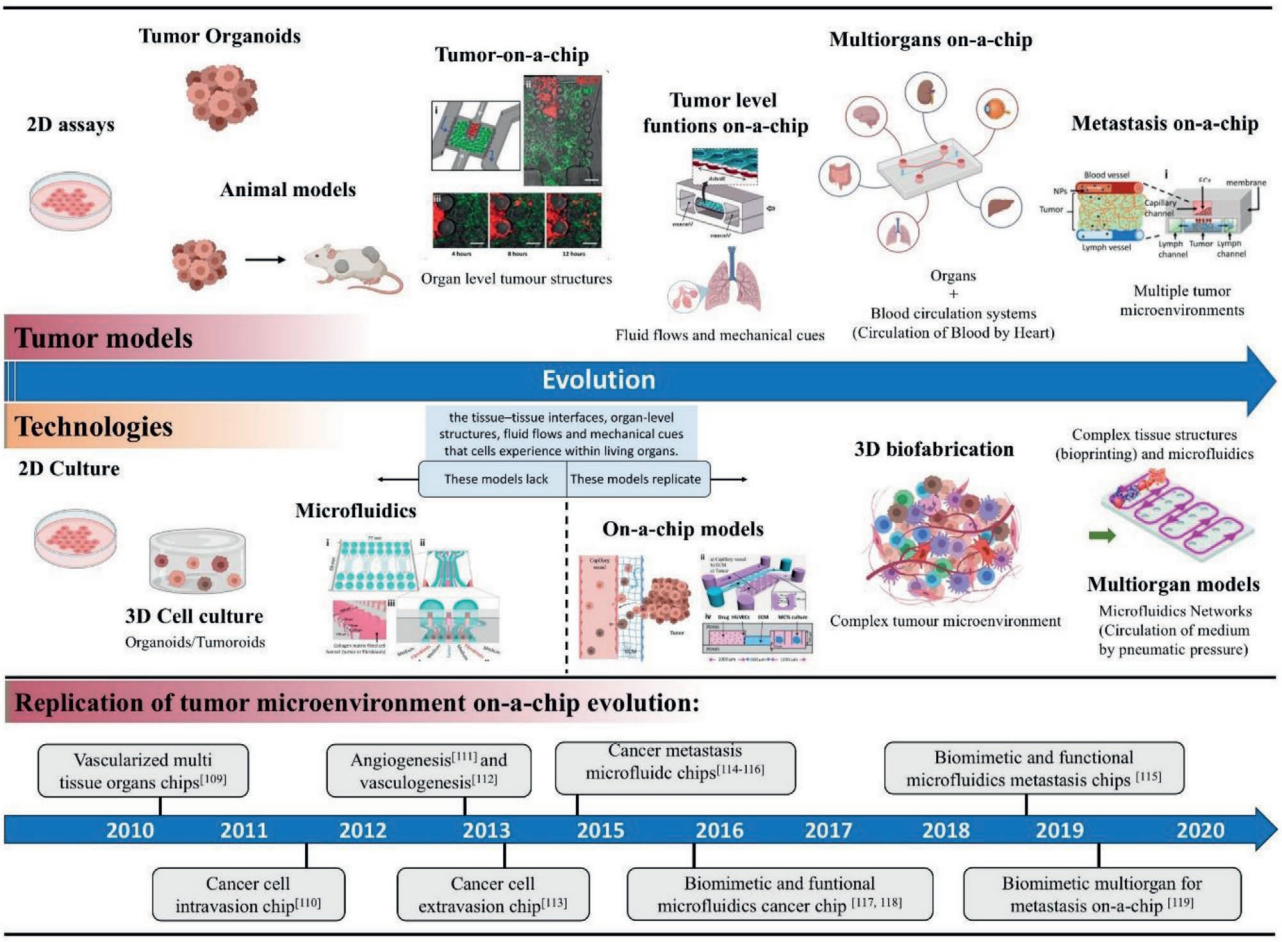


**Figure 2.** Evolution of metastasis research models. The study of metastasis encompasses a large number of models, starting with in vivo models in mice, which are still used. Subsequently, 3D bioprinting will play a fundamental role, from the most robust models through the construction of hydrogels that attempt to simulate the TME, to the most recent on-a-chip models, which are capable of recreating not only the TME but also the recreation of a vascular system that irrigates the tumor cells, thus allowing a better study of the metastatic process. Tumor-on-a-chip, Metastasis-on-a-chip, Microfluidics, On-a-chip-models: Reproduced with permission.[17] Copyright 2019, MDPI. Tumor level functions on-a-chip, Multiorgan models: Reproduced with permission.[158] Copyright 2020, John Wiley and Sons.

