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Molecular Mechanisms of Metastasis: Epithelial-Mesenchymal Transition, Anoikis and Loss of Adhesion

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1. Introduction

During the process of tumorigenesis, some cells separate themselves from the tumor to invade distant tissues. Cellular migration in this process is similar to what occurs during embryonic development and wound healing. Unlike these two healthy processes, which involve the creation of a structure or the healing of tissue, metastasis results in the formation of a cellular mass that, if not eliminated, leads to the death of the organism. The process of metastasis includes the invasion of the basal membrane and nearby tissue by tumoral cells and the intravasation towards the blood vessels or infiltration of the lymphatic vessels. This is followed by the mechanisms of survival of tumor cells in these vessels and their extravasation to different tissues of the organism, where they may be able to proliferate. This pathogenic process requires a precise coordination of various signaling pathways that allows the tumor cells to move across the cell membrane, remodel the matrix, transport themselves by circulation [1] and create the appropriate conditions, at a distance, for establishing themselves in a different organ (Figure 1).

Although exploring this complex process of motility and invasion by tumoral cells is fundamental for deepening the understanding of metastasis [2], much remains a mystery despite the enormous amount of research contributions. Both *in vitro* and *in vivo*, the time required for analyzing the evolution of this pathogenesis is excessive. Today the best way to make functional evaluations of the genetic changes that take place in humans with metastasis is with animal models. Although time consuming, a complete follow up can be carried out of the entire process, from the moment of the appearance of a primary tumor, to the strategies used by cancerous cells to escape from the controls of adhesion, their interaction with endothelial cells during their migration, and the establishment of a secondary tumor through the preparation of a new microenvironment favorable to tumor growth in the affected organ.

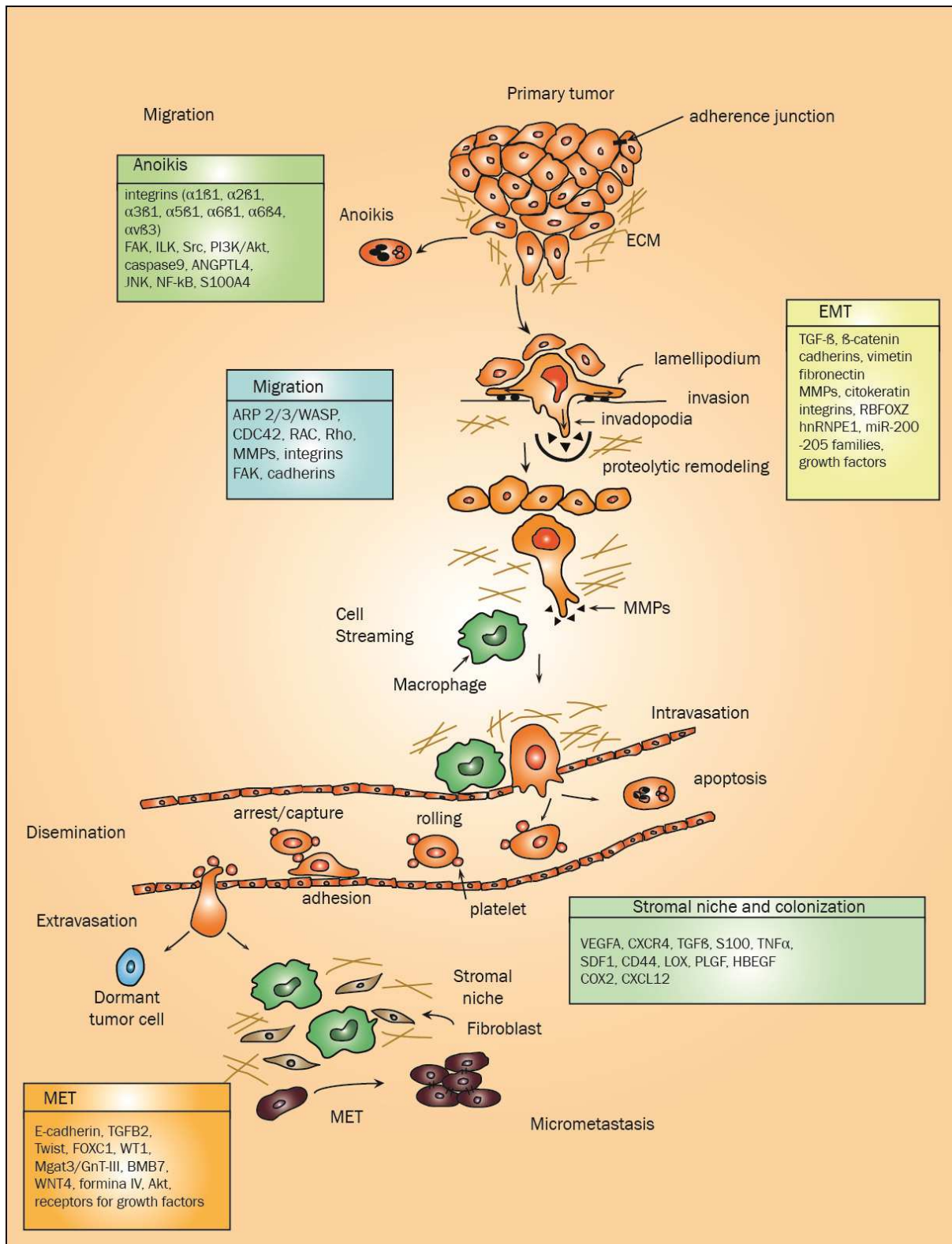


Figure 1. Separation of tumoral cells from a primary tumor and its migration to reach a blood or lymphatic vessel for dissemination to a secondary site is a very complex process that includes changes in the expression of multiple genes, which are genes involved in cell adhesion, survival, chemoattraction, growth factors, miRNAs. In the figure you can see some of the genes involved throughout this process.

2. Primary tumor migration

The majority of deaths from cancer are due to metastasis. Nevertheless, in any given moment only a small proportion of tumoral cells acquire the capacity of invasion and dissemination [3]. This capacity is favored by the activation of signaling pathways that trigger the metastatic cascade, which results from the continuous exposure to the development of the primary tumor, to growth factors, to angiogenesis and to accumulated genetic changes [4]. Since the probability that tumoral cells that travel to new sites give rise to a tumor is very small [5], the process of tumorigenesis is actually quite lengthy. Yachida et al [6], after analyzing autopsies of victims of metastatic pancreatic cancer, proposed a quantitative model of tumor development and metastasis. They consider that at least a decade must pass between the occurrence of the initial mutation and the birth of the parent cancerous cell that eventually results in a tumor. Another five years or more are required for the acquisition of a metastatic capacity. After this latter event, the process takes place very quickly, with the life expectancy of two years for the affected patient. Hence, the challenge is to detect a tumor after the onset of the parent cancerous cell but before metastasis.

