**Teaching unit 10**

**INVASIVENESS AND METASTASIS OF TUMORS**

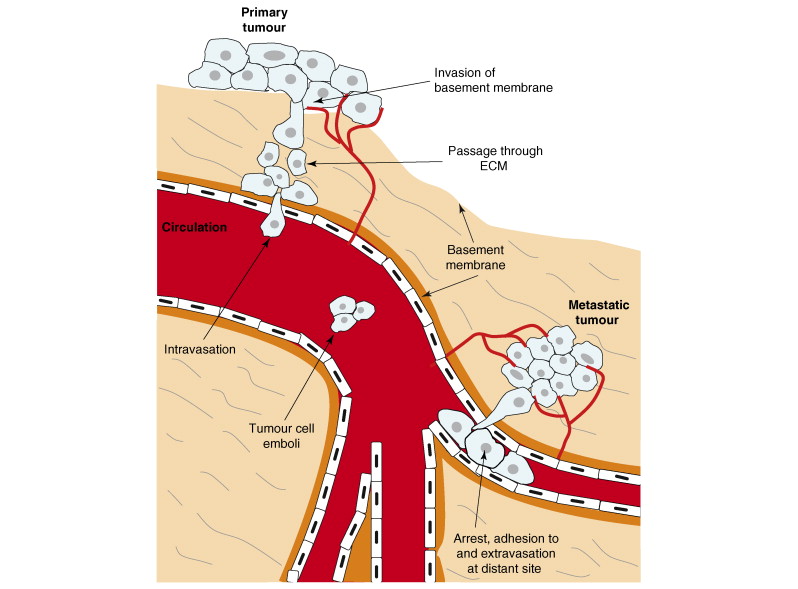
Tumor metastasis is the process of spread of malignant cells from the primary tumor to distant places. The establishment of metastases is the last qualitative step in the progression of malignant tumors. The dynamics of tumor progression depends on gene mutations, cell selection and tissue organization. Initial gene mutations, i.e. disruption of oncogene activation and anti-oncogene inactivation, provide tumor cells with a proliferative advantage and growth autonomy. The accumulation of numerous genetic changes results in a "malignant" phenotype of the tumor cell, which is characterized by insensitivity to inhibitory signals, unlimited replicative potential, avoidance of apoptosis, angiogenic potential, invasiveness and metastasis. These cells become the precursors of the cell population that dominates the tumor tissue. Any genetic change in a malignantly transformed cell is subject to clonal selection. Thus, within the tumor mass, the clone of cells that has a phenotypic advantage over other clones dominates, namely growth autonomy, insensitivity to inhibitory signals, "avoidance" of apoptosis, and others.

In 1889, Stephen Paget put forward a theory of metastasis called "seed and soil". He established this theory based on the findings that different types of tumors form metastases in specific organs and assumed that malignant cells represent "seeds" while distant organs represent the "soil" inhabited by these cells. According to this theory, the process of tumor metastasis is not random, but is the result of specific effects of the microenvironment of the target organ on the homing of metastatic cells. Contrary to this theory, James Ewing considered that the tissue tropism of malignant cells is determined by mechanical factors and the circulation pattern of the primary tumor. For example, metastatic colorectal cancer cells can enter the portal system, which explains the propensity of this type of tumor to metastasize to the liver, while prostate cancer cells can pass through the presacral plexus that connects the periprostatic and vertebral veins, which explains the metastasis of malignant cells to the lower parts of the spine and the pelvis.

**Major steps in the metastatic cascade**

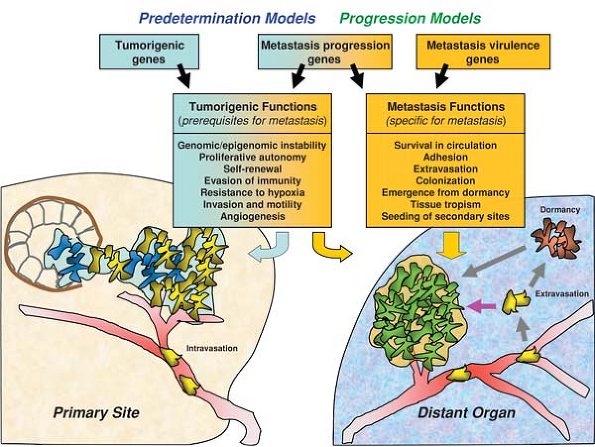
The establishment of metastases takes place in a cascade. In this metastatic cascade, several major steps are:

