Session 3. Cytoskeleton, Intracellular Transport and Cell Motility

Lectures

L3.1

Motile cilia-specific protein macrocomplexes and cilia beating

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In Eukaryotes, except fungi and higher plants, the coordinated beating of the motile cilia propels unicellular organisms and regulate the flow of the extracellular fluids in multicellular life forms. In humans proper beating of these tiny nanomachines ensures removal of the mucus and bacteria out of the airways, circulation of cerebrospinal fluid in brain ventricles, transport of the oocytes in the Fallopian tubes and sperm motility. Lack or dysfunction of motile cilia leads to multisymptom group of heterogeneous human disorders called primary ciliary dyskinesia, PCD.
Compared to immotile sensory cilia, whose scaffold is made of nine peripheral doublets composed of A- and B-tubule, motile cilia assemble additional structures indispensable for their proper beating: two centrally located singlet microtubules with associated projections, so called central pair complex (CPC), and macrocomplexes anchored on the surface of the peripheral doublets. The ciliary macrocomplexes: radial spokes (RS), nexin-dynein regulatory complex (D-NRC), modifier of inner arms complex (MIA), calmodulin and spoke associated complex (CSC) and inner (IDA) and outer (ODA) dynein arms form characteristic 96-nm periodicity on each doublet called the "ciliary unit" or "ciliary repeat".
The motor activity of the dynein arms is believed to be regulated by the mechanochemical signals transmitted from the central pair complex through radial spokes to dynein arms. Data obtained in *Chlamydomonas* and our own analysis of the ultrastructure of *Tetrahymena* cilia indicate that there is structural and most probably also functional heterogeneity of the radial spokes and dynein arms in a single ciliary repeat. In my talk I will discuss the differences in the radial spokes architecture and protein composition as well as identification and functional analysis of the radial spokes docking complexes.

Acknowledgements

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L3.2

Motile cilia disorders and approaches to their treatment

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Cilia are small projections present on the surface of most cells in the human body. While immotile cilia are playing mostly a sensory role, multiple motile cilia on the surface of epithelial cells and single flagella of the spermatozoids are involved in the transport of mucus, brain fluid or ovum, and movement of the sperm cells.
Inherited defects of motile cilia underlie the rare genetic disease, primary ciliary dyskinesia (PCD), a multisystem disorder belonging to the recently widely-studied class of ciliopathies. The molecular and genetic basis of PCD is highly heterogeneous, due to the large number of proteins involved in building the normal ciliary structure and essential to proper functioning of cilia. Mutations in 28 "PCD genes" identified so far, explain ~ approximately 65% of cases. As in most of the human genetic disorders, the majority of mutations in PCD introduce premature termination codons (PTC), which lead to the generation of a non-functional, truncated proteins.
Apart from the life-long therapy of PCD symptoms, different approaches to the treatment of the disease cause are considered. One of such strategies is the translational readthrough of premature termination codons (PTC-RT) induced by specific chemical compounds. In several genetic disorders, PTC-RT approach has been proven as an effective way of restoring functional protein expression and reducing disease symptoms. Through insertion of a random amino acid in place of PTC, PTC-RT allows translation to proceed to the true end of the transcript, often resulting in a functional protein, even when incorporated amino acid differs from the wild type. Selective promotion of the PTC-RT is therefore considered an alternative to gene therapy in genetic diseases caused by PTC, especially in cases where even low levels of physiologically functional proteins are sufficient to restore the lost function.
Efficiency of this approach depends on many factors: the compound used, PTC type and its sequence and tissue context etc. In my talk, I would like to summarize the state of the knowledge in this field and present some of the achievements of our laboratory in the studies of PTC-readthrough approach in PCD.

L3.3

Cytoskeleton dynamics contribution to neuronal defects and pathology

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Neurons are the highly polarized cells with very complex morphology. Consequently, proper development, function and wellbeing of these cells strongly rely on proper cytoskeleton dynamics and cytoskeleton-based intracellular transport. Mutations in microtubule- and actin-binding proteins as well as proteins regulating the dynamics of these two elements of cytoskeleton often cause severe neurodevelopmental (e.g., lissencephaly, Williams Syndrome) as well as neurodegenerative diseases (e.g. taupathologies, *amyotrophic lateral sclerosis. Perry syndrome*). Cytoskeleton dynamics is also important contributor to so called aberrant plasticity, a process that accompanies for example epileptogenesis. During the talk, examples of importance of cytoskeleton related proteins for proper development and neuronal function will be firstly discussed. Next, selected neuronal pathologies related to improper cytoskeleton dynamics will be described. Finally, in more detail I will present data linking a mammalian Target of Rapamycin (mTOR) to changes in cytoskeleton in neuronal physiology and pathology. mTOR is a serine-threonine kinase, forming two protein complexes – mTORC1 and mTORC2. Both of them have been shown to contribute to cytoskeleton dynamics. Both of them are also implicated in neuropathologies. For example, mTORC1 is believed to contribute to epilepsy, tuberous sclerosis and autism spectrum disorders, while mTORC2 deficient mice are models of mood disorders. Whether mTOR-dependent cytoskeleton changes could contribute to mTOR-related pathologies remains currently unknown and revealing such connection is one of the important aims of our team.

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Oral presentations

O3.1

Localization and function of phosducin-like proteins (PhLP2A and PhLP2B) in eukaryotic cells

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In order to properly perform their functions, newly synthesized proteins must achieve their native conformation. Proper folding is facilitated by the so-called molecular chaperones that prevent misfolding and formation of the aggregates as well as recognize misfolded proteins and direct them for degradation. Chaperonin containing TCP-1 (CCT) forms a large complex that is required for folding of many proteins, actin and tubulin being most important among them.

Phosducin-like protein 2A (PhLP2A) and its homolog PhLP2B in mammalian cells, are small cytosolic proteins that belong to the highly conserved phosducin-like family of proteins. It has been shown that PhLP2A, which is present in most tissues, modulates activity of CCT during folding of cytoskeletal proteins and possibly also has its own activity as a chaperone of VEGFR. PhLP2B is thought to perform similar function but its expression is limited to germ cells.