#### *4.1.2. Genetically Engineered Mouse Models*

Possible alternatives to xenografts models include genetically engineered mouse models (GEMMs), which have been developed and used to study many aspects of tumor biology.[128] The generation of these models involves alterations in gene expression (overexpression or suppression) with particular emphasis on those genes that play a major role in the type of tumor involved (Figure 2).[129,130] Such models offer several advantages, most notably the generation of orthotopic tumors in immunocompetent hosts that often reflect their respective human tumor histotypes and contain a stromal and vasculature of the same species.[106] This is why this type of model has been particularly effective in studying early events in tumorigenesis. However, they have not been able to replace xenograft models as reliable clinical predictive tools for examining the efficacy of various types of metastatic treatments. This may, at least in part, be because such models generally show a low incidence of distant metastatic disease.[131]

However, once again these models have major disadvantages: the microenvironment where the study tumor develops occurs in the mouse rather than in a human microenvironment; sometimes the promoters and oncogenes used are not truly representative of human disease, and they may not be well defined to a specific lineage; penetration is generally low, while there is a long latency in terms of metastasis development; they have poor metastasis tropism and extensive mouse breeding programs are often required, which involves a great deal of time and resources.[106]

### 4.2. 3D Bioprinting: A New Step into the Future

The field of tissue engineering, including the use of 3D bioprinting to generate complex tissues, has seen rapid advances in recent years toward the modeling of both normal tissues and disease states.[132–134] Thus, 3D bioprinting allows the generation of tissues that incorporate a variety of cell types in a complex and defined spatial architecture whose main motivation is to better mimic human physiology and functions at multiple scales, from the molecular level to the organ level across

the cellular, multi-cellular, and tissue levels.[15] The resolution of 3D bioprinting techniques is around 300 µm, which is suboptimal if precise control of cell positioning is required.[135]

As an additive manufacturing technique, 3D bioprinting is based on the deposition of biomaterials, either by encapsulating cells or by subsequently bioprinting cells on a micrometric scale to form subtle structures comparable to tissues. One of the most broadly used type of bioprinting is bioplotting, which is an extrusion-based technique consisting of a three-axis mechanical platform that controls the movements of an extruder. The bioink is then deposited following a predefined numerical code that translates into coordinates for the three-axis platform the shape desired by the designer. Due to advantages such as deposition accuracy, cost-effectiveness, simplicity, and control of cell distribution, the development of 3D bioprinting and its applications have been steadily increasing over the past few years. As a result of this development, the need has arisen to generate new bioinks that provide properties required for successful bioprinting, such as bioinks printability, fidelity, and cell viability.[14] In this sense, bioinks play a fundamental role, since they can become the TME when used for research in the fight against cancer. In this way, recent studies are aimed at generating bioinks from decellularized tumor matrix, recreating this TME in a more faithful way. Piccoli et al. (2018), for example, developed a decellularized colorectal cancer matrix for the 3D in vitro study of this cancer. In this way, they describe an innovative approach to tissue engineering applied to colorectal cancer (CRC) from decellularized human biopsies to generate a bioactive 3D organotypic model. This in vitro 3D system recapitulates the ultrastructural environment of the native tissue demonstrated by histology, immunohistochemistry, immunofluorescence, and scanning electron microscopy analysis. Thus, they demonstrated the capacity of biofabrication of 3D acellular matrices preserving their biological properties. Given the biological activity that the scaffolds maintained after decellularization, this approach is believed to be a powerful tool for future research and preclinical testing.[136]