1. **Invasion and mobility**. In physiological conditions, cells are in contact with the basement membrane and/or with neighboring cells, which provides them with the necessary signal to maintain cellular homeostasis. The first step in metastasizing is the separation of cells from the primary tumor mass. Tumor cells lose contact with each other as well as contact with the extracellular matrix and thus become mobile, which is a fundamental characteristic of metastatic cells. Such cells then enzymatically break down the extracellular matrix, which enables them to migrate further.
2. **Intravasation and survival in circulation**. Metastatic cells break through the basement membrane of local post-capillary blood vessels and thus enter the circulation. In the circulation, they become targets of immune cells, as well as mechanical influences (blood turbulence), so that only those malignant cells that have managed to survive the aforementioned influences are attached to the endothelium of capillaries in distant organs. When they reach the circulation from a solid tumor, malignant cells cannot survive as individual cells, which is why they often interact with each other, as well as with blood cells, and thus form tumor emboli in the circulation, which enables them to survive.
3. **Extravasation of malignant cells into parenchymatous organs.**When malignant cells are arrested in the capillary system in distant organs, tumor cells leave the circulation and enter the organ parenchyma. The process by which tumor cells leave the circulation during dissemination is very similar to the way leukocytes do this using the same adhesive molecules (eg selectins and integrins) as leukocytes during extravasation.
4. Growth of metastatic colonies in distant organs. Successful adaptation to a new microenvironment promotes the establishment of metastatic colonies, and implies the existence of growth factors as well as the preservation of the sensitivity of metastatic cells to those factors (Figure 1).

[](http://www.google.rs/url?sa=i&rct=j&q=&esrc=s&frm=1&source=images&cd=&cad=rja&docid=nkQeYlzSQ3CehM&tbnid=7BrXEARX0eXvuM:&ved=0CAUQjRw&url=http://www.sciencedirect.com/science/article/pii/S0167779907000212&ei=Pn85UaH8BInCO7vXgKgD&bvm=bv.43287494,d.ZWU&psig=AFQjCNGqDvS-qBN0-Nsp4Ac-t8BnzDWCpA&ust=1362808922950686)

**Picture 1.**Metastatic cascade

All these mentioned steps in the metastatic cascade are necessary for the successful formation of metastases. Thus, if only one of these steps is missing, metastasis of tumor cells is unsuccessful. Only those tumor cells that have acquired all the necessary genetic changes can successfully form metastatic foci. The inability of tumor cells to complete the metastatic cascade is also a consequence of the fact that in the circulation these cells are more susceptible to the dying process and become targets of immune cells. Circulating tumor cells are characterized by twice as much spontaneous apoptosis compared to cells in the primary tumor. Not all circulating tumor cells are metastatically competent, ie. able to colonize distant organs.

Numerous genes are thought to be responsible for each individual step in the establishment of metastasis. These genes are classified into three groups - genes for the initiation of metastases, genes responsible for the progression of metastases and genes of metastatic "virulence". The initiation of metastases depends on the newly acquired functional properties of malignant cells, some of which enable them to avoid local hypoxia by initiating neoangiogenesis, as well as the epithelial-to-mesenchymal transition (EMT) program, which involves reducing the expression of adhesive molecules and consequently increases the mobility of tumor cells. New functions responsible for metastatic progression relate to matrix remodeling, evasion of the immune response, and extravasation. Metastatic virulence is a property that provides some tumor cells with a selective advantage over other cells during adaptation to organ-specific conditions and expansion in a new environment (Figure 2).