With real-time PCR we analyzed the expression pattern of both PhLP2A and PhLP2B in rat tissues. Our results show that while PhLP2A is ubiquitously expressed, PhLP2B is expressed, as expected, in testis but also in tracheal epithelial cells, suggesting that it performs specific functions in cells with motile cilia.

To characterize the function of PhLP2A/B proteins, we attempted to identify their binding partners in NIH 3T3 fibroblasts and in cell lysates from rat sperm or tracheal epithelial cells. Using GST pull-down approach followed by mass spectrometry, we have found several proteins that may form complexes with phosducin-like proteins, such as Rab14, Rab5 and hexokinase-1, and confirmed these interactions by pull down and western blot analysis. Immunoprecipitation analysis with HA-tagged proteins expressed in NIH 3T3 cells, confirmed interactions between PhLP2A/B and Rab14 or Rab5, but not with hexokinase-1. Results of the immunoprecipitation assay are consistent with immunolocalization data obtained in NIH 3T3, sperm and tracheal ciliated epithelial cells showing that Rab14 protein colocalizes with PhLP2A in all tested cells. Hexokinase-1 and PhLP2A colocalize only along the whole length of sperm flagella and within cilia of tracheal epithelial cells. Similar localization of hexokinase-1 in sperm cells was earlier reported by Travis and co-workers (Travis *et al.*, 1998, *Mol Biol Cell* **9**: 263–276).

O3.2

Design, synthesis and biological evaluation of novel tubulin inhibitors

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Microtubules are dynamic cytoskeletal polymers that are involved in various cellular functions including cell division. The mitotic spindle, composed of microtubules generated by polymerization of αβ-tubulin heterodimers, has been considered one of the most important targets in treating many types of cancers. Combretastatin A-4 (3'-hydroxy-3,4,4',5-tetramethoxy-*cis*-stilbene, CA-4 belong to the microtubule interfering agents (MIA) that exhibit anticarcinogenic and antivascular properties. CA-4 phosphate salt is currently in III phase of clinical trials as an adjuvant in cancer therapy.

The aim of this study was to design, synthesize and characterize new derivatives of *cis*-stilbene assuming CA-4 as a leading compound. The derivatives with methylthio substituents and *cis*-restricted CA-4 derivatives; oxazoles and N-methylimidazoles were synthesized. Their anitubulin properties were studied *in vitro* with the use of tubulin polymerization assay kit (Cytoskeleton, USA). Cytotoxic activity was estimated against breast cancer cell line MCF7, human MDA-MB-231 breast cancer cells, immortalized human keratinocyte line HaCaT and human epidermoid carcinoma cell line A431 with the use of MTT assay.

In a series of studied oxazole compounds, two potent inhibitors of tubulin polymerization were found: 4-(3,5-dimethoxy-4-methylthiophenyl)-5-(3-hydroxy-4-methoxyphenyl)oxazole (KomOx3) and 4-(3-bromo-4,5-dimethoxyphenyl)-5-(4-methoxy-3-methylthiophenyl) oxazole with IC_{50} values of 1.05 and 0.80 μM, respectively, showing a stronger inhibition of tubulin polymerization than CA-4 (IC_{50} = 2.5 μM). Potency of antitubulin activity of studied compounds correlated well with their cytotoxic effect in cell lines. KomOx3, an analogue of CA-4 having methylthio substituent in position 4 instead of methoxy group exerted the strongest cytotoxic activity against all studied cell lines with IC_{50} in the range 0.21-0.71 μM. Interestingly, the derivative with three substituents in both phenyl rings, 4-(3,4,,5-trimethoxyphenyl)-5-(3-bromo-5-methoxy-4-methylthiophenyl)oxazole appeared to be non-active and its cytotoxic activity was poor. Contrary to oxazole derivatives, N-methylimidazoles with phenyl rings substituted in comparable manner to oxazoles did not affect the process of tubulin polymerization. Computational methods in molecular analysis of structure-activity relationship have been applied. Elucidation of the interaction between tubulin and inhibitors of its polymerization would provide the data that may be useful in the design of novel efficient drugs for cancer treatment.

O3.3

The mechanism of kinesin-14 Ncd localization to the microtubule growing end

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Ncd, a member of kinesin-14 family of microtubule retrograde motors participates in the organization of microtubules (MTs) during cell division. To perform its function, i.e. MT-MT sliding, the dimeric protein has two sets of MT-binding sites: one in the C-terminal motor domain and the other in the N-terminal tail. Although Ncd is a minus-end-directed motor, significant amount of this kinesin is found at the plus ends of spindle MTs where it is believed to counter plus-end-directed kinesins, such as Eg5. The plus end localization is ascribed to its interaction with the end-binding protein 1 (EB1). Details of this interaction are still missing. For example, it is not known if the kinesin-EB1 complex is formed in solution, or on the MT or what is the role, if any, of the tail diffusion along MT lattice.

To characterize the interaction between these proteins we measured the affinity of purified GFP-labeled Ncd tail (Ncd336, comprising amino acid residues 1-336) to QSY35-labeled EB1 by FRET. The dissociation constant (K_d) was high > 8 μM, indicating that Ncd-EB1 interaction in solution is weak. This suggests that Ncd interacts with MT-bound EB1 rather than with free EB1.

Next, we reconstituted the MT growth *in vitro* using purified proteins and observed fluorescently labeled Ncd fragments and MTs under the TIRF microscope. We used TIRF microscope in two ways in order to establish the role of Ncd-MT interaction and diffusion in the mechanism of plus-end tracking. First, we deleted the MT-binding site from Ncd336. Using cosedimentation assay we confirmed that modified Ncd336 does not bind to MTs. Ncd tail after deletion of MT-binding site was still able to track the growing MT ends, although the efficiency was much lower. Second, in order to distinguish between free and MT-bound motors we used Ncd336 fused to Dendra2, a photoconvertible fluorescent protein. When Dendra2-Ncd336 was bound to MTs it underwent a photoconversion by laser light and emitted red fluorescence. However, Dendra2-Ncd336 in solution was not photoconverted and emitted green fluorescence. In certain conditions, we observed a red fluorescence spot tracking the growing ends of the MT. It suggests that MT-bound Ncd could be trapped by EB1 at the MT tip and track the growing end of MT.

