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Knowledge Transfer at the RECAMO Summer School of 2013

Předávání znalostí na letní škole Regionálního centra aplikované molekulární onkologie v roce 2013

Sheard MA[1], Zdrazilova-Dubska L.[2]

[1] Children's Hospital Los Angeles, Los Angeles CA, U.S.A.
[2] Department of Laboratory Medicine and Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic

Summary
Scientists early in their careers do not often get a chance to meet with their peers in a well-structured professional environment to discuss and compare their initial experiences and results. Creating just such an opportunity, a meeting held on August 18–22, 2013 in Litohor, Czech Republic, titled 'Summer School – Methods of Basic and Translational Cancer Research', was organized by the Regional Centre of Applied Molecular Oncology at the Masaryk Memorial Cancer Institute and funded by IntegRECAMO project (EU funds, reg. no. CZ.1.07/2.3.00/20.0097). The participants included senior and junior scientists, as well as PhD students. Educational activities involved various formats: 1. hands-on activities, 2. educational talks, 3. junior and PhD student talks with many presentations in English, 4. science-related excursions, 5. an environment for informal researcher interactions, 6. reports from visits to distant laboratories. Each participant spoke for 20 minutes or more, introducing the methods performed in their laboratory and sharing their recently obtained data. The talks ranged from basic and pre-clinical research to clinical methods. In this report, basic and pre-clinical research is presented first, followed by clinically relevant methods and practices. Here we present overview of selected talks.

Key words
education – molecular biology – methodology – statistics – laboratory research – cancer

Souhrn
Na počátku své profesionální kariéry vědci často nemají příležitost k strukturované interakci se svými kolegy a možnost porovnávat a diskutovat své zkušenosti a dosavadní výsledky. Regionální centrum aplikované molekulární onkologie (RECAMO) Masarykova onkologického ústavu vytvořilo příležitost pro setkání vědců v podobě letní školy zaměřené na metody základního a aplikovaného výzkumu v onkologii. Účastníky letní školy, která se konala 18.–22. srpna 2013 v Litohoři za podpory projektu IntegRECAMO (EU fondy, OP VK, CZ.1.07/2.3.00/20.0097), byli mladí výzkumníci a Ph.D. studenti, ale také výzkumníci senioři. Letní škola se skládala z několika typů edukačních aktivit: 1. praktická výuka, samostatné práce a ukázky (workshopy „biostatistika a analýza dat" a „celogenomové sekvenování", 2. edukační přednášky, 3. prezentace výsledků mladých vědců a Ph.D. studentů, zpravidla v anglickém jazyce, 4. exkurze do technologických provozů, 5. neformální interakce, 6. reporty z pracovních cest do zahraničních a tuzemských laboratoří. Vybrané přednášky, které zazněly na letní škole, informující o aktuálních přístupech a výsledcích základního, preklinického i klinického výzkumu jsou shrnuty v tomto příspěvku.

Klíčová slova
vzdělávání – molekulární biologie – metodologie – statistika – laboratorní výzkum – nádorové procesy

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Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

RNDr. Lenka Zdrazilova Dubska, Ph.D.
Department of Laboratory Medicine and Regional Centre for Applied Molecular Oncology
Masaryk Memorial Cancer Institute
Zluty kopec 7
656 53 Brno
Czech Republic
e-mail: dubska@mou.cz

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MicroRNAs (miRNAs) are short species of RNA that are 18–22 nucleotides in length. MiRNAs regulate approximately half of all protein-coding genes. In general, any one miRNA can regulate many genes (20–200), and some miRNAs can exert oncogenic while others exert tumor suppressor activity. On this topic, Jaroslav Juracek (Masaryk University) discussed the role of miRNAs in triple negative ($ER^-PR^-Her2^-$) breast cancers (TNBC), which represent approximately 15% of all breast cancer cases. TNBC cannot be treated with therapies that target estrogen receptor, progesterone receptor, or the Her2 receptor, and therefore identification of new targets for TNBC is needed. Juracek and colleagues used quantitative RT-PCR to detect 7 miRNAs that were identified in the literature as possible targets, namely miR-205, 24–2, 99, 125b, 146, 187, and 505. Eighty-one samples from TNBC patients were evaluated for expression of the 7 selected miRNAs, and an association with improved prognosis was observed for miR-205 and miR-505. Because miR-205 has already been intensely studied by others, Juracek and colleagues decided to select the less studied miR-505 for further analysis, and included miR-187 based on findings reported in the literature. TNBC cell lines MDA-MB231 and BT474 were chosen and transfected with miR-187 and miR-505. Scratch wound assays demonstrated a suppressive effect of miR-187 and miR-505 on the migration of both cell lines. Of these two candidate miRNAs, however, only miR-505 reduced proliferation of the cell lines, and preliminary results suggest this reduction is caused by a G1 cell cycle arrest. Future plans include replication of these experiments in other TNBC cell lines, 3D *in vitro* invasion assays, and identification of target proteins using co-immunoprecipitation.