**FFigure 2**. Metastasis model

It is believed that in the tumor tissue, only a small number of tumor cells manage to accumulate all the genetic changes that are necessary for the full metastatic potential of the cells. Therefore, the primary tumor is heterogeneous in terms of metastatic capacity and in it an extremely small number of malignant cells can successfully metastasize (less than 0.01%). Thus, the dominant clone of tumor cells in the primary tumor undergoes a series of genetic changes and thereby acquires a "selective advantage" over other clones, as well as the ability to metastasize. In metastatic lesions, malignant cells are assumed to originate from a single precursor cell that possesses full metastatic potential.

**SELECTIVE PRESSURE IN THE PRIMARY TUMOR INFLUENCES THE ACQUISITION OF METASTATIC POTENTIAL**

The selective pressure in the primary tumor determines the metastatic potential. Hypoxia and inflammation play an important role in metastasis and influence the tumor to cooperate with numerous cells, such as bone marrow-derived cells (BMDCs), among which myeloid suppressor cells (MDSCs) are important. Myeloid-Derived Suppressor Cells and Mesenchymal Stem Cells (MSCs). These cells, together with the cytokines they produce, increase the ability of tumor cells to migrate, invade and overcome hypoxia. In addition, these cells participate in the maintenance of the immunosuppressive microenvironment. The ability of malignant cells to initiate the epithelial-to-mesenchymal transition (EMT) program in the primary tumor is also a result of the selective pressure they face in the primary tumor. Initiation of EMT in metastatic cells results in their migration, invasion and intravasation and is considered responsible for the development of circulating malignant cells. Events related to hypoxia and inflammation contribute to the acquisition of metastatic potential by increasing the ability of circulating malignant cells to extravasate and survive in the circulation, and participate in the formation of the premetastatic niche. Although premetastatic niches facilitate the adaptation of metastatic cells to the "new conditions" of the microenvironment in distant organs, additional selection is necessary for complete colonization of these cells.

**Hypoxia**

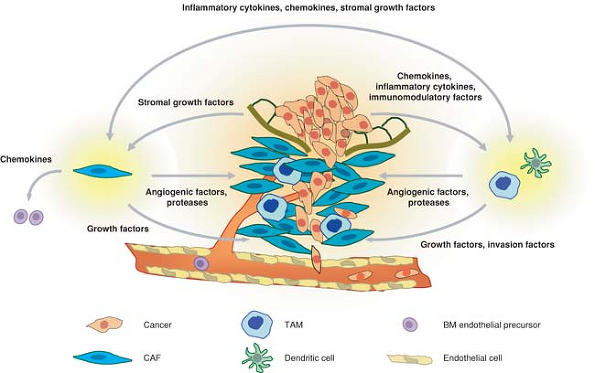
There are numerous ways by which malignant cells manage to adapt to hypoxia in the tumor tissue, of which the transcription factor HIF (hypoxia-inducible factor) occupies a central place. Under hypoxic conditions, HIF1α and HIF2α are stabilized resulting in the transcription of more than 100 genes. These target genes are involved in angiogenesis, glycolysis, and invasion, which together help tumor cells adapt to hypoxic conditions. For example, the expression of genes for angiogenesis, such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), increases. These two growth factors induce increased permeability of blood vessels resulting in the extravasation of proteins involved in the remodeling of blood vessels and extracellular matrix in the vicinity, as well as in the activation of endothelial cells involved in the creation of a new vascularized region in the tumor tissue. Various genes for glycolysis are expressed and the products of this process affect the acidification of the extracellular space. Since these conditions are toxic for cells, further adaptation of tumor cells is necessary, which includes increased expression of the H+ transporter and the acquisition of resistance to apoptosis. In order to support the invasion of tumor cells into the newly vascularized region, HIFα increases the expression of MMP1 and MMP2 (Matrix Metalloproteinase-1 and -2), LOX (Lysyl Oxidase) and the chemokine receptor CXCR4. Degradation of the basement membrane under the influence of MMP2 and changes in the extracellular matrix under the influence of MMP1 and LOX enable the physical barrier to be removed, which results in the migration of malignant cells. Thanks to the expression of CXCR4, malignant cells migrate to regions of angiogenesis. Invasion of malignant cells through the basement membrane is a characteristic of a malignant tumor.