Some years ago, Engel et al [7] found that the period from the onset of metastasis to the diagnosis of breast cancer was approximately 6 years. For the purpose of establishing such a diagnosis, two types of tumoral cells can be detected: those circulating in the blood and those disseminated in lymphatic nodes and bone marrow. Such tumor cells can persist for years, awaiting the adequate conditions in some organ to be able to establish themselves in a secondary tumor. In the event of the existence of both types of tumor cells in a patient, the likelihood of tumor development is quite high [8].

There are two proposed models for explaining metastasis. Firstly, there is the model of linear progression, which considers that tumoral cells pass through multiple successive rounds of mutations, resulting in the selection of the most apt for proliferation in a relatively autonomous manner, followed by the transport of these clones to a new site. Secondly, the model of parallel progression holds that tumor cells separate themselves from the primary tumor before the acquisition of the malignant phenotype, and that these cells then undergo a somatic progression and metastatic growth at a distant site [9, 10].

Detailed analysis of diverse data, including that from animal and computational models, suggests that dissemination is not a lengthy process, at least in breast cancer, prostate cancer or cancer of the esophagus [11,12]. Thus at least for these cancers, the model of parallel progression is best supported to explain metastasis from the primary tumor. Through the use of comparative genomic hybridization, Baudis [13] analyzed 5918 malignant epithelial neoplasias and observed typical imbalances: whereas there were recurrent findings of increases in 8q2, 20q, 1q, 3q, 5p, 7q and 17q, similar patterns of losses were seen in 3p, 4q, 13q, 17p and 18q, among others. These genetic similarities between primary and metastatic tumors tend to indicate a convergent evolution more than the result of selection starting from a clone [5]. Nowadays it is well known that tumoral cells abandon the primary tumor before cancer is diagnosed. The possibility that these cells develop a tumor in a distinct organ depends largely on the characteristics of the primary tumor.

2.1. Cell invasion

Tumor cells must acquire certain characteristics for cell migration to occur, such as polarity and disassociation from their point of origin. Afterwards, these cells must undergo cycles of extension and contraction, and at the same time they adhere to and are released from the substrate [14] to move from one site to another. During this process their form is radically modified.

The ability of cells to migrate, studied since 1863 after the discovery by Virchow, allowed them to carry out various processes including embryogenesis, angiogenesis and wound healing to immune response [15]. Cell migration can be divided into stages according to the changes observed in the morphology of the cell: polarization, protrusion, adhesion, translocation of the cell body, and retraction of the rear portion. The physiology of cell migration is diverse, depending on the type of cell involved [16]. For instance, fibroblasts and melanocytes generally migrate mesenchymally, as individual cells that are highly adhesive and require proteolytic remodeling of the matrix [17,18.]. These cells form specialized protrusions of the membrane, among which are lamellipodia, which are actin projections of the cytoskeleton formed in the extreme front part of the mobile cell, and invadopodia (invasive pseudopods), which are proteolytically active protrusions of the plasmatic membrane that are responsible for the focal degradation of the components of the extracellular matrix (ECM). The latter type, characteristic of highly invasive tumor cells, contains sites of matrix metalloproteinases (MMPs) [19]. Wolf et al [20] observed that tumor cells do not always require MMPs, since they can escape through openings in the ECM by means of a great contractile force. Also among specialized protrusions of the membrane of metastatic fibroblasts and melanocytes are filopodia and podosomes. The former protrusions are in the shape of thin bundles of actin, and are in charge of probing the environment in search of signals [21]. The latter are functionally the same as invadopodia, forming in macrophages and osteoclasts associated with a tumor, and aiding in the process of invasion. The capacity of malignant cells to migrate in a directional manner is due to the presence of receptors on their surface that allows them to follow the gradient of chemokines [22]. Among the most common type of chemokines are CXCR4 and CCR7, which have been found in diverse types of cancer and are attracted by CXCL12 and CCL21, respectively [23].

2.2. Mechanisms of invasion

Yilmaz et al [24] suggest classifying the invasion by cancerous cells as individual or collective. The use of one or another type of mobility depends on the type of malignant tumor and the surrounding tissue, defined by distinct patterns in the activity of extracellular proteases, matrix-cell adhesion mediated by integrins, cell-cell adhesion mediated by cadherins, cellular polarity and cytoskeletal arrangements [25]. Epithelial cells, which are the most common source of diverse cancers, can exhibit multiple phenotypes of migration. They commonly have a stationary behavior and form layers of cells interconnected with strong bonds. They are capable of moving individually, as a layer, or as a tubular structure. The latter type of structure is found during embryonic development [26]. Among epithelial

tumoral cells, all these three types can be found for any particular tissue, suggesting that these pathogenic cells have the capacity to change from individual to collective migration, according to the environment that they face.

2.3. Individual cell migration

The movement of cells inside of living organisms is highly complex and strictly coordinated. For instance, hematopoietic cells exhibit a highly individualized ameboid movement, with little adhesion and without causing remodeling of the matrix. Tumor cells are capable of taking advantage of the strategies of healthy cells, moving in the same individual manner for example as a leukocyte or fibroblast. Individual migration of cells can have some variants: ameboid or mesenchymal [27, 28], solitary or in single file. When cells are transported to a new location, their form is modified. They often, but not always, adopt a bottle form. The constriction of their apexes can have two functions: (i) the cells can transport much of their intracellular content and begin the movement out of the epithelium, and (ii) they can reduce the amount of non-adhesive apex membrane, since by passing through the epithelial layer to arrive to the new location the apical point breaks. Additionally, this form allows for passage through the epithelium layer with a reduced space left behind. The apical constriction is driven by contractions based on actinomyosine, while the apical membrane is reduced by endocytosis. The cells must carry out a process of de-epithelialization, which means that they lose an essential property of epithelial cells—contact with neighboring cells.

