**TEACHING UNIT 3**

**SIGNALING PATHWAYS IN THE CELL**

Essential processes for all living organisms, such as growth, proliferation, differentiation and programmed cell death, are tightly regulated by numerous control mechanisms. These processes are under the constant control of extracellular signals that include interaction with the extracellular matrix, intercellular contact and, finally, the action of soluble molecules known as growth factors. With the help of a complex network of signaling pathways in the cell, these signals are integrated and distributed to different places where the appropriate biological response to the stimulus begins.

Cell growth factors are soluble signal molecules that exert their effect by binding to specific receptors on the cell membrane. Examples of such molecules are cytokines and hormones. Growth factors affect cell proliferation in both positive and negative directions, and initiate a series of different events in the target cell that result in cell survival, proliferation, differentiation, or apoptosis. The interaction between a growth factor and a specific growth factor receptor on the cell membrane activates a cascade of intracellular signaling pathways resulting in an appropriate biological response. Several classes of receptors are involved in signal transduction in the cell. These include receptors with tyrosine kinase activity, G-protein coupled receptors and cytokine receptors.

Receptors transmit information from the outside through the cell membrane via the numerous intracellular signaling pathways in which cytoplasmic signaling proteins participate. After triggering a signal from the receptor, signaling proteins are activated by switching from an inactive to an active conformation, until they receive another signal that returns them to an inactive state. The two basic mechanisms of activation of signaling proteins are phosphorylation and guanosine-3-phosphate (GTP) binding. The largest number of signaling proteins are activated by the process of phosphorylation carried out by enzymes called protein kinases. Protein kinases are enzymes that covalently attach one or more phosphate groups to amino acid residues of proteins. On the contrary, protein phosphatases are enzymes that remove phosphate groups, i.e. perform dephosphorylation of proteins, usually returning them to an inactive state and interrupting signal transmission. Many signaling proteins are protein kinases organized as signaling cascades in which a protein kinase activated by phosphorylation subsequently phosphorylates and activates the next in the signaling sequence. In this way, the signal is amplified and occasionally distributed to other signal pathways. The two basic types of protein kinases are serine/threonine kinases, which phosphorylate proteins at the amino acid positions serine and threonine, and tyrosine kinases, which, analogously, phosphorylate proteins at the positions of amino acid tyrosine. The second group of signaling proteins includes those that are in an inactive state bound to guanosine diphosphate (GDP) and are activated by replacing guanine nucleotides (removing GDP and subsequently binding GTP).

The signals are distributed to different places in the cell, and finally reach the nucleus, where the expression of target genes is modulated by activating transcription factors. Different extracellular signals can trigger the same signaling mechanism in the cell. The efficient coordination of signaling events in the cell is controlled by molecules called adapter and scaffold proteins. These proteins integrate, connect, the intracellular components of the signaling pathway. Through protein-protein reactions, adapter proteins connect molecules of the signaling cascade with proteins such as receptors with tyrosine kinase activity. Scaffold proteins bind the different signaling proteins together in a functional complex and thereby enable the formation of multienzyme signaling complexes involved in certain signaling pathways.

During the process of oncogenesis and tumor progression, tumor cells acquire numerous properties that basically define their malignant phenotype. These properties include the ability to proliferate independently of an external signal, to invade the surrounding tissue and metastasize to distant tissues and organs, to induce angiogenesis and avoid apoptosis and replicative senescence. All these changes are the result of disturbances at the level of a complex network of signaling pathways that control cell proliferation, motility and survival. Different components of the signaling cascade, from the extracellular signal, through receptors and a series of signaling proteins, to transcription factors, can become oncogenic and participate in the malignant transformation of the cell. Excessive expression or accumulation, or hyperactivity of a certain component of the signaling pathway, caused by gene mutation, is most often involved in the constitutive activation of the signaling pathway independently of control regulatory mechanisms. Similarly, inhibitory mechanisms, which block signal transduction and continuously regulate cell growth and division, can be disrupted. By losing strict control and regulation of growth and proliferation, the tumor cell acquires independent proliferative capacity and behavior, independent of extracellular or intracellular influences and control.

