Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Konrad Kwaśniak	Year of studies: 3rd

Title of the project: Assessment expression of non-coding RNAs in patient with non-small cell lung cancer and their influence on cancer cells

Name of the supervisor: Professor Jacek Tabarkiewicz

Affiliation: Medical Faculty, University of Rzeszów

AIMS of the project:

In this study, we focused on long non-coding RNAs which belong to class large intergenic non-coding RNAs (lincRNAs) and which are transcribed from sites between coding protein sites.

The role of lncRNA is still poorly understood and more investigations are needed to get knowledge about this molecules in cancers. Our ncRNA is sense-intronic-ncRNA (which his name were lncCDH16-2:1, but now LNCpedia named this lncRNA - NONHSAT143046. Their localisation is Chr.16 66841433-66853448, before *CDH1* gene (Chr.16 68737225-68835548). In this study we would like to answer on few main questions: 1) Is a NONHSAT143046 an oncogene ? 2) is it regulating aggressiveness of NSCLC cells ? 3) how influence on expression *EpCAM*, *E-cadherin*, *CXCR-4* and *TRIM-44* ?

The aim of this project is to assess the expression of NONHSAT143046 in tumor cells and determine the influence of mentioned non-coding RNA on aggressiveness of tumor cells.

Methods: *Total RNA isolation, RT-PCR and qRT-PCR:* Total RNA was isolated from 18 patients (in I-III stage of diseases) with non-small cell lung cancer, and A549 human lung cancer cells. Next, RNA was transcribed on cDNA and evaluated expression level of NONHSAT143046. Furthermore, we also determine mRNA levels of *CXCR-4*, *EpCAM*, *E-cadherin*, and *OCT-4*. Endogenous controls in this study were *GAPDH* and *HPRT*. Additionally, we evaluated our results with the TNM stages.
Cell Culture, Transfection & Microscope analysis: A549 human lung cancer cells before all experiments seeded 24-26h prior treatment. Silencing of mentioned lncRNA was conducted using antisense oligonucleotides (AONs), 48h after transfection cells were collected and isolated total RNA and transcribed on cDNA. Expression level of *EpCAM*, *E-cadherin*, *OCT-4* & *CXCR-4* genes in transfected cells were evaluated using qRT-PCR. Moreover the subcellular distribution of AONs was examined using FITC 5` labelled antisense oligonucleotide targeting to the sequence of NONHSAT143046, control AONs contain sequence which does not match with human genome. To take a photos cells were planted into cover glasses, 24h after transfection, cells nuclei were dyed using DAPI. The localisation of AONs-FITC were assessing using fluorescence microscope.
Western blot & Flow cytometry: All experiments were performed on A549 cells which grown in 24-well plates and 48h after transfection conducting using control AON`s and specific AON`s for NONHSAT143046. The percentages of live, dead, early and late apoptotic cells; proliferating and non-proliferating and the determination of the cell cycle were measured using Muse® Cell Analyzer except analysis on FACS Aria III & Amnis® FlowSight®.By Western blot we evaluated level of β-catenin, CXCR-4, EpCAM, E-cadherin, Oct-4, Akt, phosphorylated-Akt, ERK1/2, phosphorylated-ERK1/2 and phosphorylated-β-catenin. While using flow cytometry we evaluate level of EpCAM, E-cadherin, CXCR-4 and TRIM-44 which were labeled by Zenon Alexa Fluor 488 Rabbit IgG Labeling Kit.
Flexmap 3D assay: To dectect protein level of Il-6, MMp-9, CXCL8/Il-8, MMP-1, MMP-2, CXCL-10/IP10, CCL2/MCP2, MMP-8, IL-1β, MMP-13, MMP-12, Total Cathepsin S, CXCL1/GROa, FGFbasic (FGF2), CXCL9/MIG, CRP, CXCL11/ITAC-1, MMP-10, FGFacidic (FGF1) and Proteinase3/PRTN3 in A549 culture medium and cell lysates of control and transfected cells were prepared using manufacturer`s suggestion.
Cell migration assay: In this study A549 cell lines were transfected using AONs, cultured in 24-well plates and made a straight scratch using 10µl tip. Cells migration were measured for 72 hours.