Apart from the eventual loss of the adherence of the apical point, the adjoining surfaces forming the bottle neck are also broken up [29]. Due to employment of intravital multiphoton imaging, it has been possible to observe tumor cells in movement, and therefore to see breast cancer cells utilizing ameboid movement to transport themselves rapidly (~4 μ m/min) [30]. During transport these cells acquire a polarized phenotype, bearing Ca²⁺ in front of and behind the cell, which allows for the retraction of the rear portion

[31]. This process of retraction is supported by the contraction of miosine II and by the disassembly of focal adhesion at the rear of the cell, due to the breakage mediated by calpain of the proteins of focal bonds, including integrins, talin, vinculin and focal adhesion kinase (FAK) [32]. Focal adhesion is composed of a structural point and adhesion signaling between the ECM and the cytoskeleton. The velocity of the formation and disassembly of the focal adhesions is what determines the efficiency of cellular migration [33]. In tumor cells the loss of E-cadherin facilitates the formation of focal adhesion and better communication with the ECM. The small STPases (Rho, Rac, CDC42) are among the key molecules for initiating remodeling of the cytoskeleton. The remodeling of actin is mediated by actin-related proteins (such as ARP2/3) [34]. The other type of individual migration, mesenchymal migration, can be used by cells that undergo a transition from the epithelial to mesenchymal cell type, the latter of which is present in 10-40% of carcinomas [35]. This type of migration requires proteolysis of ECM proteins so that the cells can move through the matrix-filled spaces of the tumor [19].

2.4. Collective cell invasion

Targeted multicellular migration can be of two types: (i) collective cellular migration, in which the cells undergoing transport maintain close contact with one another, and (ii) streaming, in which these cells are not always in direct contact [22, 23]. This latter type is carried out by cells during the stage of gastrulation in embryonic development and in wound healing. Collective cell migration, where the cells are bonded together in layers, is more common than individual migration under normal physiological conditions [36]. The best understood collective cell migration is that of wound healing. Neurons and smooth muscle cells also move collectively, as a cohesive group and in a coordinated and integrated manner. The cells involved in this movement retain much of their epithelial characteristics, such as the cell to cell adhesion, averting the need for an epithelial-mesenchymal transition that leads to a modification of cellular shape [37]. In all types of collective migration, the foremost cells of the migratory group actively participate in chemotaxis and degradation of the matrix in order to make way for passage. These leading cells are linked to the cells behind them, dragging them through the remodeled matrix [36].

In tumor cell collective migration, the leading cell can be a tumor cell with proteolytic activity or a stromal cell of the tumoral microenvironment [38]. Among the most relevant proteins for collective cell migration are the $\beta 1$ and $\beta 3$ integrins grouped in the ruffling edges of the cell group in movement. These proteins are responsible for providing the adhesion and dynamic strength necessary for transporting the cell group. The leading cells have the greatest proteolytic capacity in relation to the ECM, and it is their production of the metalloproteinases MT1-MMP and MMP-2 that enable proteolysis, as these proteins cut collagen fibers and form a pathway for collective migration and expansion of the migratory group [39, 40]. In squamous carcinomas of the larynx, lungs, esophagus, cervix and skin, among other tissues, collective invasion has been associated with the presence of podoplanin and CDC42 proteins [41-43].

3. The epithelial to mesenchymal transition (EMT)

Regarding the acquisition of mobility, a fundamental process in the transformation to a malignant cell, the best understood phenomenon is the epithelial-mesenchymal transition. The epithelial layer is formed by cells with apical-basal polarity that maintain the laminar structure intact through various types of adhesion, thus forming the barrier necessary for the good functioning of the epithelial layers. This barrier, known as the apical joint complex, is formed and maintained by cell to cell contact at the cell surfaces that surround the apical-lateral dominion. This complex includes the adhesive joints (AJ), which promote the integrity of the tissue by maintaining a strong bond between the cells [44] and have cadherins as their principal component [45]. The resulting tight joints (TJ) form a physical barrier to the movement of ions, macromolecules, immune cells and pathogens between the epithelial cells [46] and desmosomes. The AJ as well as the TJ are closely related with the actin cytoskeleton and are functionally regulated by filaments of the circumference of actomyosin [47]. Their dynamic interaction is important during the morphogenesis of

epithelial tissues in embryonic development, in tissue repair [48], and in the maintenance of an effective epithelial barrier in an adult [49].

During early embryonic development, there is a process of essential differentiation resulting in the establishment of the germinal layers in the epithelial-mesenchymal transition (EMT) [29]. The EMT, discovered by Frank Lillie in 1908, is an important mechanism for the reorganization of the germinal layers [50]. This process, whose essential characteristics are the interruption of intercellular contacts and the acquisition of a fibroblast-type conjugated morphology, converts well-organized epithelial cells into isolated cells with a mesenchymal morphology that are capable of transporting themselves [51] to become established in new tissues, thus allowing for inductive interactions during embryogenesis. Currently, it is thought that three subtypes of EMT exist: EMT type 1 is related to embryonic development, EMT type 2 to wound healing, and EMT type 3 to tumor cells [52]. As Thiery [53] points out, it took a long time for EMT to be recognized as a mechanism of tumor progression due to the difficulty of detecting the process in patients. Today, it is known that tumor cells utilize the EMT as a key element in the invasive process, a phenomenon very similar to embryonic EMT. There are diverse pathways in common between embryonic and tumoral EMT, including the stimulation by TGF- β that induces phosphorylation of β -catenin, which in turn activates transcription factors such as Snail [54], as well as diverse growth factors and proteins. This is accompanied by a loss of diverse epithelial proteins such as E-Cadherin, α and β -catenins, Claudin, occludin and cytokeratins, and at the same time an overexpression of mesenchymal proteins such as vimentin, fibronectin, metalloproteinases, actin and integrins α v and β 1 [55, 56]. E-cadherin is a key protein because it facilitates the adhesion of epithelial cells and desmosomes [57], which in turn prevents cellular mobility and metastatic dissemination.

The cadherin switch is essential to increase mobility, but is not always necessary for the morphological changes that accompany the EMT [58]. The expression of cadherins can be inactivated by somatic mutations, hypermethylation and desacetylation of histones or transcriptional repression. Some researchers have considered that the expression of N-cadherin is more important for metastasis than that of E-cadherin [59], based on the fact that the former is a mesenchymal protein marker found overexpressed in cells with the EMT. Camand et al [60] found that a decrease in the N-cadherin levels, through regulation of focal adhesion formation, promotes a more rapid migration in healthy and tumoral glia cells. In the latter cells, this contributes to the invasive capacity of astrocyte tumors.