By adapting to the selective pressure of hypoxia, tumor cells acquire the ability to withstand unfavorable microenvironmental conditions by inducing glycolysis switching, resistance to apoptosis, angiogenesis and invasion through the extracellular matrix. All these acquired functions enable tumor cells to metastasize to distant places. For example, anaerobic metabolism and resistance to apoptosis are essential for the survival of metastatic cells both in the circulation and in distant organs. The ability of malignant cells to invade and migrate allows them to enter and leave the circulation. Angiogenesis contributes to the successful adaptation of metastatic cells in the parenchyma of distant organs. In addition to determining the malignant potential, the genes for CXCR4, MMP1, MMP2 and LOX have additional functions that are specific for metastasis, which is why they represent genes that are also necessary for metastatic progression.

**Inflammation**

Tumor cells are often surrounded by activated fibroblasts and BMDCs. Due to the similarity between the processes that occur during carcinogenesis and the processes during wound healing, cancer resembles a "wound" that never heals. The inflammatory response in the tumor tissue induces significant selective pressure on tumor cells. Considering that many of the processes that normally occur during inflammation, namely that the breakdown of the extracellular matrix, the stimulation of cell growth and angiogenesis are simultaneously beneficial for aggressive tumor growth, it is logical that those tumor cells that have retained the ability to coordinate these inflammatory processes will manage to survive and move on. Although it was previously thought that a large number of innate immune cells in the tumor environment represent an active attempt of the immune system to reject the tumor, it is obvious that the tumor selects an immunosuppressive environment, and at the same time uses innate immune cells for its own progression.

In order to facilitate an immunosuppressive environment, the tumor microenvironment selects those cells that stimulate the production of immunomodulatory factors, such as TGF-β, COX2 (cycloxigenase-2), macrophage growth factor CSF-1 (colony-stimulating factor-1), IL-10 and IL-6. All of the above factors inhibit the maturation of dendritic cells and encourage the development of tumor-associated macrophages (TAM), which have an immunosuppressive effect. In addition, the tumor recruits BMDCs, among which MDSCs are important and also have an immunosuppressive effect. In the tumor tissue, these cells increase the local production of TGF-β, thus blocking the functions of T lymphocytes and inhibiting the activation of NK cells. That's rightin most cases, the tumor microenvironment by chronic activation, primarily cells of the innate immunity, polarizes chronic inflammation, which promotes tumor progression in various ways.



**Figure 3.**Interactions of tumor cells with the stroma promote tumor invasion and metastasis.

Instead of suppressing inflammation, tumor cells even encourage inflammatory mechanisms that they then use for their own purposes. E.g,MDSCs contribute to immunosuppression but at the same time, they facilitate tumor invasion by secreting metalloproteinases.Various incoming cells in the tumor stroma,together with tumor cells actively contribute to tumor growth. For example, MDSCs and TAMs participate in basement membrane degradation by producing uPA and MMPs that help tumor cells degrade components of the extracellular matrix or stimulate tumor growth and migration through ligands for the EGF receptor and PDGF. Growth factors secreted from TAMs activate fibroblasts. Fibroblasts activated in this way become carcinoma-associated fibroblasts (CAFs), which stimulate tumor growth by secreting CXCL12, which increases the expression of CXCR4 on tumor cells. CAFs also stimulate angiogenesis by means of CXCL12, which induces the recruitment of precursor endothelial cells. Also, these cells act on TAMs that are recruited to regions of hypoxia where they produce VEGF. It is obvious that malignant cells redirect the immune system as an "enemy" into their "accomplice" in different ways (Figure 3).