Statistical analysis: Non-parametric statistical tests were for analysis. The level of significance was set at 0.05. Statistical analyses were performed using the Statistica package version 12.

Results: Expression level of NONHSAT143046 was statistically significant higher in tumour from 18 patients with NSCLC and in A549 cell line compared to control, moreover in group of patients we do not found any statistical significant results between TNM stage and expression level of NONHSAT143046. Further, we would like to mention that very high expression of NONHSAT143046 could be correlate with patient shorter survival but in our case we have only two patients who have died so far. We think that NONHSAT143046 can be taken as an oncogene, which normally could be weaker expressed in healthy lung tissue than in tumour.

Imaging flow cytometry and microscopy analysis showed, that NONHSAT143046 is localised in nucleus (43,71%) and cytoplasm (56,29%).

After silencing using antisense oligonucleotides we detected up-regulation of genes which are regulating *ephitelial to mesynchymal transition* (EMT) such as *CDH1*, *EpCAM*, *CXCR-4* and *TRIM-44* genes. Additionally after silencing NONHSAT143046 expression level of *CHD1* and *EpCAM* increased five times compared to control, while *CXCR-4* expression were lower what could be due to faster translation process, and what we confirmed using Flow Cytometry and Western Blot analysis. Transfected cells in which NONHSAT143046 were silenced, faster undergo apoptosis, but percentage of proliferating cells (characterises by expression of Ki67) also were higher compared to control. We found that cells escape from G0/G1 phase and accumulate in G2/M phase. Moreover, experiments which determine aggressiveness capabilities of cancer cells showed that cancer cells with silenced NONHSAT143046 have limited capabilities to invasion and migration.

Results, obtained using flow cytometry analysis showed that in transfected cells population which, except higher EpCAM and CXCR-4 (what has been confirmed using western blot analysis), have also high E-cadherin what stay in accordance with results which we get after qRT-PCR. However, when we compared all events, the results were the same as which, we get using Western Blot – E-cadherin protein level were not changed statistically significant.

Western blot analysis also did not show statistical significant differences between phosphorylated β-catenin, total β-catenin, phosphorylated-AKT, total-AKT, phosphorylated-ERK1/2 or total ERK1/2 proteins concertation in control and after silencing NONHSAT143046.

Taken together, NONHSAT143046 could be considered as an oncogene and its low expression can be good prognostic factor for NSCLC patients. Because of their expression in healthy lung is significantly lower compared to patients with NSCLC with diseases stages I-III. Moreover, A549 cells are ongoing apoptosis when mentioned lncRNA is silenced in cancer cells. A549 cells in response must save itself by turn-on signalling pathway such as EGFR which regulate NF-κβ and in consequences upregulates most of pro-survival proteins which were investigated in our study, such as: IL-1β, IL-6, IL-8, CXCL-1 and MMP`s. Moreover, among cancer cells there are cells which up-regulate genes and proteins such as EpCAM, CXCR-4 and high E-cadherin which normally are not expressed as high as after silencing NONHSAT143046 and also, overexpress proteins which are able to regulate cells proliferation. However, the ability to migration of cells after silencing NONHSAT143046 is limited, despite of overexpression proteins which support migration capabilities. This results shows that NONHSAT143046 is very important molecule in NSCLC cells, which promote migration and invasion capabilities, and its lack promote apoptosis and many proteins which are responsible for proliferation to compensate the lack of NONHSAT143046.