Integrins, a determining factor in the process of cellular adhesion, can also induce EMT. Recently, Gupta et al [61] proposed that integrin α 9 β 1 is implicated in EMT by accelerating lung tumor cell migration, mediated by scr, in a TGF β -independent manner. To date, a wide variety of proteins and factors have been discovered that regulate EMT. Indeed, it is thought that hundreds of genes significantly modify their expression during EMT [52]. One example is Twist, a potent inducer of EMT that was originally identified as an inducer of the mesoderm in *Drosophila* [62]. Its expression in epithelial cells causes the loss of cell to cell adhesion mediated by E-cadherin, which leads to cell decomposition. It also activates BMI1 polycomb proteins, which induce stem-like properties and thus annul the p53- and Rb-

dependent pathways that would otherwise allow the cells to enter in premature senescence induced by oncogenes. Twist also suppresses the production of let-7i, which in turn allows cells to acquire mobility in the last stage of EMT [63, 64]. The overexpression and activation of the signaling pathways of HER-2/Neu and TGF β activate EMT in tumoral cells of breast cancer.

EMT is also regulated by alternative splicing, such as that of ENAH, a regulating protein of the cytoskeleton of actin. The transcript present in epithelial cells contains a small exon 11a, which is absent in mesenchymal cell lines and during the EMT [65]. RBFOX2 is a splicing factor which together with ESRP1 and ESRP2, proteins for binding with RNA that are specific to the epithelium, promote splicing of transcripts that are important in EMT [66]. The loss of ESRPs in epithelial cells induces morphological changes similar to EMT [67]. On the other hand, Shapiro et al [68] found a partial induction of the splicing program in mesenchymal cells by means of the ectopic expression of ESRP1 or by elimination of RBFOX2 in immortalized epithelial mammary cells. Mesenchymal isoform IIIc of FGFR2, isoform 3 of catenin p120, and isoform CD44s of CD44 are all expressed in mesenchymal cells and in tumoral cells with the EMT, as reviewed in [69].

Just as the process of EMT resembles that observed during embryonic development, it seems that both pathways are regulated by an intricate interaction of transcriptional and post-transcriptional programs, as was discovered by observing that the ribonucleoprotein, heterogeneous nuclear E1 (hnRNP E1) is a selective transductional regulator of transcripts of EMT induced by TGF β . Due to its capacity to bind to RNA, hnRNP E1 adheres to 3'-UTR BAT structural elements present in the transcripts of mRNA necessary for EMT, and upon doing so silences these elements. This silencing, mediated by hnRNP E1, occurs during the stage of elongation when hnRNP E1 impedes the release of the elongation factor eukaryote 1 A1 (eEF1A1) of the ribosomal site A. The phosphorylation of hnRNP by Akt2, mediated by TGF β , restores the transduction of white transcripts of EMT. The attenuation of the expression of hnRNP E1 in non-invasive breast cancer cell lines induces not only EMT but also metastasis [70,71]. Furthermore, the steroid hormones such as androgens have also been observed to be implicated in the maintenance of cellular characteristics, evidenced by the fact that Sun et al [72] found that the absence of androgens in prostate tumors is associated with a worse prognosis and acquisition of cell characteristics typical of mesenchymal or stem cells.

With triple negative breast cancer (ER $^-$, PR $^-$ HET2 $^-$), there is an overexpression of N-cadherin and EMT, leading to a worse prognosis. Regulation by miRNAs is not an exception in EMT, particularly the miRNAs of the miR-200 (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) and miR-205 family. These miRNAs participate in the regulation of EMT induced by TGF β by mediating the production of ZEB1 and ZEB2 [73,74]. Recently miR-29b was added to the list of miRNAs that regulate EMT, due to the fact that an increase in miR-29b in PC3 prostate cancer cells inhibits the capacity of these tumoral cells to invade and colonize [75]. In addition to TGF β , other growth factors such as EGF, HGF, PDGF, FGF2, TNF α and IGF can induce EMT by activating the expression of transcriptional repressors of E-cadherin or

by activating the transduction signaling pathways, such as MAPK, PI3K, Wnt/ β -catenin, NF- κ B and Notch [69 and references there].

3.1. EMT reversion

After tumoral cells are disseminated, they must reactivate their epithelial properties by means of the reversion of the process of EMT, known as the mesenchymal-epithelial transition (MET). In the metastatic process, just as EMT is essential for the initial transformation of cells from being part of the epithelial tissue to being released from intercellular connectedness and ready for transport, MET is critical for the later stages of metastasis. Possibly this latter change is triggered by the local environment after extravasation towards the parenchyma of the invaded organs [76]. Although EMT has been extensively studied, there is much yet to be discovered about the molecular mechanisms that regulate MET. One of the keys to the process of MET is the re-expression of E-cadherin, which enables tumor cells to interact with the tissue of the recently colonized organ [77]. Leontovich et al [78] demonstrated that the constitutive activation of Raf-1 induces the overexpression of HER-2/Neu, leading in turn to the development of metastasis in xenografts of MCF7 ER α + cells. This metastasis was linked to the activation of the MET pathway, and curiously it was characterized by a reduction in the expression of genes implicated in EMT, such as TGFB2, TWIST and FOXC1. On the other hand, Phino et al [79] found that the levels of glycosylation of E-cadherin regulated by Mgat3/GnT-III were diminished in EMT, and that they were recovered when the cells once again acquired an epithelial phenotype. Other genes identified in MET are WT1, BMB7, WNT4 and the protein formin IV of the cytoskeleton [53 and references there]. Diverse miRNAs have been found to be deregulated in tumors, particularly metastamir, which is implicated in metastasis [80]. Wang & Wang [81] summarized the types of metastamir that are deregulated in breast cancer cells: miR-9, -10b, -21, -29a, -31, -103/107, -126, -335, -210, and -373. All of these are involved in migration, aggressiveness and the worst prognoses. An increase in the levels of miR-200 induces MET by reducing motility and aggressiveness [82]. Likewise, the expression of miR124 modifies the morphology and capacity of metastasis of MDA-MB-231 cells, as well as by decreasing the levels of vimentin, a mesenchymal marker [83]. The regulation of diverse transcription factors, receptors for growth factors (including FGFR2b, FGFR2c, EGFR and HER2), and the activation of Akt are other elements in the reversion of MET.

3.2. Collective to ameboid transition and the mesenchymal to ameboid transition

Through the process of metastasis, cells can undergo changes in the way in which they transport themselves. In explants of melanoma, it has been observed that the inhibition of the integrin β 1 provokes the separation of groups of cells, which thus become individual ameboid cells. These cells acquire the capacity to migrate, independently of proteases and/or integrins, similar to that observed in ameboid movement. Hence, the transition from collective invasion to ameboid movement of individual cells is an interchangeable and bidirectional process that depends on environmental factors, making evident the great

plasticity that tumoral cells can have [84]. The mesenchymal-ameboid transition can serve as a compensatory mechanism of migration after the inhibition of pericellular proteolysis. This transition provokes a change from a cellular form similar to a fibroblast to a round or elliptical shape, and at the same time spurs a change in the cytoskeletal organization as well as the distribution of integrins [85].