**Signal transduction from receptors with tyrosine kinase activity**

Most of the extracellular signaling proteins, including cell growth factors, achieve their effect by binding to membrane receptors that possess tyrosine kinase activity, which contain so-called tyrosine kinase domains in their intracytoplasmic part. Tyrosine kinases are enzymes that reversibly bind a phosphate group to the amino acid tyrosine, i.e. perform protein phosphorylation. After ligand binding, receptors dimerize or oligomerize, which brings their tyrosine kinase domains into close proximity to each other and allows them to phosphorylate multiple tyrosines of adjacent receptor chains (transphosphorylation) or other proteins involved in signal transduction. Phosphorylated tyrosine residues further serve as anchoring sites for various downstream signaling proteins. Signaling proteins bind with high affinity to tyrosine phosphate groups using specific phosphotyrosine-binding domains. After binding to the activated receptor, the signaling proteins themselves are activated by phosphorylation, which initiates the formation of signaling complexes and the distribution of signals to different places in the cell. Different receptors with tyrosine kinase activity bind different combinations of signaling proteins and consequently induce different cell responses to a certain stimulus.

Activated receptors with tyrosine kinase activity trigger numerous signaling pathways in the cell, such as Ras/MAPK (Ras protein/mitogen-activated protein kinase), PI3K/Akt (phosphatidyl-inositol-3 kinase/Akt kinase), and JAK/STAT (Janus kinase/signal transmitter and transcription activator) that play the role of important signaling pathways in cell growth, proliferation and differentiation, but also in the processes of oncogenesis and tumor progression.

**Rasprotein**

The Ras protein superfamily contains a large number of different monomeric proteins that bind guanosine-3-phosphate (GTP) in the activated state. They are also called small G proteins or small GTP-ases. Among them, the most important in conducting signals from receptors with tyrosine kinase activity are members of the Ras and Rho families (Rac, Rho and Cdc42). The three main, structurally and functionally similar Ras proteins in the human body are K-Ras, H-Ras and N-Ras. Small GTP-ases in the cell exist in an active or inactive state. In an inactive state, they bind guanosine diphosphate (GDP), and are activated by replacing GDP with GTP. This reaction is assisted by guanine-nucleotide exchange factors (GEFs), by releasing GDP and enabling the binding of GTP.

Ras is a small GTP-binding protein that contains its own GTP-ase region. It contains one or more covalently bound lipids that enable Ras protein to be anchored in the inner part of the cytoplasmic membrane. Ras protein represents a hub from where, in a strictly coordinated action, a signal is generated to different places in the cell. Basically, Ras protein activation is based on the transition between an active and an inactive state. In the inactive state, the Ras protein binds GDP. When the receptor is activated by ligand binding, the GEF stimulates the dissociation of GDP and the subsequent binding of GTP instead of GDP on the Ras protein. Over time, the GTP-ase region of the Ras protein hydrolyzes GTP to GDP, thereby converting it back into its inactive state. GTP-ase activity is strongly activated by molecules called GTP-ase-activating proteins (GAPs). Receptors with tyrosine kinase activity activate Ras either by GEF activation or by inactivation of GAP proteins following their binding to phosphorylated tyrosine residues of the activated receptor.

Depending on the cell type and the nature of the extracellular signal, the Ras protein mediates the processes of cell growth, proliferation, differentiation and survival in different ways. Activated Ras protein can recruit a large number of effector molecules to the membrane and initiate various signaling pathways in the cell. GTP-ases from the Rho family (Rac, Rho and Cdc42), like Ras proteins, are activated by replacing guanine nucleotides, but exhibit different functions. Activated Rho proteins participate in the reorganization of the cytoskeleton of the cell and its contact with the surrounding structures. In this way, Rho proteins control the shape and motility of the cell, while in the case of malignant transformation, they affect its invasiveness.