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O3.4

Correlation of gelsolin level with invasion abilities of melanocytes and melanoma cells

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Gelsolin is one of the most extensively studied proteins among actin binding proteins. Published data suggest gelsolin is probably a tumor suppressor (reviewed by Nag *et al*., 2013). In our former studies we observed that downregulation of *GSN* (gene coding for gelsolin) expression by siRNA in A375 melanoma cells decreased the number of cells able to invade into the three dimensional extracellular matrix (Litwin *et al*., 2012). However, this approach does not fully answer the question if gelsolin is indeed promoting cell invasion of melanoma cells. Additionally, up to date there are no studies comparing gelsolin level in melanocytes and melanoma cells. Because of that we decided to estimate *GSN* expression level in both melanocytes and melanoma cells and to elucidate if *GSN* expression level is correlated with invasion abilities of these cells.
We analyzed *GSN* expression at both mRNA and protein level in melanocytes and five melanoma cells lines obtained either from primary of metastatic sites. Obtained results were correlated with invasion abilities of tested cells. Actin cytoskeleton organization in tested cells was analyzed with the help of confocal microscopy. Filamentous actin, monomeric actin, gelsolin, actin:profilin complex and thymosin beta4 were visualized by using specific dyes and antibodies. We decided to include profilin and thymosin beta4 in our analysis, since gelsolin under certain conditions competes with these proteins for actin binding as we stated in our previous work (Mannherz *et al*., 2010). This phenomenon can potentially have an influence on cells invasion potential. We additionally analyzed other parameters such as actin polymerization state in cytosolic fractions of tested cells. Because in our former studies we have tested only the effect of *GSN* expression downregulation on melanoma cell invasion abilities we have performed additionally overexpression of gelsolin to check how higher gelsolin level influences invasion potentials of tested cells.

References:

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O3.5

Bacterial cytoskeletal protein FtsZ as a target for new antibacterial drugs

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Bacterial cell division is a relatively novel target for antibacterial drugs discovery. It is an essential process which starts with polymerization of highly conserved cytoskeletal protein FtsZ in the middle of the cell. After the Z-ring assembly, several other proteins are recruited to the midcell forming a complex called divisome. The divisome ensures that cell division occurs in the correct time and place in the cell. The recruitment of all divisome components and their midcell localization depends on the assembly of FtsZ. FtsZ belongs to the tubulin family of cytoskeletal GTPases. The binding of GTP to FtsZ promotes the assembly of FtsZ monomers into long strands *in vitro*. FtsZ is conserved among bacteria and is essential for cell viability, making it a potential target for new antibiotic discovery.
Alkyl gallates are potent antibacterial agents with broad spectrum for many Gram-positive and Gram-negative bacteria. It was shown that they exhibit multifunction mode of action, including surfactant activity. However, biochemical mechanisms play a more important role in their antimicrobial activity. Here, we show that *Bacillus subtilis* FtsZ (FtsZ-Bs) is a direct target for alkyl gallates with the length of side chain C5-C8. We show that alkyl gallates are specific for FtsZ and are able to inactivate FtsZ GTPase by bundling FtsZ polymers and/or clustering monomers.

O3.6

Regulation of contractile and cytoskeletal actin filaments by cofilin

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In muscle cells actin filaments are present in contractile apparatus and are part of cytoskeletal network, which maintains cell shape and integrity. While the length of contractile thin filaments is stable, the cytoskeletal filaments are subject to dynamic polymerization and depolymerization. Actin turnover is under control of cofilin 2 (Cfl2), the muscle-specific actin depolymerizing factor. Contractile and cytoskeletal filaments are stabilized and regulated by different tropomyosin (TM) isoforms. αTM and βTM are striated muscle-specific isoforms, which form heterodimers and regulate contraction. TM5NM1 is a non-muscle isoform, which was also found in the cytoskeletal compartment of skeletal muscle. The goal of this work was to verify the hypothesis that TM isoforms affect dynamics of actin filaments by differentiation of Cfl2 activity.
The proteins were either isolated from skeletal muscle (actin and αβTM) or expressed in bacterial cells, strain BL21 (TM5NM1 and Cfl2) and used in biochemical assays. Filaments stability and binding of cofilin to the filaments were analyzed in a co-sedimentation assay. Kinetics of actin polymerization was followed by turbidimetry.
Both TM5NM1 and αβTM stabilized actin by reducing the extent of the filaments depolymerization by Cfl2. At substoichiometric Cfl2:actin ratio both TM isoforms reduced the depolymerization degree. At higher concentrations Cfl2 overcame the stabilizing activity of αβTM leading to similar degree of depolymerization as observed in the absence of TM. In the presence of TM5NM1 the filaments were stable independently on Cfl2 concentration. This difference could be explained by the effects of TM isoforms on the actin affinity of Cfl2. As compared to the affinity of Cfl2 to unregulated actin, the affinities to the filaments saturated with αβTM and TM5NM1 were reduced 2- and 3-fold, respectively.
When used separately TM isoforms and Cfl2 slowed down actin polymerization. Combining TM5NM1 and Cfl2 in the polymerization reaction further increased the time required for full polymerization. Surprisingly, the presence of αβTM along with Cfl2 significantly decreased the polymerization time.
The results suggest that differential regulation of Cfl2 activities by TM isoforms is one of the mechanisms which diversify functions of actin filaments in muscle cells.

Posters

P3.1

Phosducin-like protein 2 (Phlp2p) is essential for cilia assembly in *Tetrahymena thermophila*