**Avoiding apoptosis and aging**

Disruption in cellular homeostasis can occur due to loss of cellular contact, when a cell is damaged or aged. The main mechanism that prevents this disruption of cellular homeostasis is based on programmed cell death or apoptosis of such "deviant" cells. This form of cell death is genetically regulated and can be triggered by different signaling pathways involving different proteins that either control environmental signals or act as damage sensors. Tumor cells ignore all the mentioned signals and this resistance to apoptosis is one of the prerequisites for their successful metastasis. The most common internal cellular triggers of apoptosis are excessive activation or loss of function of some proteins. For example, inadequate activation of c-MYC or loss of Rb protein function results in programmed cell death, which can be prevented by enhanced expression of the antiapoptotic gene (Bcl-2) or inactivation of the proapoptotic gene p53. External triggers of apoptosis are hypoxia, low pH, reactive oxygen radicals, loss of cellular contact and killing mediated by immune mechanisms. Ectopic expression of antiapoptotic genes in malignant cells, such as BCL2 and BCL-XL, not only renders these cells resistant to a wide range of insults, including hypoxia, low pH, and reactive oxygen radicals, but also increases their metastatic capacity.

In addition to apoptosis, cellular senescence represents another important barrier in carcinogenesis. Cellular aging is a consequence of telomere erosion and is dependent on the protein p53. Pressure on tumor cells to escape this cellular senescence results in either inactivation or mutation of the p53 gene in malignant cells.

**The ability to self-renew**

Most malignant cells retain some resemblance to the tissue from which they originate thanks to the property of persistently differentiating, albeit in an abnormal manner. Many tumor cells may have limited proliferative potential and some cells acquire the ability to self-renew, similar to normal cells. Tumor stem cells are a small subpopulation of tumor cells that have the ability to self-renew. The presence of tumor stem cells was first registered in acute myeloid leukemia, and more recently in breast cancer and glioblastoma, as well as in other types of malignancies.

**PROCESSES IMPORTANT IN THE INITIATION OF METASTASIS**

The epithelial-to-mesenchymal transition (EMT) program is one of the most significant characteristics of cancers that tend to metastasize. Malignant cells use the EMT program to initiate metastasis. Hypoxia increases the activity of β-catenin which then promotes the expression of the transcription factor, Snail, and consequently the initiation of EMT. In addition to hypoxia, the inflammatory microenvironment also stimulates EMT. Thus, TNF-α, which is secreted by TAMs, participates in the stabilization of β-catenin and Snail, and thus this inflammatory mediator increases the motility of malignant cells. Tumor cells that use the EMT program are also characterized by increased resistance to apoptosis, which is probably related to the transcription factor Snail. Tumor cells are thought to activate EMT genes to escape the selective pressure of apoptosis and cellular senescence. In addition, the transcription factors of this program can directly increase the number of tumor cells with stem cell characteristics.

EMT genes are essential for metastatic processes. The transcription factor Twist (involved in the EMT program) is thought to be responsible for the increased number of circulating metastatic cells by increasing their intravasation and/or survival in the circulation. Using the EMT program, malignant cells enter the circulation by reducing the expression of E-cadherin, which causes them to lose intercellular contact and then invade the basement membrane.

Tumor invasion is initiated by loss of cell adhesion. In most cases, decreased expression of E-cadherin is responsible for the loss of cell adhesion. Well-differentiated tumors tend to maintain the expression of E-cadherin, while poorly differentiated tumors decrease the expression of this adhesive molecule. Many growth factors, such as EGFR, FGFR, Src-family kinases and IGF-1R (Insulinlike Growth Factor-1R) can stimulate tumor cells to reduce E-cadherin expression. The E-cadherin gene promoter can also be suppressed by specific transcriptional repressors, such as SNAIL and TWIST. These transcriptional repressors are involved in the EMT program. Thus, loss of E-cadherin expression disrupts intercellular adhesion and promotes detachment of tumor cells from the epithelium. Tumor cells altered in this way gain the ability to invade and metastasize.

After separating from neighboring cells, malignant cells induce degradation and remodeling of the extracellular matrix and invade this structure. Malignant cells tend to alter integrin expression as well. During migration, malignant cells express filopodia (protrusions at the cell periphery). Some integrins support the mobility of malignant cells through the locally degraded extracellular matrix.

**PROCESSES IMPORTANT IN METASTATIC PROGRESSION**

Among the genes that are selected to support the growth of the primary tumor, some of these genes are also necessary in the later processes of tumor progression, that is, for the dissemination of malignant cells. These genes provide functions that are specific for metastasis and have been designated as genes responsible for metastatic progression.