Hydrogels with 3D hydrophilic polymer networks are one of the most promising and most widely used biomaterials in 3D bioprinting (Figure 2) thanks to their high biocompatibility, exceptional permeability and appropriate rigidity, and are recognized as preferable options for in vitro tissue and organ model engineering. In cell culture, hydration and porosity of hydrogels are indispensable parameters to provide a suitable environment for the proper functioning of cells.[137] Likewise, hydrogels also allow the exchange of nutrients, gases, and metabolic waste derived from the cell metabolism itself. In this way, there are different types of hydrogels (natural, synthetic, and hybrid) that have robustly allowed the study of the cellular microenvironment.[15] Thus, for example, Luker et al. (2018) generated a hybrid hydrogel system composed of collagen and alginate to model the environments of tumors in breast cancer and other malignancies where all the material properties of the hydrogel were taken into account, including rigidity, microstructure and porosity, covering parameters present in normal organs and tumors. By embedding multicellular tumor spheroids, a 3D model of tumor invasion was constructed, demonstrating the effects of CXCL12-CXCR4 signaling, a pathway involved in tumor progression and metastasis, in a 3D hydrogel double tumor spheroid invasion model.[138]

As a result, and in combination with this 3D bioprinting, new models have emerged for the study of different pathologies, the on-a-chip models (Figure 2), as an effective methodology for mitigating the major disadvantages of animal models to complete the understanding of different pathologies, including tumors and, therefore, metastasis.

### 4.3. Lab-On-Chip Technology Applied in Cancer

#### *4.3.1. Tumor-On-A-Chip Technology*

Organs-on-a-chip are microfluidic cell culture devices composed of optically clear plastic, glass, or flexible polymers, such as PDMS, which contain perfused hollow microchannels populated by continuously perfused living cells that recapitulate in vivo the physiology and physiopathology at the organ level, recreating in vitro structures and functions of tissues and organs.[13] The objective is not to build a complete living organ, but to synthesize minimal functional units that recapitulate the functions at the level of tissues and organs. The simplest organ-on-a-chip systems are composed of a single, perfused microfluidic chamber containing a type of cultured cell that exhibits functions of a tissue type. In more complex designs, two or more microchannels are connected by porous membranes, lined on opposite sides by different cell types, thus recreating interfaces between different tissues, such as an endothelial barrier, while allowing analysis of specific organ responses, including recruitment of circulating immune cells, in reaction to drugs, toxins, or other environmental disturbances.[139] These devices can also be flexible and contain hollow side chambers through which cyclic suction can be applied to rhythmically stretch and relax the organotypic tissue interfaces, thereby mimicking the mechanical signals relevant to the organs, and replicating the air–liquid interfaces (ALI), fluid flow, and associated physiologically important shear stresses.[13]

As seen so far, the organ-on-a-chip has great potential for research into the basic mechanisms of organ physiology and disease. Therefore, these models are optimal for the study of biological phenomena that depend on tissue microarchitecture and perfusion, as well as physiopathological processes.[139] Although the culture of a single cell type can mimic some characteristics of the tissue microenvironment, it is not usually sufficient to generate functionality similar to that of the organs. An organ is a hierarchical structure composed of two or more different tissues, which in turn are formed by groups of different cell types. Therefore, to fully replicate this functionality, it is necessary to combine two or more different tissue types. With all this and thanks to the evolution of these models in recent years, it has been possible to mimic the TME for the study of this pathology in the named as tumor-on-a-chip. These devices allow the study of the specific steps of the cancer cascade, such as growth and expansion of the tumor, angiogenesis, progression of lesions from early stage to late stage involving EMT, invasion and metastasis of tumor cells.[13]

Tumor-on-chip models provide a unique biomimetic environment to recapitulate all the events mentioned in metastatic screening and can be used to help us better understand the

behavior of new drugs in cancer. These models can easily recreate cell–cell or cell-ECM interactions, chemical/physical gradients, space-time or the (hydro)dynamic properties of the cellular microenvironment.[140] Also, these models solve the important limitation of in vitro models, such as the lack of a functional network of blood vessels to transport nutrients and gases, and to remove toxic products generated by the cells. Recent studies have provided evidence that these on-a-chip systems can provide a low-cost and physiologically relevant study model as an alternative to standard in vitro and in vivo models for clinical applications.[139,141–143] In this way, several research groups are developing better tumor-on-a-chip models.