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The phosducin-like protein 2 (PhLP2) is a conserved protein, involved in the regulation of CCT, a chaperonin whose activity is essential for folding of cytoskeletal proteins, tubulin and actin. The precise molecular function of PHLP2 is still not clear. Our studies revealed that Phlp2p is essential for cilia assembly and function.
To better understand the importance of Phlp2p in cilia, we analyzed the localization and function of Phlp2p in *Tetrahymena thermophila*, an excellent model organism to study cilia and ciliogenesis. Both GFP or HA-tagged Phlp2 proteins are localized in cilia, basal bodies and cytosol. However, while GFP-Phlp2p is distributed evenly along the entire cilia length, Phlp2p-HA is observed in cilia as patches and resembles the localization of the components of the intraflagellar transport. Although both GFP- and HA tagged PhLP2 had similar localization, fusion of PhLP2 to the relatively large tag, as GFP, affected protein function. GFP-Phlp2p overexpressing cells have fewer cilia and showed decreased rate of cells motility, proliferation and phagocytosis compared to wild type or Phlp2p-HA overproducing cells. Moreover, after deciliation GFP-PhLP2 cells were unable to regenerate cilia.
The negative effect of GFP-Phlp2p overexpression on ciliogenesis was abolished by simultaneous overexpression of Phlp2p-HA. Thus, elevated level of non-functional GFP-Phlp2p inhibits ciliogenesis presumably by displacing endogenous Phlp2p or by titrating out key components for cilia assembly. High level of functional HA-tagged protein can "rescue" dominant negative effect by changing the ratio between functional (HA-tagged) and non-functional (GFP-tagged) forms of Phlp2 proteins. Interestingly rat orthologs of Phlp2p, PhLP2A/PhLP2B proteins, localize in cilia of rat tracheal epithelial cells. Additionally, rat PhLP2A and PhLP2B proteins expressed in *Tetrahymena* cells, localized in cilia, basal bodies and cytosol. Thus ciliary localization of PhLP2 orthologs is evolutionary conserved. Immunoprecipitation assay followed with mass spectrometry analysis indicated that *Tetrahymena* HA-tagged Phlp2p protein, may form functional complexes with proteins involved in vesicular transport (clathrin, Sar1or kinesin-like protein), which is necessary for cilia formation and function (Garcia-Gonzalo & Reiter 2012; Kaplan *et al.* 2012), further supporting the hypothesis that the activity of PhLP2p in *Tetrahymena* cells is essential for cilia assembly.

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P3.2

Deletion of CCDC113p protein affects cilia function in a ciliate *Tetrahymena thermophila*

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Cilia are microtubule-based external cells protrusions that originate from the basal bodies. They are present in nearly all types of organism from protists to mammals, with the exception of fungi and higher plants. There are two types of cilia, immotile sensory cilia and motile locomotory cilia that enable cells motility and flow of extracellular fluids in multicellular organisms. Lack or dysfunction of cilia leads to severe human disorders called ciliopathies. The ultrastructure of cilia is well evolutionary conserved. Numerous proteomic analyses indicate that cilia may be composed of even 600-800 proteins, however function of most of these proteins remains unknown or is not well understood.
Here we present data indicating that the putative ciliary protein CCDC113p (coiled-coil domain containing), an ortholog of *Chlamydomonas* protein FAP263, is a basal body / ciliary protein and its absence affects cilia function in a ciliate *Tetrahymena thermophila*. CCDC113p protein is evolutionary conserved and *CCDC113* gene orthologs were found in the genomes of organisms assembling cilia from protist to mammals, with the exception of *C.elegans*, which forms ultrastructurally unique basal bodies and only sensory cilia.
While expressed as N-terminally GFP- tagged protein, CCDC113p co-localizes with basal bodies. The elevated level of CCDC113p does not affect cilia assembly or function however *Tetrahymena* cells with knocked out *CCDC113* gene swim with reduced speed compare to the wild type cells, indicating defects in the function of the locomotory cilia. Moreover, deletion of CCDC113p results in the reduction of the proliferation rate and food vacuole formation, processes known to depend upon the activity of the locomotory and oral cilia, respectively. Immunofluorescence analysis showed that the number and length of cilia in *Tetrahymena CCDC113* knockout cells are similar as in wild type cells. Moreover deletion of CCDC113p does not affect cilia assembly and regeneration. Thus, taken together our data suggest that CCDC113p participate in the regulation of cilia motility.

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P3.3

The effect of long and short beta-glucan on the cytoskeleton and manganese superoxide dismutase expression (MnSOD) in the human normal and malignant cells

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β-Glucan is widely applied in medicine, cosmetics and the food industry. Glucans can activate the immune system, exhibits antitumor, anti-inflammatory and antioxidant activities. Immunomodulatory and anti-cancer properties of beta-glucans result from their structure, molecular weight, degree of branching, conformation and its behavior in gastrointestinal tract. These properties depend on an isolation procedure of β-glucan. Many studies have been conducted on b-glucan extracted cereals, fungus, seaweed, yeasts and bacteria, which generally have low purity and yield or are characterized by high cost of manufacturing and by poor water-solubility. Nowadays, easy obtaining and water-soluble glucans are requested.
The aim of the study was evaluation of the changes in the cytoskeleton and expression of MnSOD in the human normal keratinocytes cell line (HaCaT) and human epithelial lung cancer cell line (A549) after incubation with β-glucan derived from oat.
The obtained results indicate that low (50 μg/ml) as well as high (200 and 400 μg/ml) concentrations of low-molecular-weight beta-glucan caused dramatic changes in cell morphology manifested by nucleus perturbations such as nuclear blebbing and abnormal chromatin condensation in both examined cell lines. In case of a high-molecular-weight beta-glucan similar abnormalities were observed at high (200 and 400 μg/ml) concentrations only in HaCaT cell line. The changes in actin filaments were not observed in opposite to low β-glucan. Immunocytochemical ABC analysis showed that both high- and low-molecular-weight β-glucan induced strong expression of manganese superoxide dismutase (MnSOD) in both tested cell lines. The both compounds derived from cereals revealed anticancer activity.

Keywords: oat β-glucan, manganese superoxide dismutase, cytoskeleton, keratinocytes, lung cancer.