Participation in conferences:

Publications, scientific presentations as a leading author:

1.Kwaśniak, K., Myszka, A., Czarnik, J., & Tabarkiewicz, J. (2018). The contribution of selected variants in ACE, MSTN and ADRB2 genes in the achievements of judo practitioners. ARCH BUDO, 14, IF-1,551; MNiSW-100.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Piotr Wysocki	Year of studies: 3rd

Title of the project: Functional study of *DIRC3* gene in differentiated thyroid cancer.

Name of the supervisor: Prof. Krystian Jażdżewski, Dr Monika Kolanowska

Affiliation: CENT University of Warsaw / Medical University of Warsaw

AIMS of the project:

Differentiated thyroid cancer (DTC), a common endocrine malignancy, has poorly explored hereditary component. A few of DTC predisposing variants were found to cluster in a functionally uncharacterized long non-coding RNA (lncRNA) gene called *disrupted in renal cancer 3* (DIRC3). It has been recently demonstrated that DIRC3 germline variants not only increase the lifetime DTC risk, but also alter the overall DTC patient mortality. In this project, we aim to describe the influence of the DIRC3 transcript and its top exonic DTC risk variant, rs11693806, on thyroid carcinogenesis. By elucidating the role of DIRC3 in the molecular processes orchestrating thyroid cancer, we hope to uncover novel prognostic or therapeutic possibilities.

The specific aims of the project include:

1. Analysis of DIRC3 expression in DTC tissue and correlation of the results with transcriptomic, germline genotyping and clinicopathological data.
2. DIRC3 silencing experiments aiming to probe biological and phenotypic function of DIRC3 transcript(s) *in vitro*.
3. Cloning of DIRC3 transcript isoforms and profiling their function in *in vitro.*
4. Genomic editing of DTC risk variant, rs11693806, to reveal its impact on DIRC3 function.
5. RNA-seq of cell lines with overexpressed DIRC3 to attain insights into transcriptomic effects induced by the lncRNA.

Methods:

1. We analyzed RNA-seq datasets to identify the DIRC3 expression profile in normal and cancer thyroid tissue. We also explored a possible transcriptomic mechanism of DIRC3 action by seeking correlations in the expression of DIRC3 and other genes. Next, we utilized patient-matched DTC/normal thyroid tissue pairs to analyze the DIRC3 expression with custom qRT-PCR assays. DIRC3 expression results were confronted with patients' clinicopathological data.
2. We obtained a panel of thyroid cancer (MDA-T32, MDA-T68, MDA-T120, K1) and breast cancer (MCF7) cell lines. Expression of major DIRC3 isoforms was evaluated using RT-PCR. Additionally, the genotype of rs11693806 in the cell lines was identified with PCR-based rhAmp SNP Genotyping Platform and Sanger sequencing. Next, we designed antisense oligonucleotides (ASOs) capable to selectively silence two major DIRC3 isoforms (DIRC3-202 & DIRC3-203). The ASOs were transfected into the cell lines and successful gene silencing was confirmed.
   After confirming successful DIRC3 knock-down, we will evaluate its phenotypic effect on cell viability (MTT assay), anchorage-independent growth (soft agar test), motility (scratch and Transwell tests), invasivity (Matrigel chambers) and apoptosis (Caspase-Glo 3/7 Assay).
3. We plan to isolate full-length transcripts of DIRC3 in cell lines utilizing 5'- and 3'-RACE System for Rapid Amplification of cDNA Ends. The products of RACE-PCR will be cloned and sequenced. Afterwards, full-length products (possibly originating from different DIRC3 isoforms) will be cloned into pcDNA3 expression plasmid. The biological effect of DIRC3 overexpression will be studied using a panel of functional assays described above.
4. We synthesized single guide RNA (sgRNA) targeting rs11693806 locus. Cas9/sgRNA ribonucleoprotein (RNP) complexes were delivered to thyroid cell lines via lipofection.