Carvalho et al. (2019) managed to develop a tumor-on-a-chip model on a CRC chip for precision medicine, recreating a hydrogel with colon cancer cells covered with endothelial cells on a PDMS chip. Model validation was performed through integrated feasibility studies with in vivo imaging to confirm the dose-response effect of cells exposed to the carboxymethyl chitosan-grafted-terminal carboxyl group-poly(amidoamine) (CMCht/PAMAM) nanoparticle gradient. This platform also allows the analysis of gene expression, where a down-regulation of all the genes studied related with invasion, proliferation and apoptosis was observed (MMP-1, Caspase-3, and Ki-67). The developed tumor-on-chip platform, which comprises a nucleus similar to that of a human CRC and the surrounding vascularized microtissue, is a promising tool that presents numerous advantages over conventional models, whether for high-content image-based examinations or for analysis of gene expression to study responses to drug doses, as well as all the advantages presented by these models that will be discussed below.[144]

Another recent study by Lu et al. (2018) developed a 3D biomimetic liver tumor-on-a-chip with the integration of essential components derived from the decellularized liver matrix (DLM) and GelMA, generating a dynamic 3D cell culture system based on microfluids. The biomimetic liver tumor-on-a-chip achieved greater cell viability and improved hepatocyte function under flow conditions. Thus, this improvement in tumor performance based on DLM-GelMA can be attributed to the supply of biochemical factors, the preservation of scaffold proteins and the restoration of biophysical signals for better mimicry of the 3D liver EMT. In addition, this chip model exhibited linear dose-dependent pharmacological responses to paracetamol and sorafenib toxicity. Therefore, this study demonstrated that the liver tumor model faithfully mimicked the liver TME, making it possible to use this tool for a wide range of pathological and pharmacological studies.[145]

On the other hand, Aung et al. (2020) described the development of a multicellular perfusible tumor-on-a-chip platform involving different cell populations. Breast cancer cells, monocytes and endothelial cells were spatially confined within a gelatin hydrogel in a controlled manner by 3D bioprinting. Using this platform, they examined cancer cell-myocyte interactions in the recruitment of T cells, which were allowed to infiltrate. In turn, a hypoxic environment was provided by the culture of tumor spheroids, demonstrating increased recruitment of T cells versus scattered cells with a less hypoxic environment. The addition of monocytes to the cancer cells improved T-cell recruitment. Thus, this study tested how effective was the development of a tumor-on-a-chip model involving heterotypic cells, as well as mimicking the recruitment of immune cells by the TME.[146]

#### *4.3.2. One Step Further: Metastasis-On-A-Chip Technology*

All these findings have allowed to go a step further in the study of metastasis. As mentioned above, the first events in the metastatic cascade are tumor growth and invasion. Recently, some metastasis-on-a-chip (MoC) devices have been described for the analysis of these phenomena, which differ in the origin of the cancer cells, the architecture of the chip or the composition of the cellular microenvironment.[147,148] Some of these devices use SGC-7901 human gastric cancer cells[149] or NPC-BM1 nasopharyngeal carcinoma cancer cells,[150] with high metastatic capacity to obtain representative results. In the first of these, a microfluidic system was developed to detect highly metastatic sublines through the differential resolution of cell invasion. The system consisted of a PDMS glass device connected with a syringe pump and a petri dish. To facilitate the selection process, a long-term driving force based on a gradient of chemotactic factors was generated and the invasive cells were collected for selection through an open region on the chip. Using this system, a subline of SGC-7901/B2 of the human gastric cancer cell line SGC-7901 was established. In vitro tests showed that SGC-7901/B2 cells were superior to parental cells in terms of proliferation and invasion. In addition, an in vivo tumorigenicity trial showed that, compared to parental cells, the subline had a higher spontaneous metastatic and proliferative capacity, which translated into a shorter survival time. This revealed the differences in protein expression, including E-cadherin and Smad3, between the subline and the parental cells. In conclusion, this microfluidic system proved to be a very effective tool for selecting highly metastatic sublines, and this SGC-7901/B2 cell subline could serve as a potential model for tumor metastasis research.[149]