Ras protein is hyperactive in many tumor cells. Mutated forms of Ras protein, resistant to the stimulation of GTP-ase activity induced by GAPs, are in a constantly activated state in the cell and are responsible for the formation of tumors. Mutations in genes encoding Ras proteins are present in about a third of all tumors. Mutated forms of K-Ras protein are detected in about 90% of pancreatic adenocarcinoma, 40-45% of colon adenocarcinoma, lung cancer, melanoma and numerous other tumors. Mutated forms of the H-Ras protein are represented in a significant percentage in bladder cancer, while mutations of the gene encoding the N-Ras protein are mainly present in hematopoietic tissue tumors (various forms of leukemia and lymphoma), anaplastic thyroid cancer, melanoma and other tumors.

**Ras/Raf/MAPK signaling pathway**

The cell cycle is controlled by hormones and growth factors in a paracrine manner. Receptors of these molecules transmit signals to the nucleus. When the growth factor binds to the receptor on the target cell, a cascade of phosphorylation is triggered by the receptor, via the Ras protein and a series of mitogen-activated protein kinase kinases (MAPKs). These kinases enter the nucleus where they phosphorylate and activate transcription factors that regulate the expression of target genes. The binding of various extracellular signaling molecules stimulates a highly conserved kinase cascade system, culminating in the activation of a specific MAPK. In other words, different MAPKs regulate different cell responses, including morphogenesis, cell death, and stress responses.

The first and most important effector molecule downstream of the Ras protein is the serine/threonine kinase Raf. Raf is a protein kinase that phosphorylates proteins at serine and threonine amino acid positions. The activation of Raf kinase, mediated by the Ras protein, is based on the attraction of Raf kinase from the cytoplasm to the inner side of the cytoplasmic membrane of the cell where the Ras protein is located. Activated Ras protein that has bound GTP has a higher affinity for binding Raf kinase. After phosphorylation and dephosphorylation of various amino acid residues and subsequent conformational changes, Raf kinase engages, phosphorylates and activates the next components of the signaling cascade, protein kinase MEK (Mitogen/Extracellular signal-regulated kinase). MEKs are protein kinases that have a double specificity, they phosphorylate serine/threonine and tyrosine residues of downstream protein kinases in the signaling cascade, ERK (Extracellular signal-regulated kinase). ERKs are further released from the complex and transported to the nucleus. MAPKs regulate the activation of several transcription factors important for cell cycle regulation, either by direct phosphorylation or activation of other protein kinases. Among the most important transcription factors are Fos and Jun, which together form activation protein 1 (AR-1), hyperactive in many tumor cells. ERKs stimulate the transcription of genes encoding heparin-binding EGF, cyclin D1, Fos, p21.

In addition to ERK, there are other MAPKs, such as JNKs (Jun N-terminal kinases) and p38 kinase, which become active under stress conditions. All these enzymes are serine/threonine kinases, which are activated in the cytoplasm, in response to a specific extracellular signal, and then transported to the nucleus. Also, all eukaryotic cells contain several members of the MEK superfamily, which phosphorylate different members of the MAPKs superfamily.

The Ras/Raf/MAPK signaling pathway is overactivated in many tumors. Even in the absence of proven mutation of genes encoding components of this signaling pathway, its hyperactivity is detected in more than half of cases of acute myeloid and acute lymphocytic leukemia, as well as in a large percentage of breast and prostate tumors. Raf kinase plays an important role in the pathogenesis of melanoma. The mutated form of B-Raf kinase is detected in about 50% of cutaneous melanomas and represents the most common genetic anomaly responsible for the onset of this disease. A mutated form of Raf kinase has also been detected in a large number of cases of different types of glioma, almost all cases of hairy cell leukemia, colon cancer, lung cancer, thyroid cancer and other tumors. Although MEK kinase inhibitors are used in the therapy of some tumors, their mutated forms are rarely encountered. Similar to MEK, mutations in the genes encoding ERK are rarely present in tumors, although hyperactivity of this signaling pathway is one of the most important factors in malignant cell transformation.