Jitka Mlcochova (Masaryk University) and colleagues have been working to determine miRNAs expression profiles of metastatic colorectal carcinoma patients with wt-*KRAS* who were treated with cetuximab and to identify those of predictive significance. Using microarrays and qRT-PCR, they compared 21 patients who responded to anti-EGFR therapy versus 23 who did not. They found that miR-31 and miR-31* were significantly lower in responders compared to non-responders, and a combination of miR-31 plus miR-31* was able not only to stratify responders from non-responders, but their higher level also correlated with shorter time to progression and lower overall survival.

The famous tumor suppressor gene *TP53* has two other family members, *TP63* and *TP73*. Paulina Orzol (Masaryk Memorial Cancer Institute – MMCI) presented a review of this gene family, revealing that all three family members can regulate cell cycle arrest and apoptosis, but to different degrees and in different tissues. The *TP53* gene is the most frequently mutated gene across all cancer types, in slightly more than 50% of all human cancers. Unlike *TP53, TP63* is rarely mutated in the small subset of cancers in which it is expressed; e. g., B-cell lymphomas. This is in spite of the fact that p63 can induce apoptosis and inhibit metastasis. In normal development, p63 is involved in epidermal development. In contrast, p73 is involved in neural development and participates in determining neural structures. Like p63, p73 is expressed in only a subset of cancers. There are several isoforms of the p73 protein. Among the isoforms, the TAp73 isoform is overexpressed in several cancers (egg. breast, ovary, melanoma) and can act as a tumor suppressor by inducing cycle arrest and apoptosis. In contrast, the ΔNp73 isoform is considered to be an oncogene owing to its ability to interfere with p53 and TAp73 activity. The *p73* gene can be methylated and is thereby down-regulated in many hematological malignancies, possibly preventing its tumor suppressor activity. Research into p73-based therapies includes modulation of the TA/ΔN ratio of p73, and development of miRNAs to inhibit ΔNp73.

Cancer cell lines grown *in vitro* are routinely used as models intended to represent cancer cells *in vivo*. Michael Sheard (Children's Hospital Los Angeles) gave a presentation in favor of establishing all future cell lines in physiologic levels of oxygen (O_2) such as 5% O_2 rather than the common practice of using room air (~ 20% O_2). Specimens from 12 acute lymphoblastic leukemia (ALL) patients were obtained and three grew successfully as newly established cell lines in both 20% and 5% O_2. Whole genome analysis indicated that the cell lines established in 20% O_2 had lower expression of glycolysis genes than their sister cell lines in 5% O_2, which was confirmed by RT-PCR and immunoblotting. Cell lines established in 20% O_2 also consumed less glucose and produced less lactate, indicative of lower glycolysis. These effects could only be partially reversed by moving cell cultures from 20% to 5% O_2 or from 5% to 20% O_2 for eight weeks. Since aerobic glycolysis is a hallmark of cancer, the lower level of glycolysis in cells established in atmospheric O_2 suggests that these cells are less representative of the original cancer. These results argue that ALL cell lines should be established at the onset in physiologic levels of O_2 to preserve aerobic glycolysis. It has been previously described how researchers working with cell types other than ALL identified reasons for preferring physiologic O_2 rather than atmospheric O_2, such as improved outcomes in cell-type specific functional assays [1].

The high rate of proliferation of many cancer cells identifies proliferation itself as a therapeutic target. Proliferating cells must generate new copies of their DNA, and the targeting of DNA with compounds such as cisplatin has long been an established therapeutic approach. Lucie Koubkova (MMCI) presented an introduction to organometalic compounds. While cisplatin is the most well-known of these compounds, it has substantial side-effects. Carboplatin has fewer side effects than cisplatin and oxaliplatin and has found its way into the therapeutic regimen of some cancer types. Other drugs are still in investigational stages, including satraplatin, lipoplatin and non-platinum organometallic drugs (metallocenes), such as the ruthenium-based drugs NAMI-A, DW12, and its derivative NP309. Koubkova and colleagues are investigating the titanocene family of drugs. Titanocene dichloride was previously tested in phase I and II clinical trials, but was even-

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tually found to be too reactive for therapeutic use. Koubkova and colleagues are continuing the search for titanocene derivatives exhibiting an improved therapeutic index.