4. The tumor microenvironment

During the process of migration, cancerous cells pass through different microenvironments, including the stroma, the endothelium of blood vessels, the lymphatic vessels and the tissue of the organ where the secondary tumor forms [86]. In each of these sites, the microenvironment exerts a strict control over the behavior of tumor cells [87].

Cells can cross the basal membrane (BM) during embryonic development and immune vigilance, either as part of the embryonic process or more routine morphological processes such as leukocyte activity [88]. The BM is a dense layer of the extracellular matrix that is highly reticular and rich in laminina, collagen type IV, perlecan, nidogen-1 and nidogen-2. Its structural function is to delimit epithelial and endothelial tissue, and to give polarity to cells [89, 90]. After the loss of intercellular adhesion, cells acquire a mobile phenotype and produce metalloproteinases that allow them to digest the BM. Hence, the BM is the first barrier that tumor cells must cross [2]. As a tumor invades an organ, there is a dynamic interaction between invasive cells, the invaded tissue and the BM that separate the two. Although it has not been easy to study this process *in vitro*, the transmigration of leukocytes has proved to be a very useful model, allowing for the identification of regions of the perivascular BM that have less resistance to invasion. These regions contain reduced levels of laminin and collagen IV [91]. It is not yet clear how carcinoma cells initiate the invasive process after abandoning the primary tumor, but there is evidence that points to the participation of invadopodium in the proteolysis of vascular BM.

4.1. Interactions with the extracellular matrix

A critical component of the cellular microenvironment is the extracellular matrix (ECM), a biopolymer complex that provides healthy and tumor cells with biophysical and biochemical signals that influence their function and survival [92]. Cells are capable of sensing and responding to changes in the conformation of the ECM through a process of mutual feedback that involves cellular contractile mechanisms. Each cell type responds to the rigidity that is characteristic of its host [93]. Integrins are crucial participants in the communication of cells with the ECM, because they act as a conduit between extracellular ligands and the cytoskeleton. Therefore they have the capacity of responding to the rigidity of the external substrate through a counter-response exerted by the actomiosin network. They act as mechanosensors that undergo conformational changes in response to mechanical forces, resulting in increased adhesion between the cells and the ECM, the formation of focal adhesions, and the dispersion of the cells [94]. The extracellular signals mediated by integrins are transduced internally through focal adhesion components [95].

Tumor cells have the capability of ignoring the conformational changes that take place in the ECM, and this capability is associated with the activation of integrin $\beta 1$ and the phosphorylation of FAK in a manner dependent on fibronectin and the overexpression of $\alpha v\beta 3$ [96]. Lysyl oxidase (LOX), a copper-dependent amine oxidase that catalyzes the crosslinking of collagen, elastin and fibrin in the ECM, can modulate metastasis by facilitating extravasation or by conditioning the metastatic niche, the latter done by increasing the rigidity of the ECM and fibrillar collagen [97]. As can be seen, although the ECM is considered a barrier for the dissemination of tumor cells, its remodeling by tumor cells actually converts it into a promoter of the same process. The release of proteins from the ECM by tumor MMPs can create a promigratory stimulus, in part by increasing the rigidity of the tissue, which in turn augments the motility of tumor cells [98].

4.2. The cancer-associated fibroblast and the tumor associated macrophages and immune cells

Diverse signals emanating from tumor cells allow them to recruit and activate host cells, particularly monocytes, fibroblasts and mesenchymal stem cells, all of which are abundant in the microenvironment of a tumor. Among these cell types, fibroblasts are the most abundant and important in the interaction with the tumor. The carcinoma associated fibroblasts (CAFs) are miofibroblasts with contractile properties and a staining capacity for alpha-smooth muscle actin (α -SMA). Several studies have reported that they can potentiate tumorigenesis by expressing proteins that favor migration or by altering the stromal environment in a way that facilitates colonization. Brentnall et al [99] point out that the overexpression of paladin by the activation of k-ras can transform normal fibroblasts into miofibroblasts in 5 days. As a result, the expression of paladin alters the morphology and behavior of cells, leading to an increased cellular migration, the degradation of the ECM by the formation of invadopodia, and the creation of tunnels through which cancerous cells can transport themselves. Malanchi et al [100] found that to facilitate the process of colonization, infiltrating tumor cells need to induce the expression of stromal periostin (POSTN) in the target organ for a secondary tumor. POSTN is a component of the ECM that is expressed by fibroblasts, both in healthy tissue as well as in the stroma of a primary tumor. Møller et al [101] reported that fibulin 5, whose expression is downregulated by factors released by fibroblasts in tumors, is capable of suppressing metastatic colonization of the lungs and liver by inhibiting the production of metalloproteinase 9 and by reducing the invasive behavior of fibroblasts. Hence, tumor cells avoid the expression of this protein.

Epithelial cells produce multiple factors that recruit various cells able to facilitate the infiltration of tumors, including tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), lymphocytes, mesenchymal stem cells, endothelial cells and the aforementioned CAFs. In the case of macrophages, experimental evidence exists supporting the idea that they can differentiate into pro-inflammatory macrophages (M1) or anti-inflammatory macrophages (M2), depending on the stimulus [102,103]. TAMs are similar to M2 macrophages, promoting tumor progression by their incapacity to induce T cells, their increased expression of manose and scavenger receptors, as well as their release of pro-

tumorigenic factors (e.g., TGF- β 1, IL-10 and MMPs) and pro-angiogenic factors [104]. As they generally accumulate in hypoxic zones, the pro-angiogenic program of TAMs is triggered, resulting in the secretion of VEGF, IL-1b, TNF-a, angiogenin and semaphorin 4D. By secreting urokinasetype plasminogen activator (uPA) and its receptor, uPAR, TAMs can participate in the degradation of the ECM [105].

The non-neoplastic cells present in the stroma of the tumor produce a series of chemokines and growth factors that form a complex network of communication within the tumoral microenvironment. One factor produced by the CAFs is CXCL12, which promotes the migration of tumor cells towards blood vessels [106]. TAMs co-migrate with tumor cells in a paracrine-dependent manner. They produce EGF, which increases the migration of breast cancer cells that express EGFRs. In response, these tumor cells secrete CSF1, thereby attracting the TAMs that express CSF1Rs [107]. Campbell et al [108] found that the TAMs positive to CD68 and PCNA are associated with a worse prognosis for breast cancer patients.