**The premetastatic niche**

Before the tumor colonizes distant organs, it is necessary that this "soil" ie. the new microenvironment in the target organs prepares for the settlement of disseminated metastatic cells. This is accomplished by the formation of a premetastatic niche that is under the control of the primary tumor. Premetastatic niches are often localized around terminal veins in distant organs and contain recruited hematopoietic precursor cells of the myeloid lineage from the bone marrow as well as stromal cells. These niches provide cytokines, growth factors, and adhesive molecules that support the arrival of metastatic cells. It is assumed that secreted cytokines and growth factors, in the inflammatory and hypoxic microenvironment of the primary tumor, coordinate the formation of premetastatic niches. For example, malignant cells in the primary tumor by secreting VEGF and PlGF can mobilize VEGFR1+ myeloid cells from the bone marrow to specific target tissues. If the primary tumor secretes VEGF, this growth factor promotes the deposition of fibronectin in the lungs, and together with PlGF determines the formation of a premetastatic niche at this site. Similarly, VEGF, TGF-β and TNF-α originating from the primary tumor specifically induce the synthesis of inflammatory proteins, S100A8 and S100A9, in the lung parenchyma. This results in the infiltration of myeloid cells into the lungs and the subsequent formation of a premetastatic niche. In addition to these pro-inflammatory signals, LOX (eng. Lysyl OXidase) synthesized in the hypoxic microenvironment of the primary tumor can also direct the formation of the premetastatic niche.

When myeloid and activated stromal cells form premetastatic niches, the local environment in distant organs is altered and pro-inflammatory cytokines and MMPs are produced in it, causing this environment to resemble the site of the primary lesion. Consequently, when malignant cells detach from the primary tumor and enter the circulation, target organs with established premetastatic niches become "better soil" that facilitates the anchoring, retention, survival and growth of metastatic cells. If the formation of the premetastatic niche is disrupted, tumor metastasis is inhibited. Cytokines and growth factors that accompany inflammation and hypoxia in the primary tumor not only stimulate the growth of the primary tumor, but also participate in the creation of the microenvironment in distant organs after the dissemination of malignant cells.

**Survival of metastatic cells in the circulation**

It has been experimentally shown that tumor metastasis is a rather unsuccessful process, because despite the large number of circulating tumor cells, only a relatively small number of cells successfully form metastases. When malignant cells are separated from the primary tumor and enter the circulation, they become the target of mechanical influences in small blood vessels. In the presence of mechanically retained metastatic cells, the hepatic sinusoids become activated and begin to secrete nitric oxide. Nitrogen monoxide can cause apoptosis of tumor cells trapped in this place. Immune system cells can also actively attack circulating malignant cells. For example, NK cells kill tumor cells using TRAIL and CD95L molecules.

The question arises: how do circulating malignant cells avoid apoptosis and how do they increase their metastatic potential? The growth of the primary tumor is the result of the selection of those tumor cells that are more resistant to apoptosis, and these cells have an advantage and proliferate intensively. Increased expression of antiapoptotic genes BCL2 and BCLXL or decreased expression of proapoptotic genes, as well as genes for TNF-family receptors result in an increase in the metastatic capacity of tumor cells. The expression of αVβ3 integrin on circulating tumor cells and on platelets stimulates the aggregation of these cells, which results in the formation of tumor embolus. These tumor emboli not only facilitate the retention of tumor cells but also protect them from the action of NK cells.

**Extravasation and colonization of metastatic cells**

When malignant cells are arrested in the capillary system in distant organs, these cells leave the circulation and enter the organ parenchyma. During extravasation, tumor cells mimic leukocytes because they use the same adhesive molecules as leukocytes. For example, tumor cells as well as leukocytes bind to E- and P-selectin expressed on the endothelium in target organs. Additionally, VEGF released from tumor cells disrupts intercellular connections between endothelial cells and thus increases vascular permeability, which all together facilitates extravasation of tumor cells. The expression of CXCR4 on circulating tumor cells enables selective extravasation into certain organs. This selective extravasation is due to the production of the corresponding cytokine CXCL12 in certain organs, such as the lungs, bones and lymph nodes. The four genes responsible for lung metastasis, namely EREG, MMP1, MMP2 and COX2, are also important for tumor cell extravasation. The results of some studies indicate that mesenchymal cells recruited in the stroma of the primary tumor produce CCL5, which by its paracrine effect increases the mobility, invasion and extravasation of tumor cells into the lung parenchyma.