Generation of indel in the rs11693806 locus was confirmed with T7 endonuclease I cleavage assay. Next, we plan to deliver RNP together with single strand donor oligonucleotide (ssODN) and employ Homology-Directed Repair to generate alternate rs11693806 allele in cell lines.

5. We will perform RNA-seq of cell lines with overexpressed DIRC3 lncRNA. The generated data will be utilized for pathway and gene set enrichment analysis in iDEP software aiming to pinpoint the putative transcriptomic mechanisms responsible for DIRC3 functionality.

Results:

Evaluation of RNA-seq datasets revealed that DIRC3 is pervasively expressed in DTC. *In silico* analysis identified that DIRC3 is co-expressed with *IGFBP5* (insulin growth factor binding protein 5), a nearby gene with a putative tumor suppressive role. Study performed in 75 DTC/normal thyroid tissue pairs demonstrated that DIRC3 expression in cancer is downregulated, and confirmed a strong co-expression of DIRC3 and IGFBP5. We also observed a tendency for low DIRC3 lncRNA levels in DTCs manifesting with metastasis, mortality and angioinvasion.

Next, we characterized expression of two major isoforms of DIRC3 (DIRC3-202 and DIRC3-203) and rs11693806 genotype in a panel of cancer cell lines, detecting a range of expression levels and variety of genotypes. Basing on the data we hypothesized that rs11693806 genotype might influence isoform-specific expression of DIRC3. Accordingly, we will plan to profile isoform expression in DTC/normal thyroid tissue pairs and confront it with rs11693806 genotype.

We successfully transfected cancer cell lines with ASOs selectively silencing MALAT1 (a positive control gene) and DIRC3 isoforms. Importantly, knock-down of DIRC3-203 isoform induced downregulation of IGFBP5, in agreement with a hypothetical crosstalk between these two genes. We also performed preliminary MTT cell viability assay observing enhanced cell growth after DIRC3 knockdown. Nevertheless, due to concerns about Lipofectamine 2000 toxicity, the experiments will be replicated after transfection with alternative reagent (Fugene 6).

We also performed RACE-PCR of MDA-T32 cell line RNA generating 5'- and 3'- cDNA products corresponding to the expected DIRC3 transcript isoforms. After validation of their identity, we plan to clone them into pcDNA3 vector and characterize their function *in vitro*.

We successfully utilized Cas9 RNP to edit K1 cell line (genotype rs11693806[G/G]) generating indel in rs11693806 locus. Next, we plan to introduce ssODN carrying alternative rs11693806[C] allele and isolate modified clones. The phenotypic and transcriptomic effects induced by introduction of rs11693806[C] allele into K1 will be profiled.

Participation in conferences:

1. American Association for Cancer Research, Annual Meeting, March 29-April 3, 2019, Atlanta, USA

2. European Society of Digestive Oncology MASTER CLASS, October 5-6, 2018, Marseille, France

Publications, scientific presentations as a leading author:

1. *Hassan C, Wysocki PT et. al.* Endoscopic surveillance after surgical or endoscopic resection for colorectal cancer: European Society of Gastrointestinal Endoscopy (ESGE) and European Society of Digestive Oncology (ESDO) Guideline. Endoscopy. 2019 Mar;51(3). (IF = 6,381)

2. *Ren S, Gaykalova D, Wang J, Guo T, Danilova L, Favorov A, Fertig E, Bishop J, Khan Z, Flam E, Wysocki PT, DeJong P, Ando M, Liu C, Sakai A, Fukusumi T, Haft S, Sadat S, Califano JA.* Discovery and development of differentially methylated regions in human papillomavirus-related oropharyngeal squamous cell carcinoma. Int J Cancer. 2018 Nov 15;143(10). (IF = 4,982).