Macrophages are capable of regulating other stromal cells and of exerting influence through them. For instance, the loss of signaling by TGF- β in mammary fibroblasts produces an increase in the secretion of CCL2, which in turn results in a progression of 4T1 tumors, either through direct action on cancerous cells or through the recruitment of macrophages [109]. The infiltration of lymphocytes is associated with metastasis and an increased expression of the activator of the NF- κ B (RANK) receptor and of its ligand (RANKL). In particular, T regulatory cells (Treg) infiltrated in a breast cancer tumor stimulate metastasis in breast cancer through the signaling by RANKL-RANK to IKK- α , causing the suppression of the inhibitor of the antimetastatic serine proteinase and maspin, and favoring the survival of circulating tumor cells [110]. It is possible that CAFs express CCL5, thus attracting Treg cells that express CCR1 and produce RANKL. Treg cells secrete IL-4 and IL-13, which in this case would result in the activation of TAMs and consequently the promotion metastasis in RANK-positive tumors [111]. Neutrophils, on the other hand, participate in the preparation of the tumoral microenvironment. They produce enzymes that remodel the ECM, and also produce ROS, which in turn activates NF- κ B and in this way allows tumor cells to attenuate their apoptotic response and increase their mutation rate [112].

5. Survival of tumoral cells and modulation of cell survival

The metastatic process has various rate-limiting steps, and consequently only a minority of tumor cells is able to reach distant sites [86]. It has been suggested that approximately 0.01% of circulating tumor cells can manage to produce a metastasis [113]. Talin 1 is one of the proteins whose altered expression allows for the survival of tumor cells. It can recruit focal adhesion proteins ILK, FAK and Src through its interaction with integrin β , resulting in the promotion of the survival, invasion and angiogenesis of tumor cells. Its overexpression allows prostate cancer tumor cells to activate survival signals and resist death by anoikis [114]. In this sense, the PI3K/Akt and Wnt/ β -catenin signaling pathways, along with mutations in p53 and other genes, play an important role in the avoidance of death by metastatic cells.

The adhesion of tumor cells to the ECM is not sufficient for inducing a survival signal. The changes in the cytoskeleton associated with adhesion signaling are critical for tumor cell survival. Halder & Johnson [115] indicate that the newly established Hippo suppressor pathway (which regulates the size of organs by maintaining a balance between proliferation and apoptosis in physiological conditions) might be involved in tumor proliferation. The Hippo pathway phosphorylates and inhibits the transcriptional coactivator Yes-associated protein (YAP), a key oncogene for the regulation of the size of organs. This inactivation is triggered by the reorganization of the cytoskeleton. Once the cell has separated, the Hippo kinases Lats 1/2 are activated, causing the inhibition of YAP, and therefore resulting in anoikis. It has been found that with metastatic prostate cancer, the levels of expression of Lats 1/2 are significantly downregulated [116], whereas, Lamar et al [117] demonstrated that increased YAP activity promotes metastasis and tumor growth at both the primary site and the metastatic site.

5.1. Integrins in cell survival and apoptosis (anoikis)

A loss of balance between cell division and cell death is common in cancer, due to a decrease in apoptotic cell death that leads to the progression of a tumor. There are various ways by which a malignant tumor cell averts or resists apoptosis.

The distinctive morphological characteristic of apoptosis is the condensation of chromatin and the posterior fragmentation of the nucleus, accompanied by a reduction of cell volume, a retraction of pseudopods, and the formation of vacuoles, all leading to a loss of integrity of the cell membrane [110]. The biochemical changes that occur during apoptosis are: (i) the activation of caspases, (ii) the rupture of DNA and proteins, and (iii) changes in the membrane related to its recognition by phagocytes [111].

Tumor cells can avert apoptosis by altering the balance of pro-and anti-apoptotic proteins, decreasing the function of caspases, and altering the signaling of receptors related to cell death [112]. A cancerous cell in an ectopic site, whether near a primary tumor or in route to a secondary organ, can employ various mechanisms to avert the process of apoptosis known as anoikis [113]. Through anoikis, a healthy cell in an inappropriate location activates its programmed death by separating itself from its neighbors and its microenvironment, and thus is eliminated. Anoikis is therefore a barrier against the formation and survival of potentially oncogenic clones. Only non-adherent cells, such as leukocytes and mature hematopoietic cells, are protected from anoikis [114].

The principal effector mechanisms of anoikis are autophagy and apoptosis [115]. A critical step in the series of changes through which a tumor cell passes to avoid apoptosis is a change in the expression of integrins, which are a family of receptors that receive signals from the ECM. This change is based on genetic and epigenetic alterations that can only occur in the microenvironment of a tumor [116], allowing tumor cells to ignore signals by the ECM and act as if they were in the appropriate microenvironment. Besides this extrinsic pathway, tumor cells can also avoid apoptosis by damaging the mitochondria, an intrinsic pathway allowing these cells to hyperactivate mechanisms of survival and proliferation

[117]. Another important factor in anoikis is the integrity of the cytoskeleton [118], since many pro-apoptotic proteins, including BIM and BMF, co-locate with the cytoskeleton.

In vertebrates, integrins are a family of receptors composed of 18 subunits α and 8 subunits β , whose combination gives rise to 24 types of these receptors [119]. Changes in the integrins that act as receptors for the ECM are essential for the avoidance of anoikis. The normal functioning of these receptors not only provides a physical bond with the ECM, but also establishes a platform that depends on signals from adhesion molecules, including adaptor proteins and kinases [120, 121]. For instance, the integrins $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha5\beta1$, $\alpha6\beta1$, $\alpha6\beta4$ and $\alpha v\beta3$ have a profound impact on cell survival [122].

A particularly important structure in this sense is hemidesmosome, in which there are complexes of integrin-talin-paxilin-actin that receive signals from the ECM and transduce these signals through various proteins such as focal adhesion kinase (FAK) in order to avoid anoikis. FAK, the kinase linked to the integrin ILK, tyrosine kinase Src, PI3K, the extracellular signal-related kinase (ERK) and the adaptor protein Shc are key to the transduction of signals mediated by integrins for protection against anoikis [123]. The FAK binds to the cytoplasmic tails of the integrins and is autophosphorylated at the Y397 residue in order to transmit a survival signal [124]. After the adhesion of integrins with the appropriate proteins of the ECM, FAK and ILK recruit and activate PI3K/Akt, ERK and the Jun kinase (JNK) pathway [125].