P3.4

PhLP3 protein in *Tetrahymena thermophila*

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Phosducin-like proteins (PhLP1, PhLP2, PhLP3), a conserved protein subfamily, participate in the regulation of CCT-mediated protein folding. Both, PhLP2 and PhLP3 play role in the biogenesis of cytoskeletal proteins, however available data are not consistent. *In vitro* studies suggest that yeast PhLP3 plays an inhibitory role in β-tubulin folding, while conversely *in vivo,* the studies in *C. elegans* point to the supporting role of PhLP3 in tubulin biogenesis. siRNA-mediated silencing of PhLP3 caused defects in microtubule architecture and aberrant cytokinesis. Recent data obtained by Hayes and co-workers support the hypothesis that PhLP3 is important for the maintenance of β-tubulin level in mammalian cells but also that its modulation can promoteactin-based cytoskeletal remodeling (Hayes *et al.* 2011).
Ciliated protozoan *Tetrahymena thermophila* assembles a great variety of microtubule arrays and therefore is a splendid model to investigate the role of PhLP3 in tubulin biogenesis. In this study we examined the molecular mechanism, by which PhLP3 affects CCT activity and/or determines microtubule assembly in *Tetrahymena*. We approached these problems using standard biochemical, molecular biology techniques and bioinformatics. Phlp3p expressedin *Tetrahymena* cells as GFP or HA-tagged fusion protein, localized in cilia, basal bodies and cytosol. Overexpression of the GFP- or HA-Phlp3p resulted in mild defects in cilia function. Mutant cells showed decreased rate of cells motility, proliferation and phagocytosis compared to wild type cells. Contrary to the effect caused by the elevated level of the PhLP3 expression, cells with knocked out *PHLP3* gene (*PhLP3-KO*) divided and moved faster than wild-type cells. To investigate if *PHLP3-KO* affects microtubule dynamics *in vivo*, we treated wild type and mutant cells with microtubule-stabilizing agent, paclitaxel. The paclitaxel induced hyperelongation of ciliary axonemes in both wild type and knockout cells, but the effect on *PHLP3-KO* cells was stronger. To characterize the function of Phlp3p, we searched for potential binding partners of this protein. Our results of immunoprecipitation experiments followed with mass spectrometry analysis indicate that Phlp3-HAp forms complexes with some axonemal dyneins.

Reference

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P3.5

The transition zone of cilia in *Paramecium* and *Tetrahymena* contains nucleoporins but no septins

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The complex structure of the transition region of locomotory cilia participates in docking young basal bodies to the surface, stabilizes the basal body-membrane connection, forms a diffusion barrier and modulates intraciliary transport. In some ciliated protists, the transition zone includes three plates: the terminal plate, the intermediate plate and the axosomal plate (Aubusson-Fleury *et al.*, 2012, Sattler & Staehelin, 1974). The three plates span the whole proximal part of a cilium from the interior of the cilium to the membrane where they are continuous with various elements of the membrane skeleton (epiplasmins). The plates differ in substructure and show a porous aspect.
In vertebrate ciliated cells, nucleoporins and septins together with other proteins were suggested to be present in the transition zone of locomotory cilia (Kee *et al.*, 2012).
Here we present data that suggests the presence of nucleoporins in the transition region of cilia in *Paramecium* and *Tetrahymena* as well as in nuclear pores. Using both the monoclonal antibody 414, which binds to FG-containing nucleoporins, and specific anti-tubulin antibodies,we detected labeling at the distal part of basal bodies and a spotted pattern in nuclear envelopes.
Previous studies established that in *Tetrahymena* GFP-tagged septins (Sep1, Sep2 and Sep3) localize to the mitochondrial/ER compartment but localize neither to the basal bodies or cilia nor to either macro- and micro-nuclei. In *Tetrahymena,* the septins are not essential for viability; however, ultrastructurally studied cells with knocked-out septins display disrupted nuclear (Mac and Mic) membranes but unaffected ciliary transition zones (Włoga *et al.*, 2008). Similarly, ultrastructural studies of *Paramecium* fixed after RNA silencing of the expression of the *SEP2* gene also revealed affected nuclear membranes but normal transition regions. Immunolabeling of *SEP2*-silenced cells with mAb 414 and DAPI showed normal labeling of the ciliary base, but abnormal nuclei with patches of missing nuclear pores in macronuclei.
Our studies support the conclusion that some nucleoporins are present in both the nuclear pores and the ciliary transition region, although there are no definite similarities in the ultrastructure of the nuclear and "ciliary" pores. In addition, the nuclear pore proteins seem to interact with septins, yet the lack of any effect of silencing of septins on the immunolacalization of mAb 414 in the ciliary terminal plate suggests that nucleoporins may have different binding partnetrs in nuclear pores and in the plates in the transition region.

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P3.6

Gamma-tubulin phosphorylation on specific residues is correlated with the regulation of the basal body duplication and positioning

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Basal bodies and centrioles are evolutionary conserved homologous structures. Their scaffold is composed of nine triplets of microtubules. γ-Tubulin (Gtu) is an essential component of centriole and basal body. In *Tetrahymena*, knockout of γ-tubulin (GTU-KO) is lethal due to the inhibition of basal body duplication and destabilization of the existing basal bodies that leads to the progressive degeneration of microtubule-based cortical structures correlated with loss of cell polarity.
Little is known about the regulation of Gtu function by posttranslational modifications. To date, only ubiquitination and phosphorylation of Gtu were described and shown to play an important role in the regulation of centrosome duplication in mammals and microtubular network formation in yeast. Till now, it is unknown if posttranslational modifications of Gtu have any effect on basal bodies.
We used mass spectrometry analysis to identify posttranslational modifications of *T. thermophila* Gtu. We found several sited of Gtu phosphorylation. These data together with bioinformatic analysis of available phosphoproteomes allowed us to select several serine and threonine residues that were found to be phosphorylated, and lysine residues known to be ubiquitinated. Using side-directed mutagenesis we mutated Gtu and introduced single amino acid substitutions: lysine (ubiquitination site) by unmodifiable arginine and serine and threonine by alanine (mimicking permanent dephophorylation) or aspartic acid (permanent phosphorylation). Mutated HA-tagged Gtu was expressed in *Tetrahymena* cells.
γ-Tubulin with replaced two lysines known to be ubiquitinated in mammals rescued GTU-KO *Tetrahymena* cells and restored wild type phenotype. This suggests that in *Tetrahymena* ubiquitination of Gtu on these residues is dispensable for basal body function.
Tetrahymena mutants carrying mutations mimicking permanent phosphorylation or dephosphorylation of serine or threonine residues showed abnormal phenotypes: reduction of the number of basal bodies and basal body rows (BBRs) and misposition of basal bodies with abnormal location of accompanying structures.
Our findings suggest that phosphorylation of γ-tubulin on several residues could regulate basal body duplication and positioning within cell cortex and thus influence the formation of the cell cortex polarity.