**PI3K/Akt signaling pathway**

Some of the most important intracellular signaling pathways in the regulation of cell growth and proliferation involve the activation of the enzyme phosphatidyl inositol-3-kinase (PI3K). PI kinases are lipid kinases that catalyze the transfer of a phosphate group from the ATP molecule to membrane-bound phosphatidyl inositol (PI). The most important of them phosphorylates phosphatidyl inositol-2-phosphate (PIP2), resulting in phosphatidyl inositol-3-phosphate (PIP3). PI3K can be activated by various signals such as binding of growth factors to receptors with tyrosine kinase activity or directly, through activated Ras protein. The Ras protein attracts PI3K to the inside of the cell membrane and brings it close to the substrate. Once activated, PI3K initiates several signaling pathways in response to various growth factors (PDGF, NGF, IGF-1, IL-3) or adhesive molecules (integrins) of the extracellular matrix. PI3K activation is under very tight control. In the absence of growth factors as a stimulus, the level of PIP3 in the cell is very low, which is a consequence of the action of numerous phosphatases that remove phosphate groups from previously phosphorylated PI. One of the most important phosphatases is PTEN (Phosphatase and tensin homolog), which dephosphorylates PIP3 and thereby inhibits signal transduction.

The most important cytoplasmic protein that binds to the resulting PIP3 is the serine/threonine kinase Akt, also known as protein kinase B (PKB). By binding to the cytoplasmic membrane and subsequent phosphorylation, the functional activation of Akt kinase occurs, which further engages numerous cytoplasmic proteins with multiple effects on the cell. These effects include stimulation of cell survival, growth and proliferation. Akt kinase prevents cells from entering programmed cell death by inhibiting several important proapoptotic proteins. At the same time, Akt kinase stimulates cell proliferation by promoting its passage through the cell cycle.

Independent of the effects on cell survival and proliferation, the activated PI3K/Akt signaling pathway exerts a strong effect on the stimulation of cell growth by stimulating protein synthesis. PI3K, through the activation of Akt kinase, engages the central molecule of this signaling pathway, the kinase mTOR (Mammalian target of rapamycin). mTOR is a serine/threonine kinase that plays one of the key roles in the regulation of metabolism, growth and cell division. In human cells, mTOR kinase is present in the form of two protein complexes: mTORC1 and mTORC2. mTORC1 consists of two main subunits: mTOR kinase and a regulatory protein called Raptor. mTORC1 regulates gene transcription, ribosome formation, protein synthesis, cellular metabolism, autophagy, and cell growth and division. mTORC1 activity depends on the availability of growth factors, nutrients and energy in and around the cell. Thus, amino acids, especially leucine, directly activate mTORC1. Protein kinase AMPK (AMP-activated protein kinase) inhibits mTORC1. Cellular stress, hypoxia or DNA damage inhibit mTORC1 via AMPK.

The main substrates of mTORC1 are p70 S6 kinase (S6K) and 4E-BP (Eukaryotic translation initiation factor 4E (eIF4E)-binding protein). mTORC1 phosphorylates S6K and thus activates it. S6K increases the synthesis of ribosomal proteins, elongation factors and other proteins necessary for the translation process. In addition, mTORC1 phosphorylates 4E-BP and thus prevents the inhibition of eIF4E. Free eIF4E further stimulates translation. mTORC1 regulates the transcription of genes involved in metabolic and biosynthetic processes, the uptake of glucose, amino acids, lipoproteins and iron into the cell, as well as autophagy and apoptosis.

In addition to cell growth, mTOR kinase stimulates cell transition from G1 to S phase of the cell cycle. In contrast, inhibition of mTOR kinase by rapamycin inhibits cell growth and proliferation by slowing or completely abrogating G1 phase progression or inducing apoptosis. Recent research has shown that mTORC1 activation is not limited to the G1 phase of the cell cycle. Increased mTORC1 activity stimulates both G2 and M phases of the cell cycle.

Various components of the PI3K/Akt signaling cascade, particularly PI3K, Akt and PTEN, are overexpressed or mutated in numerous tumors. In addition to mutations in genes encoding Ras proteins, mutations in genes encoding PI3K are among the most common genetic abnormalities in tumorigenesis. Mutated forms of PI3K, responsible for increased activation of the PI3K/Akt signaling pathway, are present in a number of tumors such as: colorectal and hepatocellular carcinoma, endometrial, breast and prostate tumors, glioblastoma and other. Increased expression or activation of Akt kinase, resulting from a gene mutation, has been detected in liver tumors, pancreatic cancer, hepatocellular and colorectal cancer, glioblastoma or hematological malignancies. The phosphatase PTEN is a key negative regulator of the PI3K/Akt signaling pathway. Loss of PTEN function as a result of gene mutation is responsible for the constitutive activation of this signaling pathway in prostate and endometrial tumors, gliomas, melanoma, etc.