In the second one, the NPC-BM1 nasopharyngeal carcinoma cancer model, a microfluidic device was generated for nasopharyngeal carcinoma that incorporated an impedance system for cell invasion quantitative measurement. The device consisted of two tanks that were connected to a microchannel filled with a hydrogel. The malignant cells began to invade the microchannel and the impedance was simultaneously measured through electrodes located at the bottom of the microchannel. In this way, the process of cell invasion could be monitored in real time and in a non-invasive way. In addition, the cell invasion rate was also calculated taking into account the correlation between cell invasion and extracellular stimulation by IL-6 cytokine, which showed that, indeed, the cell invasion rate was directly proportional to the IL-6 concentration, providing a reliable platform for future metastatic cell-based assays.[150]

Other research focused on the description of MoC system that would allow real-time monitoring of colon cancer through the fluorescence of cells migrating from 3D intestine hydrogel constructions (of hyaluronic acid) to another structure that mimic the liver, building a device with a circulatory fluid system that represents the TME, as well as a system to measure the effect of different drugs. The devices consisted of two chambers in which the intestine and liver were housed

independently, but which were in turn connected by the continuous flow of circulation. They showed how different tumor regions lost their membrane adhesion markers, and expressed mesenchymal and proliferative markers, suggesting a metastatic phenotype.[10] These metastatic foci grew in size, eventually spreading from the intestine construction and entering the circulation, later reaching the liver construction, thus mimicking some of the migratory events observed during metastasis. Finally, the system's manipulative capabilities were also demonstrated, including chemical modulation and modifications of the mechanical properties of the hydrogel and the administration of chemotherapeutic agents, and the effects of these on the migration of invasive tumors were evaluated. Thus, these results describe the capacity of this MoC device, at an early stage of the tumor, to model several important characteristics of metastasis, also demonstrating the potential to make significant advances in cancer research and the discovery of a drug effective against cancer.[10]

In a biochemical way, several studies of MoC have focused primarily on oxygen requirements, and some have looked at acidity and lactate levels. Such research has indicated that aerotaxis (an active cell movement along gradients of oxygen)[151] is a relevant mechanism in cancer cell migration, and that acid and lactate gradients determine the direction of cancer cell invasion.[3] However, most experiments have focused on the study of metastatic invasion and intravasation. Liu et al. (2010) developed a microfluidic model that reconstitutes and is representative of the metastatic process, containing the main components of biological blood vessels, including vascular cavity, endothelium and perivascular matrix, which in turn contain chemokines. Using this model, the transendothelial invasion of the tumor aggregates can be observed and recorded in real time. In this study, the process of extravasation of the cystic adenoid carcinoma cell of the salivary gland (ACC) was analyzed. The ACC aggregates transmigrated through the endothelium under CXCL12 chemokine stimulation, observing how the integrity of the endothelium was irreversibly damaged at the site of the transendothelial invasion. In turn, it was shown that the transendothelial invasion of ACC aggregates was inhibited by AMD3100. Thus, this model allowed a detailed study of the process of transendothelial invasion of tumor aggregates, being therefore a useful tool for the analysis of the underlying mechanisms of metastasis and for testing new anti-metastatic agents.[148]

Ultimately, body-on-a-chip models represent the most developed systems for making a multi-organ model that mimics the interactions between multiple organs.[152] These recent advances aim to study organ–organ interactions as well as to investigate the ADME-tox methodology (absorption, distribution, metabolism, and elimination) of different drugs that can be used to fight against several pathologies, including metastasis.[153]