The separation of these cells, which move towards the vascular lymphatic space, as well as their implantation in a site with an unknown ECM can cause the separation of the α and β subunits of the heterodimeric receptors of integrin, which in turn leads to the deactivation of FAK, of the family of tyrosine kinases Src, and of ILK. Whereas this attenuates pro-survival pathways, including the mitogen-activated protein kinase/ERK, Akt/phosphatase, tensin homologue (PTEN), and the nuclear factor κB (NF- κB) [126], it at the same time stimulates other mechanisms of survival. The latter mechanisms include the activation of PKB/Akt, which can inhibit anoikis at multiple levels. This inhibition takes place by the inactivation of caspase 9, by a scavenging process that is dependent on the phosphorylation of the pro-apoptotic Bad protein by the 14-3-3 protein, by the activation of NF- κB , and by the inhibition of transcription factors Fork-head [127-130].

It has been seen that the response to anoikis can be recovered by silencing FAK with siRNAs in pancreatic cancer cells [131]. Recently it was found that the inhibition of the proteins of the subfamily of Rho and the expression of Akt in the B16F10 cell line inactivates the FAK pathway and induces anoikis in resistant cells [132]. Whereas healthy epithelial cells express the receptor for collagen $\alpha2\beta1$ and receptors for laminin $\alpha3\beta1$ and $\alpha6\beta1$, hyperproliferative cells overexpress integrins $\alpha v\beta5$ and $\alpha v\beta6$ and squamous carcinoma cells overexpress $\alpha v\beta6$. The latter integrin is required for the adhesion of melanoma cells to dermic collagen and for survival in the environment of the new tissue [133-137]. Goldstein et al [138] found that the substitution of valine by glutamic acid at aminoacid 600 of B-RAF (B-RAFFV600E), which is found in 66% of melanomas, is an event that leads to resistance to anoikis by continuous activation of MEK/ERK and the inhibition of BIM, a pro-apoptotic protein. Whereas in a

healthy cell BIM levels are increased upon its separation from its tissue as a mechanism to activate its programmed death, this does not occur in tumor cells. Although the overexpression of integrin $\alpha v \beta 3$ is associated with an increase in invasiveness, it has also been implicated in the induction of anoikis, which suggests that it has a dual role in tumorigenesis [139, 140].

ANGPTL4, a regulator of the metabolism of lipids, leads to the activation of Rac1 and FAK by binding to integrins $\beta 1$ and $\beta 5$. This phenomenon protects against anoikis through the activation of Src, Akt/PKB and ERK. Hence, the binding of ANGPTL4 with integrins is capable of deceiving the mechanism of programmed death by generating a false impression of anchorage [141]. The phosphorylation of ERK2 by FAK, though, leads to the phosphorylation of BIM, marking the latter for ubiquitination and degradation. Another critical protein for resisting anoikis is the $\alpha 5$ integrin, which as shown by Shen et al [142], has its expression in gastric cancer regulated by the S100A4 protein. S100A4 permits the survival of tumor cells and favors the overexpression of the $\alpha 5$ integrin.

By interacting with integrins, the microenvironment modulates their function in an important way. Marchan et al [143] found that $\beta 3$ expressed in pancreatic cancer cells has important effects, including the promotion of migration in single-layer cell cultures, the induction of anoikis *in vitro*, and the suppression of tumor growth *in vivo*. At the same time that tumor cells avoid programmed death by anoikis, they acquire a mesenchymal phenotype that allows them to begin migration.

6. Transendothelial or lymphatic migration and extravasation

Tumor cells must go in and out of blood vessels by crossing endothelial layers as part of the metastatic process, and in order to do this they undergo dramatic changes in their shape, driven by a reorganization of their cytoskeleton. The most difficult part of these changes in malignant cells, which adopt elastic and viscous properties for this purpose, is the deformation of the nucleus in interphase, because it is 10 times more rigid than the cytoplasm [144].

Intravasation is the principal route for the dissemination of tumor cells coming from carcinomas. Once inside of a blood vessel, the survival of tumor cells is affected by various factors, including hemodynamic forces, immunological stress, and collisions with blood cells. Consequently, only a very small number of tumor cells manage to leave blood vessels [145]. The majority of tumor cells that enter the bloodstream are detained in the microvasculature of the first organ they pass through [146].

It has been demonstrated that in breast cancer tumors, intravasation depends on the paracrine loop between tumor cells and TAMs [100]. In one clinical study of patients with breast cancer, the half-life of tumor cells in the bloodstream was from 1 to 2.4 h [147]. Png et al [148] pointed out that endothelial cells play an important initial role in metastasis by providing signals that promote this process. These researchers identified molecules such as secretory IGFBP2, the transference protein PITPNC1, and the kinase MERK as necessary for mediating the recruitment of metastatic endothelial cells.

Brown & Ruoslahti [149] suggested that there are molecules that favor the capture of tumor cells in blood vessels. In the case of breast cancer cells, the expression of metadherin favors homing in the pulmonary vasculature. In the majority of tumors of epithelial origin, the first sign of metastasis is found in regional lymphatic nodes. Once inside the lymphatic vessels, tumor cells can produce local or regional metastasis, or can enter in a quiescent state, whose duration depends in great part on the immune system. Immune vigilance can induce quiescence in individual cells by detaining the cell cycle through signals mediated by cytokines [150, 151]. It is estimated that 80% of metastasis of solid tumors, such as in breast cancer and melanoma, are disseminated through the lymphatic system [151].

In the bloodstream a tumor cell can be detained or can succeed in passing through the wall of a blood vessel. If the tumor cell has a diameter greater than that of a given blood vessel, mechanical or occlusive detainment will occur. This mechanism of detention has been observed in mouse models of brain cancer metastasis [152]. On the other hand, if the tumor cell has a diameter less than that of the blood vessel, it must bond to the wall of this channel in order to be able to leave the bloodstream. There is evidence that tumor cells are associated with platelets, which can mask and protect these cells from being located by natural killer cells [153]. Platelets can also facilitate the accumulation of VEGF at the bond between a tumor cell and the endothelium tissue, favoring vascular hyperpermeability and consequently extravasation [154].

7. Colonization

During the progression of cancer, the colonization of distant organs by circulating tumor cells marks the difference between a possibly curable tumor and a systemic and generally incurable disease [155]. After an analysis of breast cancer tumors, Paget [156] concluded that the local microenvironment of some organs must be favorable for the dissemination of tumor cells or their progenitors. On the one hand, the microenvironment of the target organ is a determinant for the survival of tumor cells, and on the other hand the accumulation of these malignant cells in a given organ can create an adequate stromal microenvironment to allow metastasis [157].