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P3.7

Effect of the entomopathogenic fungus *Conidiobolus coronatus* infection on morphology and cytoskeleton of hemocytes isolated from the greather wax moth, *Galleria mellonella* (Lepidoptera)

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Invertebrate model hosts represent valuable tools for the study of host-pathogen interactions because they facilitate the identification of parasitic virulence factors and allow the discovery of novel components involved in host immune system responses. In this work, we determined that the greater wax moth, *Galleria mellonella,* is a convenient non-mammalian model host for studying the effects of pathogenic fungus *Conidiobolus coronatus* infection on insect's immunocompetent blood cells (hemocytes), and described infection-induced changes in hemocytes' morphology and cytoskeleton organization.
In the hemolymph of *G. mellonella* last-instar larvae five types of hemocytes are present: granulocytes (Gr), plasmatocytes (Pl), oenocytes (Oe) sferulocytes (Sf) and prohemocytes (Pr). Two of them, Gr and Pl possess the ability to adhere to foreign surfaces and participate in cellular innate immune mechanisms (phagocytosis, encapsulation, and nodulation).
C. coronatus, a soil cosmopolitan pathogenic fungus easily attacks *G. mellonella* larvae and demonstrates high efficiency in induction of insects' mortality. Insect larvae were exposed to fully grown and sporulating *C. coronatus* colonies for 18 hours. Hemolymph was collected 24 and 48 h after exposure, stained with phalloidin – FITC and Hoechst 33342, and analyzed under fluorescence microscope. As controls served hemocytes obtained from healthy larvae.
At 24h post infection, no significant changes in the adherence ability of Gr and Pl were observed but first changes in the cytoskeleton organization began to appear. Gr started to form thin and long fibers around the entire cell, Pl extended and became 'hyperspreading', some of Pls contain a lot of actin spots and reminded detaching cells. However, 48h after infection only few cells were able to adhere but not to spread, actin distribution was disturbed, in some cases 'naked nuclei' were visible which suggests that cells have lost membrane integrity. Cells in controls looked normal – Pl had a fibroblastic shape and form regular filopodia, in Gr actin cytoskeleton and cytoplasm spread symmetrically around the cells.
In previous works we demonstrated that *C. coronatus* is able to overcome *G. mellonella* immunity mechanisms alerted by intruder invasion (increased phagocytic activity of Pl, elevated lysozyme level) chiefly by releasing toxic proteins disintegrating Sfs and decomposing hemocytic nets. Fungal metabolites seem to be also responsible for suppression of the prophenoloxidase-activating system in *G. mellonella*. Since none of 4 toxic proteins we have isolated from *C. coronatus* provoked in *G. mellonella* hemocytes cultured *in vitro* cytoskeleton changes similar to those described here, it proves that *C. coronatus* owns abundant toxic metabolites in its repertoire.

P3.8

Role of HAX-1 protein in cell migration and invasion

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It was proposed that the HAX-1 protein was engaged in many cellular processes like apoptosis, cell migration and adhesion. To further investigate its biological function we established the pairs of stable cell lines with silenced or normal expression of HAX-1 gene in HeLa, HEK293 and MDA-MB-231 cells. Through such a diversity of cell lines we provide the opportunity to test the influence of HAX-1 on various type of cells (epithelial and mesenchymal-like). Our results show that stable cell line with silenced HAX-1 derived from HeLa cells (miH1) is more invasive than the respective control cell line (mineg). Moreover, there are also differences between those cells in scratch assay tests and migration tests. In conclusion, our results support the thesis that HAX-1 protein is involved in processes like cell migration and invasion.

P3.9

Electrotaxis of two sublines of Walker WC256 carcinosarcoma cells representing blebbing or lamellipodial strategy of movement

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Although sensing of the electric fields (EF) is important for directional cell migration during wound healing, embryonic development, nerve growth and cancer metastasis, the underlying mechanism still remains unclear. Additionally there is no research addressing the question on the difference in electrotactic motility of cells representing various strategies of cell movement — specifically blebbing vs lamellipodial migration.

In the current study we arranged an unique experimental model which allows us investigation of the electrotactic movement of cells of the same origin but representing different modes of cell migration depending on the strength of adhesion to the substrate. By epigenetic selection we obtained two adherent sublines of Walker carcinosarcoma cells, representing different modes of cell migration: weakly adherent, spontaneously blebbing WC256 cells (BC) and lamelliopodia forming WC256 cells (LC). Visualization of F-actin in living cells (LifeAct) revealed that in blebbing (BC) WC256 cells, expanded blebs were devoided of actin and the cell membrane detached from the cortex. In contrast, in lamellipodia forming (LC) WC256 cells the protrusion was seen as wide band of fluorescent labeled F-actin. Here, we report that both BC and LC sublines show robust cathodal migration in a physiological EF (1-3 V/cm). The directionality of cell movement was completely reversible upon reversing the field polarity. However, the observed reaction was much faster in the case of BC (5 minutes) than LC cells (30 minutes) after the change of dcEF polarity.

We also investigated distinct requirements for Rac, cdc42 and Rho pathways and the role of extracellular Ca^{2+} ions in guidance by electric fields of WC256 sublines forming different types of cell protrusions. Significant differences in the reaction were observed after Rac, cdc42 and ROCK inhibition. It was found that Rac GTPase is required for directional movement of LC but not for BC cells. In contrast ROCK and cdc42 activity was necessary for electrotaxis of BC but not LC cells. The obtained results also showed that Ca^{2+} ions are essential only for the electrotactic reaction only of BC cells.

In conclusion, our results reveal that both lamellipodia and membrane blebs can efficiently drive electrotactic migration of WC 256 carcinosarcoma cells mediated by different signalling pathways.