Thus, Oleaga et al. constructed a functional human model capable of evaluating multiorgan toxicity in a 4-organ system under continuous flow conditions in a defined environment without serum using a platform without a pump for 14 days. With this, they demonstrated the viability of the system, as well as the functional activity of the cardiac, muscular, neuronal, and hepatic modules. In addition, pharmacological relevance was evaluated in terms of their response at 7 days to 5 drugs with known side effects, doxorubicin, atorvastatin, acetaminophen, *N*-acetyl-*m*-aminophenol and valproic acid, after a 48 h drug treatment regimen. The results of all the pharmacological treatments coincided with published toxicity results from human and animal data, making the next generation of in vitro systems increasingly realistic. This study has been a step toward an in vitro human on-chip assay for systemic toxicity detection.[118] In the same way, Skardal et al. (2017) developed a set of bioengineered organoids and tissue constructions that were integrated in a closed system of circulatory perfusion, allowing and facilitating the investigation of interorganic responses. In this system, a three-tissue organ-on-chip was accomplished, composed of liver, heart, and lung, showing responses to drugs that depend on tissue interaction and illustrating the value of multiple tissue integration for in vitro study of both efficacy and side effects associated with candidate drugs. Investigating the potentially toxic effects of bleomycin on the lung organ model, adverse effects were observed in the cardiac organoid, which were not present when the cardiac model was treated with the same compound alone.[119]

Observing the advantages provided by the body-on-a-chip, its use in metastasis is a powerful study tool. Aleman & Skardal (2019) described a metastasis-on-a-chip device that hosts multiple 3D organoids created by a 3D photodesign technique using biomaterials from hydrogels derived from the ECM. Specifically, colorectal cancer cells, which resided in a single microfluidic chamber, were connected to multiple posterior chambers in which the rest of the organ constructions such as the liver, lung, and endothelium, were developed. In this way, the tumor cells grow at the primary site, under the flow of a recirculating fluid, to which they eventually enter into circulation, and could be tracked by means of fluorescent imaging. It is important to note that they were able to demonstrate that in the current version of their system, the CRC HCT116 cells preferentially harbored the liver and lung constructs, the corresponding organs from which most CRC metastases arise in human patients, demonstrating once again the potential of these systems to mimic the metastatic process as it occurs in vivo.[116] In addition, Xu et al. (2016) reported the design and construction of a multi-organ-on-a-chip that mimics the in vivo microenvironment of lung cancer metastasis. This multiorgan-on-a-chip includes an ascending "lung" and three descending "distant organs", with three layers of PDMS and two thin PDMS microporous membranes joined together to form three parallel microchannels. Bronchial epithelial, lung cancer, microvascular endothelial, mononuclear and fibroblast cells separated by the barrier in the ascending "lung" were cultured, while astrocytes, osteocytes, and hepatocytes were grown in distant chambers, thus mimicking the metastatic process of lung cancer cells in the brain, bone, and liver, respectively. After culture in this system, the lung cancer cells formed a "tumor mass", showed an EMT (with altered expression of E-cadherin, N-cadherin, Snail1, and Snail2) and a high invasive capacity. A549 lung cancer cells that were cultured together with astrocytes overexpressed the CXCR4 protein, indicating the presence of astrocyte damage after metastasis of the cancer cells in the brain. On the other hand, the osteocyte overexpressed RANKL protein was also an indication of damage into bone due to metastasis of the cancer cells, as well as the overexpression of the AFP protein

in hepatocytes. Finally, in vivo imaging of cancer growth and metastasis in a mouse model validated the performance of metastasis in this multi-organ-on-a-chip system.[117]

#### *4.3.3. The Great Potential of Tumor/Metastasis-On-A-Chip. Will They be Able to Replace the Current Models?*

Conventional systems for metastasis analysis in mice and 3D cultures, including methods based on hydrogels, tissue engineering, static cocultures, and bioreactors, have proved very useful for the study of certain behaviors at tissue and organ levels and for developing disease models. However, their major limitations have meant that new models have begun to emerge to fill these gaps and, in this regard, microfluidic culture devices have much to offer. A disadvantage of 3D macroscale models without the addition of a chip is the difficulty of obtaining high-resolution images that allow to determine where in the tissue to look, in the same way, that it is difficult to visualize the processes in living organs. In addition, in organ-on-a-chip, cell types in one tissue can be positioned precisely and consistently concerning those in another, which has made it possible to integrate these systems with fluorescence confocal microscopy, microfluorimetry, transepithelial/transendothelial electrical resistance (TEER) measurements, multiple electrode configurations, and many other analytical tests.[139] The screening of new drugs is also a strength of these models through high-throughput screening (HTS), allowing the generation of a large number of chips with samples of a tumor to test the effectiveness of different drugs on them.