**Signal transduction from cytokine receptors**

**JAK/STAT signaling pathway**

A large number of local mediators of intercellular communication, such as cytokines, but also some hormones and growth factors, exert their effect on the cell by activating receptors that are grouped into a large family of cytokine receptors. Cytokine receptors do not contain intracellular tyrosine kinase domains, although their activation depends on tyrosine phosphorylation. Key molecules in signaling from cytokine receptors are tyrosine kinases that belong to the group of so-called Janus kinases (JAK). To date, four JAKs have been identified: JAK1, JAK2, JAK3 and TYK2. JAKs are non-covalently bound to the intracytoplasmic parts of cytokine receptors. After interacting with the ligand, cytokine receptors form dimers and activate JAKs, which perform transphosphorylation, as well as phosphorylation of the intracytoplasmic part of the receptor. The phosphorylated tyrosine residues of the receptor further serve as anchoring sites for molecules called signal transducers and activators of transcription (STATs). To date, 7 STAT molecules have been identified. JAKs phosphorylate STAT molecules that form homodimers or heterodimers and are transported to the nucleus where they further regulate the expression of target genes. STAT molecules stimulate the transcription of several hundred genes important for the processes of cell proliferation and survival, such as myc, the genes encoding cyclins D2 and D3 or the gene encoding the antiapoptotic protein Bcl-XL.

Continuous activation of STAT molecules, induced by phosphorylation mediated by JAKs or other tyrosine kinases or reduced activity of phosphatases that remove phosphate groups, may be an important factor of enhanced proliferation or reduced apoptosis in the process of malignant cell transformation. STAT3 molecule is constitutively activated in many tumors such as breast tumor, head and neck tumors, melanoma, glioblastoma and lung and stomach tumors. Mutation of the gene encoding JAK2 has been identified in a large number of patients with different types of myeloproliferative neoplasms.

**Wntsignaling pathway**

Wnt proteins are secreted signal molecules that act as local mediators in the control of many aspects of organism development such as cell proliferation, stem cell survival and their differentiation. They are structurally distinctive and contain fatty acids covalently bound to the N-terminal part of the protein, which increases their ability to bind to the cell surface. There are 19 known Wnt proteins in the human body. Wnt proteins activate several signaling pathways in the cell, of which the Wnt/β-catenin signaling pathway is the best described. Signal transmission in the cell begins with the binding of Wnt proteins to membrane receptors that belong to the so-called family of Frizzled receptors. Frizzled receptors are seven transmembrane proteins in structure.

Wnt/β-catenin signaling pathway regulates the proteolytic degradation of the multifunctional molecule β-catenin, which plays an important role in the processes of intercellular adhesion and the regulation of gene expression. In epithelial cells, β-catenin is bound to the transmembrane adhesive protein cadherin and participates in the formation and stabilization of intercellular connections. If it is not bound to cadherin, β-catenin is rapidly degraded in the proteasomes. The degradation of intracytoplasmic β-catenin depends on the so-called protein degradation complex that binds it and accelerates its destruction. The protein degradation complex is stabilized by two scaffold proteins, axin and APC (Adenomatous polyposis coli) protein. Intracellular signals triggered by Wnt proteins by binding to their receptors inhibit the function of the protein degradation complex and the consequent degradation of β-catenin. This results in the accumulation of β-catenin, which is transported to the nucleus where it binds to transcription factors and stimulates the transcription of target genes. Genes whose expression is controlled by β-catenin include c-myc, which is a strong regulator of cell growth and proliferation, and the gene encoding cyclin D1. Thus, in the presence of Wnt signaling, β-catenin accumulates in the nucleus and stimulates the transcription of c-myc and other genes, which results in uncontrolled cell growth and proliferation and consequent tumor formation.