P3.10

Synaptic podosomes - actin-rich organelles involved in muscle postsynaptic machinery remodeling

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Neuromuscular junctions (NMJ) are specialized cholinergic synapses that form between motor neurons and skeletal muscle fibers. Like synapses in the brain, NMJs undergo intense synaptic remodeling. In the course of postnatal development, acetylcholine receptors (AChR), major components of the postsynaptic machinery at the surface of the muscle fiber aggregate into a plaque-like assembly, which is subsequently reorganized into a complex structure that resembles a pretzel. The molecular mechanism that underlies this so-called "plaque-to-pretzel" transition of the NMJ is poorly understood, but it has been thought to be guided exclusively by the presynaptic nerve terminals. Recent studies show that the reshaping of the postsynaptic machinery in cultured muscle cells can occur in muscle cells cultured in the absence of neurons suggesting that muscle cells may have their intrinsic mechanism for the remodeling of the AChRs assembly. Previously, we have reported an unexpected discovery that the remodeling of the postsynaptic machinery in cultured myotubes is dependent on podosomes, actin-based protrusive structures involved in cell adhesion and extracellular matrix degradation. We further show that the scaffold protein Amotl2 is associated with podosomes in various cell types and that it regulates the development of the postsynaptic specialization in cultured muscle cells through the regulation of synaptic podosomes. Furthermore, we demonstrate that both actin and Amotl2 are present *in vivo* in postsynaptic areas of the NMJ predicted to contain synaptic podosomes, supporting our model in which muscle cells utilize podosomes as part of a muscle-intrinsic program that influences NMJ remodeling. Additionally, we provide evidence that Amotl2 binds directly to actin and regulates its nucleation, suggesting a potential biochemical mechanism for AChR cluster remodeling.

P3.11

Microscopy techniques and image analysis in glioma C6 migration studies

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Gliomas are a group of tumors characterized by strong dependence of tumor invasion on cell motility due to the isolation from circulation by the blood-brain barrier. Regulation of their movements involves chemotaxis driven by extracellular nucleotides which act on specific GPCRs from P2Y group. One of them, the P2Y2 receptor may be activated with ATP or UTP and regulates multiple signaling pathways in the cell, such as calcium response and Rac1 and RhoA proteins activation.
In our study we applied microscopy techniques to research glioma C6 cells migration and morphological properties which depend on nucleotide signaling from P2Y2 receptor. The aim of this study was the optimization of time-lapse live cell tracking. Glioma C6 cells chemotactic migration was examined with time-lapse DIC microscopy. Obtained data was processed with ImageJ plugins to perform time lapse images registration which improved the reliability of cell tracking and to stitch the adjacent fields of view to enable tracking of cells localized near the border of the field of view. Subsequently Metamorph software was used to track cells using direct tracking method and then cell trajectories were analyzed with a Matlab script to calculate migration parameters. Developed procedures eliminated random component of trajectory which was dependent on mechanized stage inaccuracy and allowed following cells crossing frame edges during experiment.

P3.12

Tetrahymena proteins FAP251 and FAP61 build the base of the radial spoke 3 and are essential for cilia motility

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Cilia are tiny cells protrusions, common in Eukaryotes except higher plants and fungi. Based on the ability to generate movement, cilia are divided into two categories: motile and immotile. Besides a scaffold made of nine peripheral microtubule doublets, characteristic for both types of cilia, motile cilia have two additional, centrally positioned single microtubules and complexes associated with peripheral doublet microtubules including inner and outer dynein arms, radial spokes, and nexin-dynein regulatory complex. Mechanochemical signals transferred from the central microtubules through radial spokes to the dynein arms regulate ciliary waveform and ciliary beat frequency.

In motile cilia there are three types of radial spokes (RS1, RS2 and RS3) that repeat as triplets along peripheral microtubule doublets. In *Chlamydomonas* RS3 is reduced to short knob while in other analyzed organisms (e.g. *Tetrahymena*) RS3 assembles as full-size structure.

Recently, in *Chlamydomonas*, it was shown that three evolutionary conserved proteins FAP91, FAP251 and FAP61, together with calmodulin, form a CSC complex (**c**almodulin and **s**pokes associated **c**omplex), located near the base of the RS2 and RS3. Depletion of either FAP61 or FAP91 results in lack or reduction of the RS2 and RS3.

Our analysis further elucidates the localization and role of FAP61 and FAP251. Using polyclonal antibodies, we showed that murine orthologs of FAP61 and FAP251, C20orf26 and WDR66, respectively, localize in motile cilia of multiciliated tracheal epithelial cells but not in non-motile primary cilia of IMCD-3 cells, suggesting that studied proteins are motile cilia-specific proteins.

In *Tetrahymena* cells both, FAP61 and FAP251 also localize in cilia. Deletion of either protein does not affect cilia assembly and stability but results in strongly reduced cell swimming velocity, compared to wild type cells. Video recording revealed that cilia in *FAP61-KO* and *FAP251-KO* mutants are less flexible and display modified beat waveform. The ultrastructural analysis showed that in *Tetrahymena* CSC complex is positioned only at the base of the RS3 and knockout strains lack or assemble only partial RS3 while RS1 and RS2 are unaltered. Thus, heterogeneity of the RS3 morphology in *Chlamydomonas* and *Tetrahymena* correlates with the differences in the positioning of CSC complex.

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P3.13

Tubulin glutamylation affects localization of katanin p60 but not its regulatory subunit p80 in *Tetrahymena* cells

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Microtubule severing enzyme, katanin p60 controls microtubule dynamics by generating internal breaks in microtubule lattice leading to it fragmentation. Katanin plays an important role in various cellular processes. In a green algae *Chlamydomonas reinhardtii* and in a ciliate *Tetrahymena thermophila* it was shown that mutation or deletion of katanin p60 or its regulatory subunit p80, affects assembly of the central pair microtubules in cilia, leading to cells immobility. Moreover, *Tetrahymena* cells with deleted p60 or p80 proteins are unable to finish cytokinesis and form chains composed of two to four subcells.

Katanin p60, overexpressed in *Tetrahymena* cells, localizes near basal bodies and weakly in cilia (Sharma *et al.*, 2007, J *Cell Biol*). We showed that HA-tagged p80, either overexpressed or expressed at the native level, accumulates in the region of basal bodies, contractile vacuoles pores, division furrow and weakly in cilia. Interestingly, in some interphase cells, a short p80-positive line-like structure was visible at the posterior end of the cells, presumably a remains of the p80 accumulation in the cleavage furrow during very recent cell division.

The co-localization of the HA-tagged p80 with ciliary, basal body and cortical microtubules was confirmed at the ultrastructural level using immunogold labeling. Interestingly p80 was also found in the region of the transition zone between cilia and basal bodies. This suggests that katanin may affect cilia assembly by the regulation of the microtubule dynamics at the basal body distal end.