Determining the dynamic structural changes in proteins can aid our understanding of protein architecture and protein : protein interactions, but such changes are poorly resolved by classical crystallographic methods. Multidisciplinary approaches involving NMR, small angle X-ray scattering, and hydrogen/deuterium (H/D) exchange may offer increased resolution of protein structural and conformational models. Filip Trcka (MMCI) and colleagues are using H/D exchange methodology to elucidate the binding properties of tetratricopeptide repeat (TPR) domains in the TOMM34 protein. TOMM34 appears to have TPR domains with conserved amino acid positions responsible for formation of a dicarboxylate clamp. Dicarboxylate clamps represent electrostatic modules capable of binding to the C-terminal aspartate residues of the chaperone proteins HSP70 and HSP90. H/D exchange experiments showed a decreased level of deuteration of TOMM34 peptides corresponding to the TPR domains after addition of HSP70/90 C-terminal peptides, suggesting that the dicarboxylate clamp in the TOMM34 TPR domains is functional. Characterization of protein structural changes using the H/D exchange method and nano-machinery such as dicarboxylate clamps will expand our ability to understand the dynamic nature of the proteome and will extend our ability to develop targeted applications and therapies.

Electrochemistry allows analysis of proteins and nucleic acids in an inexpensive format. Martin Bartosik (MMCI) reviewed the strengths (low cost, simple instrumentation and short time of analysis) and challenges (sensitivity, reproducibility) of this methodological approach, giving a description of some current trends, including DNA hybridization detection employing ELISA format as well as utilization of nanotechnologies, protein-ligand interactions, and detection of miRNAs. A current trend in electrochemistry is DNA hybridization assays, construction of microarrays and chips and detection of biomarkers [2]. Recent results with DNA methylation analyses, protein-ligand interactions and detection of miRNAs were also mentioned.

In oncology, proteomics can focus on functional studies, protein : protein interactions, protein structural characterization (glycosylation, acetylation, phosphorylation), search for biomarkers, and verification and quantification of biomarkers. Pavel Bouchal (MMCI, Masaryk University) reviewed the options for protein fractionation, mass spectrometry and software relevant to proteomics. The general workflow is to generate the initial biological or clinical idea, design experiments, extract the protein of interest, cleave the protein into peptides, fractionate, analyze by mass spectrometry, thereby identifying and quantifying the protein, and statistical analysis.

Giving an overview of sample preparation options for mass spectrometry, Lenka Hernychova (MMCI) provided a broad overview of methods for relative and absolute quantification of proteins in complex biological samples. When preparing samples for quantitative mass spectrometry measurement, a researcher can choose between enzymatic, metabolic or chemical labeling of proteins and/or peptides.

Cell surface proteins are easily accessible targets and therefore are of interest therapeutically. Jakub Faktor (Masaryk University, MMCI) and colleagues compared differences in surface proteins of MDA-MB-231 breast carcinoma cells with a variant clone that exhibits increased migratory ability. Surface proteins generally have high hydrophobicity and are therefore somewhat difficult to isolate in solution for mass spectrometry. Faktor labeled the extracellular domains of surface proteins with a reactive ester of biotin and then purified them on streptavidin sepharose, followed by fractionation by gel electrophoresis. Proteins were identified and quantified using an Orbitrap mass spectrometer. One hundred and thirty-nine proteins were found to be up-regulated in the more migrating variant (fold change > 1.5), and 38 of these proteins contained a transmembrane domain. Two hundred and twenty-eight proteins were down-regulated (fold change < 0.7), 91 of which contain a transmembrane domain. Several integrins were found among the down-regulated proteins, which might help explain the increased migratory capacity. Future work will include examination of whether any of these proteins are similarly affected in other cell lines upon selection of variants with increased migratory ability.

Cancer stem cells have been a topic of much interest in recent years. Alena Nunukova and colleagues are investigating putative stem cells in rhabdomyosarcoma. Four rhabdomyosarcoma cell lines expressing the myogenic marker Myo-D1 were examined for stem cell markers and a subpopulation of each cell line was found to express CD133, AC133 and nestin. These cell lines are being used in ongoing studies of rhabdomyosarcoma stem cells, are awaiting examination for expression of ABC drug transporters and might benefit from correlative methods, such as side population analysis that functionally measures drug efflux, a well-studied trait of stem cells. Other future plans include sorting of stem cell marker-expressing cells for functional progenitor cell assays such as the limiting dilution sphere-forming assay and *in vivo* tumorigenicity assay.