Wnt signaling pathway is constitutively autocrine activated in different types of tumors such as breast tumors, prostate cancer or sarcoma. Much more often, mutations in genes encoding various components of the Wnt/β-catenin signaling pathway, such as β-catenin or APC protein, may be involved in tumorigenesis. The gene encoding the APC protein belongs to the group of tumor suppressor genes and controls the availability of intracytoplasmic β-catenin. Hereditary mutations of the gene encoding the APC protein are responsible for the occurrence of familial adenomatosis of the colon, which is characterized by the appearance of multiple adenomatous polyps of the colorectal mucosa, which eventually undergo malignant transformation and the development of colorectal cancer. Mutations of the gene encoding the APC protein are also detected in 80% of cases of sporadic colon cancer.

**Hedgehog signaling pathway**

Hedgehog proteins are secreted signaling molecules that are structurally modified by covalently bound lipids. They represent important molecules that control the transcription of numerous genes. Hedgehog proteins exert their effects by binding to the twelve transmembrane Patched receptor. Another protein, important for the implementation of the signal induced by Hedgehog proteins, is the seven transmembrane receptor Smoothened. In the absence of a signal induced by Hedgehog proteins, the Patched protein prevents the activation of the Smoothened protein and keeps it sequestered in intracytoplasmic vesicles. By binding to the Patched protein, Hedgehog proteins inhibit its activity, which results in the phosphorylation and translocation of the Smoothened protein to the cell surface in primary cilia, present on many mammalian cells. Downstream of the Smoothened protein, Gli proteins participate in signaling, acting either as repressors, in the absence of Hedgehog proteins, or as inducers of transcription of target genes when this signaling pathway is activated. In the presence of Hedgehog proteins, Smoothened and Gli proteins accumulate and interact in primary cilia. The consequence of this is the prevention of proteolytic degradation of Gli proteins that are further transported to the nucleus where they activate gene transcription.

Hedgehog proteins are important stimulators of cell proliferation, so increased activation of the Hedgehog signaling pathway may represent one of the molecular mechanisms for tumor formation. Mutations in genes encoding inactive Patched proteins are the basis for increased activation of this signaling pathway, which is often the underlying mechanism of basal cell skin cancer.

**The role of the transcription factor NF-κB in oncogenesis**

NF-κB (Nuclear factor-κB) is a transcription factor that plays a central role in the cell's response to infection and injury. It regulates the expression of about 500 different genes that encode proteins involved in inflammation and the cell's response to stress. It is activated by numerous extracellular stimuli such as structures of microorganisms, pro-inflammatory cytokines, reactive oxygen mediators and others, by binding to specific receptors on the cell membrane. In chronic inflammation, NF-κB participates in tumor formation by stimulating cell survival and proliferation.

NF-κB is a heterodimer composed of subunits p65 and p50. In its inactive form, it is sequestered in the cytoplasm by binding to the inhibitory polypeptide IκB (inhibitor of NF-κB). Activation of NF-κB depends on the protein kinase IKK (IκB kinase), which performs phosphorylation and subsequent dissociation and degradation of IκB. NF-κB is released and transported to the nucleus where it regulates the expression of numerous genes. In the context of oncogenesis, NF-κB stimulates the expression of anti-apoptotic proteins, the myc oncogene and cyclin D1 and thus prevents tumor cell apoptosis and promotes its proliferation.

Constitutive activation of the NF-κB signaling pathway is common in tumors, although mutated forms of components of the NF-κB signaling cascade are rarely present. In breast tumors, the NF-κB signaling pathway is often hyperactive, which is most often the result of increased expression of IKK. NF-κB plays an important role in the malignant proliferation of cells of the immune system. Increased expression of NF-κB was detected in a large percentage of B and T lymphocyte lymphomas and myeloma.

**Literature:**

Robert A. Weinberg. The biology of cancer. Garland Science, 2014.

John Mendelsohn, Peter M. Howley, Mark A. Israel, Joe W. Gray. The Molecular Basis of Cancer. ELSEVIER, Expert Consult, 2014.