**GROWTH OF METASTATIC COLONIES IN DISTANT ORGANS**

One of the main limiting steps in the metastatic cascade is the ability of metastatic cells to continue to grow in distant organs after extravasation. Successful adaptation to the new microenvironment promotes the establishment of metastatic colonies in distant organs, and implies the existence of growth factors, as well as the preservation of the sensitivity of metastatic cells to those factors. In many types of malignancies, the completion of the metastatic cascade is still not successful despite the ability of tumor cells to initiate the EMT program (which induces their invasion, intravasation and self-renewal capacity), as well as the systemic impact of the primary tumor on the survival of circulating metastatic cells, extravasation and the formation of a premetastatic niche . Thus, a metastatic cell that has reached the target organ has the task of establishing metastatic colonies at that site and continuing to proliferate. This is possible only in the case of the presence of growth factors and preserved sensitivity of metastatic cells to these factors.

Bones are one of the most common sites of metastasis of many types of malignancies, such as lung, kidney and breast cancer.

Lungs are one of the predilection sites of metastasis of malignant melanoma, breast cancer, colon cancer, bladder cancer... The results of some studies indicate that cells in the primary tumor lesion emit endocrine prometastatic signals that act on the endothelium of blood vessels in distant organs, significantly earlier than which begins the dissemination of tumor cells. The production of MMP-9 in the lung, during the premetastatic phase, is critical for the invasion of disseminated tumor cells into the lung tissue. It is assumed that this molecule, among other functions, enables the release of chemotactic factors that then target circulating malignant cells. In addition to MMP-9, it has been shown that integrins participate in the "guiding" of malignant melanocytes to the lungs.

In colorectal cancer, the establishment of metastases in the liver is characteristic, where the malignant cells reach this place through the portal circulation. On the other hand, tumor cells of melanoma, lung and breast cancer reach the liver using the systemic circulation. The microenvironment of the liver is particularly favorable for the establishment of metastases in gastrointestinal cancer. In addition to the local microenvironment of the liver, signaling molecules expressed on the metastatic cells themselves are also involved in the process of metastasis.

Brain is the site of metastasis of lung, breast, kidney, colorectal cancer, melanoma... It has been shown that malignant melanocytes that metastasize to the brain have increased activity of the transcription factor STAT3. Altered expression of this transcription factor affects the expression of FGF, VEGF and MMP2 responsible for angiogenesis and invasion.

In addition to angiogenesis, advanced tumors are characterized by lymphangiogenesis (the process of creating new lymphatic vessels). Lymphatic vessels can be detected both around the tumor and in the tumor itself. Lymphangiogenesis involves VEGF-C and VEGF-D, which bind to the VEGFR-3 receptor. The expression of VEGF-C and VEGF-D is induced by inflammation, but not by hypoxia.

Tumor metastasis to regional lymph nodes is one of the early signs of metastatic potential and/or spread to distant organs. Lymph nodes secrete CXCL12 which interacts with CXCR4 expressed on malignant cells. In addition, other chemokine receptors, such as CXCR3, play a significant role in tumor metastasis to lymph nodes.

**Tumor dormancy**

Dissemination of tumor cells does not only occur in the later stages of tumor development, but malignant melanocytes as well as breast and prostate cancer cells can metastasize early during the evolution of the tumor, i.e. long before the primary tumor is clinically detectable. It is considered that these early disseminated cells are "sleeping" (tumor dormancy), and the period of their dormancy lasts for varying lengths of time depending on the conditions of the microenvironment in distant organs. These dormant malignant cells are most likely responsible for the establishment of late metastases after surgical removal of the primary tumor.