To date, although a great advance has been made in the development of tumor-on-a-chip models, there are still important limitations to be solved and which are being taken into account in future steps (**Table 4**). For example, tumor-on-a-chip platform commonly uses cell lines, resulting in inconsistencies between the model and an original tumor. However, this limitation is being solved with the use of patient biopsies that, on a small scale, would generate PDX-tumor-on-a-chip models that, without a doubt, would represent more powerful models than the current ones, avoiding the use of established cell lines.[154] The evolution of these models into more complex ones would finally solve the rest of the problems and, besides, this is being continuously observed: the change from tumor-on-a-chip to body-on-a-chip has been a matter of time and the implementation of increasingly complex vascular networks and target organs will be thanks to the development of new and more powerful instrumentation. Even, cell composition is not only the most important issue when designing a realistic model, but the general microenvironment and the ECM that makes it up are increasingly being taken into account. Thus, for example, although synthetic materials need to be used for the synthesis of the microenvironments, most of them have high biocompatibility and they do not stop faithfully recreating the microenvironment with human cells. In addition, a new approach based in obtaining the ECM from decellularized human biopsies (both from healthy and tumor tissue) instead of using materials from another natural or synthetic source, is already being recreated in chip models.[145,154]

Additionally, it is true that it is not possible to recreate tumors at real size (typically $>10^9$ cells); however, it is possible to reach a cellular range of $10^6$ cells per mL,[155,156] which already

**Table 4.** Advantages and disadvantages that present the current metastasis models depending on each step of it.

| Metastasis steps | 2D/3D cell culture | | Mouse models | | Tumor/metastasis-on-a-chip | |
|---|---|---|---|---|---|---|
| | Advantages | Disadvantages | Advantages | Disadvantages | Advantages | Disadvantages |
| Local invasion and intravasation | Decellularized tumor matrix recreate the human TME<br>Cell heterogeneity<br>Accurate cell distribution | Presence of synthetic materials (transwell cell invasion assay)<br>2D models cannot mimic the real TME | Human-like cell transport dynamics<br>Cell heterogeneity<br>Immunocompetent host if allograft and GEMM | Mouse TME<br>Applicable to limited number of cell lines<br>Immunocompromised host if xenograft | Faithfully recapitulate the TME (including immune cells)<br>Cell heterogeneity<br>Accurate cell distribution<br>Easy study thanks to coupled techniques (microfluorimetry, TEER measurement...) | Use of established cell lines<br>Impossibility of generating full-size tumors |
| Circulation | It is not possible to mimic the metastatic process since there is no circulatory system in this model | | Circulation process occurs in a living organism<br>Presence of a complex natural vascular network<br>Presence of blood circulation cells | Asynchronous metastatic development | Possibility of simulating the endothelial network<br>Adjustable circulation parameters | Difficulty in creating very complex vascular networks |
| Niche premetastatic | | | Site-specific development of metastasis in induced models | Poor tropism | Possibility of study thanks to body-on-a-chip<br>Secretome easy study | Impossibility to simulate all the organs at the same time |
| Extravasation and colonization | | | Presence of the complex structure of the target organ | Low penetrance<br>Long latency | Good extravasation due to permeable materials<br>Easy study thanks to coupled techniques (fluorescence confocal microscopy, multiple electrode configurations ...) | Difficult to simulate the complex structure of the target organ |

allows for considerable cellular heterogeneity thanks also to the coupling with other techniques such as 3D bioprinting. This heterogeneity is the one, which really determines the behavior of the tumor and its replication on a small scale facilitates the study of them. Moreover, the inclusion of immune cells in the TME facilitates the correct mimicry since, as mentioned above, they play an essential role in the tumor development and metastatic process.