In the preparation of an adequate niche, the primary tumor secretes VEGFA to mobilize progenitor hematopoietic cells from bone marrow (HPCs) toward the bloodstream, and from there to the site of metastasis. The same process occurs with fibronectin, metalloproteinases and growth factors, all of which can be synthesized by the primary tumor and released to the bloodstream, later to accumulate in the target organ. In this way, these molecules prepare a premetastatic microenvironment, to which the HPCs arrive and continue this process of adaptation for the purpose of their own survival.

Tumor cells and macrophages are recruited towards the premetastatic niche due to the chemokines synthesized by cells associated with cancer [158]. It has been shown that chemokines are chemotactic molecules important for colonization of a given organ [159, 160]. Wendel et al [161], by utilizing intravital observation techniques, were able to

demonstrate that the receptor of chemokine CXCR4 plays an important role in the extravasation of breast cancer tumor cells towards their target organs.

The anatomy of some organs provides a barrier to metastasis. In the case of the lungs, endothelial capillaries are surrounded by a basal membrane [162]. In the blood-brain barrier, capillaries have tight junctions and astrocyte foot processes [163]. Capillaries in bone marrow and the liver, on the other hand, are fenestrated, making them susceptible to invasion [162].

Another important factor in the ability of a tumor to invade a distant organ is the type of primary tumor. Breast cancer tumors positive for estrogen receptors, as well as ocular prostate cancer tumors and melanoma tumors, can give metastatic signals decades after elimination of the primary tumor [7, 164-166]. This is due to the fact that tumor cells are disseminated from the primary tumor various years before detection of the primary tumor. Adenocarcinoma of pancreatic and lung cancer have malignant cells that rapidly acquire the capacity to infiltrate and colonize without requiring a process of quiescence to generate a macrometastasis [167, 168]. Furthermore, tumor cells in general can adopt two distinct states of quiescence while awaiting a favorable microenvironment. They can exist as individual cells (cellular quiescence) or as small indolent groups (tumor mass quiescence), in either case maintaining a balance between proliferation and death during the period of latency [169].

7.1. Most frequent organs for metastasis

Although no organ is totally protected from the development of metastasis, this process tends to take place more frequently in certain regions of the body, including the lymphatic nodes (lymphatic metastasis), lungs, liver, bone marrow, brain (haematogenic metastasis), peritoneum and pleura [145]. Each type of cancer is more prone to certain organs. When it occurs, metastasis exists in at least 2 organs, with an average of 5.6 metastases per patient.

With breast cancer, metastasis generally is a slow process that occurs in the lymph nodes, lungs, bone marrow, liver or brain. Only in 6-18% of breast cancer patients does metastasis takes place in the gastrointestinal tract, with the stomach and small intestine being the most frequent hosts. On the other hand, colorectal cancer rarely metastasizes to bone marrow [170]. Adrenal glands are the preferred site for metastasis of lung cancer tumors with small cells [171]. In North and South America, lung and breast cancer are the first and second source of metastasis to the brain. Generally the earliest metastasis takes place with lung cancer [172], with 80% of such events occurring in the cerebellar hemispheres [173]. However, the most frequent metastasis to the cerebellar hemispheres occurs in breast cancer patients [174]. This frequency can be explained in part by the production of osteoclast-activating factors, such as the parathyroid hormone related protein (PTHrP), IL-1, IL-6, IL-11 and GM-CSF by breast cancer tumor cells [175]. These osteoclasts release growth factors derived from bones, such as TGF- β and IGF-1 [176]. Contrarily, the metastasis of tumor cells from prostate cancer to bone marrow generally stimulates the formation of bone, regulated by the production of osteoblast-activating factors such as endothelin-1, BMPs and PDGF. In

this way, the activated osteoblasts can maintain the proliferation and survival of the tumor cells [177].

Cyclooxygenase COX 2, EGFR, the ligand HBEGF and α 2,6-sialyltransferase ST6GALNAC5 are mediators of the passage of cancerous cells through the blood-brain barrier. Nevertheless, only ST6GALNAC5 mediates metastasis of breast cancer tumor cells to the brain, facilitating the both the passage through the blood-brain barrier and the adhesion of these cells to brain endothelial cells [178]. A recent study [179] reported that the greatest number of metastasis to the brain occur in breast cancer patients of African origin. The least frequent teratogenic metastases are found in the kidneys, gonads, spleen, subcutaneous fat and especially in the walls of the gastrointestinal tract, uterus, heart and skeletal muscle [180].

The gradients of chemokines can explain the tropism of some types of cancer. It has been suggested that in organs with high levels of chemokine expression, these latter molecules can attract metastatic tumor cells that express the corresponding receptor. This model has been demonstrated in the metastasis of breast and prostate cancer tumors to bone marrow due to the presence of CXCR4-CXCL12. With solid and hematopoietic tumors, chemokines CCL21 and CCL19 are present in metastasis to lymphatic nodules with the corresponding receptor CCR7 [18].

8. Conclusion

The capacity of tumor cells to be transported to organs distant from the primary tumor is driven by two types of factors: the complex interactions between tumor cells and their surroundings, and the changes in such cells that allow them to adapt to their environment. Among the latter factors is the hallmark of tumor cells: their capacity to avoid apoptosis. At the onset of the metastatic process, the epithelial-mesenchymal transition is critical for a tumor cell to escape the controls of adhesion and be released for transport from the primary tumor. It must then be able to move across the cell membrane, remodel the extracellular matrix, transport itself by circulation, and establish itself in a distant organ. The mesenchymal-epithelial transition is critical for this last stage in which the tumor cell survives in a new organ. Although very few tumor cells are able to achieve this task, metastasis unfortunately is a common occurrence with cancer patients due to the continuous exposure to the development of the primary tumor, to growth factors, to angiogenesis and to accumulated genetic changes. The great complexity of the regulatory network that is implicated in the susceptibility of an organ to metastasis, as well as the capacity of tumor cells to dodge the immune response and influence the microenvironment of a given organ to make it favorable to metastasis, makes the study of metastasis for the purpose of drug design for cancer treatment a titanic task. The advantage is that there are now thousands of researchers and hundreds of thousands of reports in the field of metastasis. Furthermore, metastasis is a lengthy process, often taking a decade. Hence, the challenge is to detect a tumor after the onset of the parent cancerous cell but before metastasis.

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