Resistance of cancer cells to drugs remains a major obstacle for cancer therapy. Multidrug-resistant cancer cells can be rendered drug-sensitive by inducing release of proteases from lysosomes. Blanka Jancekova (Masaryk University) reviewed how lysosomal membrane permeabilization can induce apoptosis. The lysosomal protease cathepsin D is ubiquitously expressed and can be released from lysosomes following treatment with specific drugs. MDA-MB-231 breast cancer cells were engineered to overexpress cathepsin D; among a group of therapeutic modalities examined, the TRAIL death ligand was the most active in inducing apoptosis in cathepsin D overexpressing cells but not in non-transfected parental cells. For this reason, Jancekova and colleagues decided to study TRAIL fur-

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ther. Bafilomycin, an inhibitor of lysosomal activity, was found to suppress both caspase-8 activation and apoptosis downstream of treatment with TRAIL. Interestingly, a low dose (10 ng/ml) of TRAIL was sufficient to induce release of cathepsin D, which is notable given a recent report that natural killer cells obtained from cancer patients and activated *ex vivo* can release soluble TRAIL at concentrations of 22 ng/ml in an NK cell expansion system that is being developed for adoptive cell therapy in phase I clinical trials.

Replication-deficient adenoviruses are expected to become increasingly useful for delivery of vaccines and immunotherapeutics. However, the effects of adenoviruses on inflammation *in vivo* are poorly understood, and attack by natural killer cells could undesirably dampen the effects of therapies that utilize adenovirus delivery systems. To address this issue, Mariana Pjechova (MMCI) and colleagues are using the replication-deficient adenovirus vector AdZ2 produced in HEK-293 embryonic kidney cells or 911 embryonic retinoblasts cells. AdZ2 has deletion of regions E1, E3, and E4 (ex. E4ORF6). By comparing AdZ2 with the adenovirus AdEasy that has deletions in only regions E1 and E3, Pjechova and colleagues found that deletion of the E4 region causes a decrease in activation of natural killer cells, measured as decreased expression on 293 and 911 cells of MICA, a ligand for the NK cell-activating receptor NKG2D. Further changes in signaling pathways caused by these vectors were revealed by examination of the phosphoproteome in cells infected by AdEasy and AdZ2 using an Orbitrap Elite mass spectometer. Future plans include cytotoxicity assays examining the effect of decreased MICA.

The Bank of Biological Material (BBM) which was established at MMCI in 2000 is nowadays the national coordinator of EU Biobanking and Biomolecular Resources Research Infrastructure (BBMRI) in the Czech Republic. As described by Kristina Greplova (MMCI), collection of clinical specimens is currently being performed in collaboration with institutes in Prague, Hradec Kralove and Olomouc. In the BBM, primary tumors, operable metastases, and (when appropriate) healthy tissues are cryopreserved in liquid nitrogen for future analyses. To provide material for quantification of biomarkers, specimens are stored short-term; for example, serum is stored for up to one year. To enable the potential for follow-up analysis, long-term storage of tumor material from each patient is also done. For assays examining RNA, specimens are first preserved in either liquid nitrogen or in RNAlater solution, and then RNA is purified at a later date. For genetic studies, an aliquot of genomic DNA and whole blood is archived for later examination. These approaches enable long-term studies and correlation with subsequently acquired clinical data, providing an invaluable resource for researchers and clinicians.

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Erratum

V Klin Onkol 2013; 26 (Suppl): S7–S12 byli v článku „Petráková K et al. Prekurzory karcinomu prsu“ chybně uvedeni spoluautoři článku. Na žádost autorů uvádíme správnou verzi.

Petráková K.[1], Nenutil R.[2], Palácová M.[1]
[1] Klinika komplexní onkologické péče, Masarykův onkologický ústav, Brno
[2] RECAMO, Masarykův onkologický ústav, Brno

The inccorect version of the names of co-authors were published in „Petráková K et al. Precursors of Breast Cancer“, Klin Onkol 2013; 26 (Suppl): S7–S12. At the request of the authors, we publish the correct version.

Petráková K.[1], Nenutil R.[2], Palácová M.[1]
[1] Klinika komplexní onkologické péče, Masarykův onkologický ústav, Brno
[2] RECAMO, Masarykův onkologický ústav, Brno