In order to investigate if the level of tubulin glutamylation affects localization of katanin subunits in *Tetrahymena* cells, we co-expressed either katanin p60 or its regulatory subunit p80 with a potent tubulin glutamylase TTLL6A (Tubulin tyrosine ligase-like protein) (Janke *et al.*, 2005, *Science*). The glutamylase overexpression-driven increase of the level of tubulin glutamylation on the cytoplasmic, subcortical and ciliary microtubules caused mislocalization of the katanin p60 but did not affect distribution of the p80 in *Tetrahymena* cells.

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P3.14

Effect of cGMP and dexamethasone on high glucose-dependent changes in podocytes

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Introduction: Podocytes are cells responsible for maintaining the kidney filtration barrier and their impairment leads to proteinuria. Permeability to proteins is associated with changes of podocyte motility. Vasorelaxing mediator cyclic GMP may modulate podocyte cytoskeleton and adhesion. Matricellular protein thrombospondin-1 (TSP-1) that is upregulated in diabetic kidney, may be another factor affecting podocyte adhesion and integrity. Podocytes are a direct target for glucocorticoids which are commonly used to treat nephrotic syndrome and other proteinuric diseases.
Aims: In the present study we examined (1) the effect of high glucose and cGMP on podocyte-dependent TSP-1 synthesis and (2) the effect of cGMP and glucocorticoids on podocyte motility in the presence of high glucose.
Methods: Immortalized mouse podocytes were preincubated in media containing normal (NG, 5.6 mM) or high (HG, 30 mM) glucose for 5 days. Then, 1 μM dexamethasone (DEX) or 8 bromo-cGMP (non-metabolizable cGMP analog) were added for next 24 hours. Total RNA was isolated from the cells and RT-PCR analysis was performed. Resulting bands were visualized on agarose gel and gene expression was assessed using densitometry. Migration was investigated by wound–healing assays. Some cells were cultured on the glass cover slips and TSP-1 protein together with F-actin were visualized by immunofluorescence.
Results: Podocytes cultured in both, HG and NG media express the TSP-1 gene. High glucose enhanced TSP-1 expression by 55±3%, as compared to NG. Within NG group, stimulation of cGMP did not evoke any significant changes in TSP-1 gene. In HG cells, 8 Br –cGMP inhibited the TSP-1 expression by 56±11%. High glucose increased the motility of podocytes and the effect was enhanced by 8-Br cGMP. DEX inhibited the glucose- and cGMP-stimulated motility of podocytes.
Conclusions: Elevated TSP-1 may trigger signaling pathways leading to podocyte injury in diabetes.
Increased by cGMP motility of podocytes may result in disruption of filtration barrier and proteinuria.
Glucocorticoids may protect the integrity of renal filter by stabilizing the structure of podocytes.

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P3.15

Two populations of mouse bone marrow-derived cells migrate differently in a physiological direct current electric field

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Transepithelial potential (TEP) is established in a skin and other tissues as a result of unequal distribution of ions across the epithelial layer. After wounding the conductive extracellular pathway is created and ion flux generate endogenous extracellular direct current electric field (DC EF) with the negative pole located at a wound center. These naturally-occurring EFs last during healing and may guide cell migration by process known as an electrotaxis. To date, it was shown that many cell types respond to physiological levels of EFs with accelerated, directed migration mostly toward the cathode. Mounting of evidence suggest that bone marrow-derived cells may be involved in healing of cutaneous wound, contributing to skin cells or releasing regulatory cytokines.
In our study, application of DC EF of physiological level (100–300 mV/mm) to the adherent fraction of mouse bone marrow cells results in their accelerated migration parallel to the EF lines. Further analysis lead us to discrimination of two cell subpopulations migrating to the opposite poles. These populations exhibit significantly different morphological and migratory features. The anodal cells are relatively small, elongated and they form a narrow lamellipodium at the anode-facing site of the cell setting their long axis square with the EF lines. At the same time, the cathodal cells are larger, more flattened and they tend to elongate perpendicular to the field lines. Both populations show most directed migration in 300 mV/mm with the directional cosinus γ values of 0.84±0.04 for cathodal, and -0.90±0.02 for anodal cells. Moreover, these two cell types utilize different cellular pathways in their response to DC EF. Phosphatydyloinositol-3 kinase/Akt kinase seem to be more essential for efficient migration of cathodal cells under EF, whereas small GTPases Cdc42 and Rac1 are more involved in electrotactic response of anodal cells. Interestingly, cell markers analysis lead us to conclusion, that cells migrating toward the anode are of hematopoietic origin, mostly they comprise bone-marrow macrophages, while the cathodal cells contain the fraction of mesenchymal stem cells.
Taken together, our results suggest that response to physiological DC electric field may be one of the factors that discriminate bone marrow mesenchymal stem cells attraction to the site of wounding simultaneously excluding other cell types like macrophages from the wound bed.

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P3.16

The studies on the presence of functional P2X7 receptors at different cell lines

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P2X7 receptors (P2X7Rs) are members of the family of ionotropic ATP-gated receptors. Activation of P2X7Rs by extracellular ATP triggers two distinct fluxes. The first reflects a rapid and reversible current through the channels permeable to small mono- and divalent cations, while the second one allows to open a nonselective pore suitablefor the passage of the molecules up to a molecular mass 900 Da. Additionally, the activation of P2X7Rs by ATP may induce the programmed cell death. The P2X receptors are distributed in many tissues, and P2X7Rs are primaryily localized in epithelial and immune cells, particularly in the antigen-presenting cells.

The P2X7 receptor channels are used to transport into a cell certain membrane-impermeable compounds, for example analogs of components of nucleic acids. Because active, functional P2X7Rs are not widely distributed, we decided to measure the level of mRNA (by the real time RT PCR) and protein (by western blot) for the P2X7 receptors in several cell lines. In addition, functional activity of the P2X7 receptors was determined in some cell lines expressing the P2X7 receptor gene.

The measurements were based on the fluorescence of ethidium bromide and propidium iodide uptaken by the cells following ATP-dependent formation of cell pores. In these experiments, the buffer was used that fosters ATP-induced permeabilization, with simultaneous high cell survival.

We assume that channels of this type, reversibly open/close by extracellular ATP, might serve as a mean to introduce membrane-impermeable analogs of components of nucleic acids into the cytoplasm.

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