Despite these disadvantages, the fact that these models present microfluidic systems providing perfusion (continuous, cyclic, or intermittent) offers a major advantage over static models, including spheroids and organoid cultures. This is especially important since it allows studies to focus on the neovascularization, invasion, and dissemination of cancer cells, as well as the possibility of maintaining cell viability and functionality for extended periods. The inclusion of endothelial cells in the form of vasculature also offers greater clinical relevance for drug delivery studies, as well as for the development of pharmacokinetic and pharmacodynamic (PK-PD) models,[157] where vascular tissue plays a key role. Thanks to this vascularization, the tumor model more accurately represents the TME, allowing in turn to observe invasion kinetics, tumor-blood vessel interaction, as well as drug interactions with endothelial barriers that represent a front to reach the tumor. The ability to establish controlled chemical gradients, achieve air liquid interface cell culture (ALI) to replicate the mechanical environments relevant to organs, and to model the fluids, shear stresses and hydrostatic pressures that exist in the ECM, represents an important additional advantage of these models.[13]

The real power of these systems lies in the ability to design synthetic culture method in which a large number of different parameters can be controlled: cell types and positions; molecular and specifically, oxygen gradients; precise 3D orientation of tissue/tissue interfaces; levels and flow patterns and mechanical forcing regimes among others. All these variables can be varied independently while simultaneously obtaining high quality images in real time. This unprecedented level of control makes it possible to replicate different functional units of different organs that, sometimes and each on its chip, could be linked by vascular or interstitial channels to create synthetic models of whole organs.[139]

Although there are several works and research on chips, because of their potential, more areas of these tools need to be thoroughly investigated. With all this, future perspectives should focus on the use of decellularized matrix as bioinks for 3D bioprinting. As discussed above, this decellularized matrix more accurately represent the TME of the tumor cells themselves, even with the same immune cells that, as discussed in this review too, play an important role in the development of the metastatic process. However, it would be important to take into account the variability among donors and therefore the need to establish a common protocol. Also, HTS would also be a good field to explore, which would be a further step toward precision medicine, allowing the verification of the effectiveness of a large number of drugs for the same tumor, not only for the discovery of one, but for the personalized study for each patient.

## 5. Conclusions and Future Perspectives

Metastasis is a highly complex process that, despite being very inefficient, represents the major cause of cancer deaths. This is the reason why the need for metastatic study models has become very relevant in recent times. The progression of these models has been highly remarkable, going from studies in mice, which do not faithfully represent the human tumor behavior, to improved metastasis-on-a-chips-models that accurately and faithfully represent the TME, being a powerful device for the fight against cancer. These devices allow the control of a large number of parameters that improve the study of metastasis, as well as the representation of functional units of different organs to study cell migration, being at the same time low-cost models. However, many aspects of metastasis are not well known at present, so the evolution of these models must remain constant to finally understand all the steps that come into play in the metastatic process, as well as to obtain powerful tools for the screening of future drugs and toxicological studies.

Currently, these models are not applied in clinical practice and, observing all the advantages and opportunities they present, it is a considerable challenge to implement them in daily clinical practice. Despite the disadvantages or limitations they may have (i.e., the use of established cell lines or the impossibility of generating full-size tumors), it has already been observed how they are overcome by their potential and how they could be a clear evolution in the way of treating patients, with a clear personalized approach. Thus, the tumor-on-a-chip and metastasis-on-a-chip models would be a great and modern tool for the development of personalized treatments that, fortunately, will manage to realize a previously therapeutic HTS from a small patient biopsy that allow to implement more precise treatment regimens that will result in the reduction of deaths due to metastasis.

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## Conflict of Interest

The authors declare no conflict of interest.

## Author Contributions

J.R.E., G.J., and J.A.M. design the study and J.R.E. wrote the manuscript. G.J., J.A.M., D.N., and L.M. revised critically the

manuscript for important intellectual content. All authors read and approved the final manuscript.

## Keywords

3D bioprinting, cancer, metastasis, metastasis-on-a-chip, tumor-on-a-chip

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