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Anna Biernacka	Year of studies: 4th

Title of the project: Searching for genetic background of hyperdiploidy in leukemia

Name of the supervisors: Prof. Rafał Płoski, MD, PhD; Grażyna Kostrzewa, PhD

Affiliation: Department of Medical Genetics, Medical University of Warsaw

AIMS of the project:

Hyperdiploidy is described as a gain of chromosomes, generally +X, +4, +6, +10, +14, +17, +18, and +21, and it is characteristic feature of leukemia cells, especially presented in ALL (25-30% cases of ALL). Genetic and molecular basis of hyperdiploidy in leukemia are still unknown, however recently introduced Next Generation Sequencing (NGS) gives a unique opportunity for wide searching of the mechanisms underlying this phenomenon. Our previous comparative, bioinformatic whole exome sequencing (WES) data analysis of coupled DNA samples from hyperdiploid ALL patient revealed, in sample from leukemia, a nonsense mutation NM_001024674.2:c.253C>T (p.Arg85*) in *LIN52* gene, whereas it was not observed in germline DNA. According to predictions algorithms this variant is damaging.

The aim of the study is to searching for the molecular background underlying the mechanism of hyperdiploidy in leukemias.

Methods:

WES performed on HiSeq 1500 (Illumina, San Diego, CA) using SureSelectXT Human All Exon V5 (Agilent) and SeqCap EZ MedExome (Roche) reagents for DNA library preparation. In-silico analysis was performed using MS Access files generated for each sample, which subsequently was filtered for each analysis in different way, to reveal somatic or germline mutations. Moreover .bam files were used to compare results from germline and leukemia samples and to ensure appearance of detected variants.

Results:

For all 19 samples average mean coverage was 87x, ge10 – 98 and ge 20 – 95. Comparative analysis of coupled samples was provided to assess presence of somatic and germline mutations in studied pairs. Germline variants were searched among known genes involved in cancerogenesis (TCGA) and also among rare variants with high probability of being pathogenic (using known programs to predict pathogenity). Somatic variants were searched using appropriate filters od Access files and also using MuTect algorithm.

Performed WES analysis did not reveal any clear candidate gene(s) or mutation(s) which underlie the mechanism of hyperdiploidy. Obtained results are promising, however there is no plausible candidate gene or mutation to disclose mechanism of hyperdiploidy in ALL.

Participation in conferences: none

Publications, scientific presentations as a leading author:

1. Novel COL12A1 variant as a cause of mild familial extracellular matrix-related myopathy. Jezela-Stanek A, Walczak A, Łaźniewski M, Kosińska J, Stawiński P, Murcia Pienkowski V, Biernacka A, Rydzanicz M, Kostrzewa G, Krajewski P, Plewczyński D, Płoski R. Clin Genet. 2019 Jun;95(6):736-738. doi: 0.1111/cge.13534. Epub 2019 Mar 28. PMID: 30920656
2. Mapping of breakpoints in balanced chromosomal translocations by shallow whole-genome sequencing points to EFNA5, BAHD1 and PPP2R5E as novel candidates for genes causing human Mendelian disorders. Murcia Pienkowski V, Kucharczyk M, Młynek M, Szczałuba K, Rydzanicz M, Poszewiecka B, Skórka A, Sykulski M, Biernacka A, Koppolu AA, Posmyk R, Walczak A, Kosińska J, Krajewski P, Castaneda J, Obersztyn E, Jurkiewicz E, Śmigiel R, Gambin A, Chrzanowska K, Krajewska-Walasek M, Płoski R. J Med Genet. 2019 Feb;56(2):104-112. doi: 10.1136/jmedgenet-2018-105527. Epub 2018 Oct 23. PMID: 30352868
3. Neurodevelopmental phenotype caused by a de novo PTPN4 single nucleotide variant disrupting protein localization in neuronal dendritic spines. Szczałuba K, Chmielewska JJ, Sokolowska O, Rydzanicz M, Szymańska K, Feleszko W, Włodarski P, Biernacka A, Murcia Pienkowski V, Walczak A, Bargeł E, Królewczyk K, Nowacka A, Stawiński P, Nowis D, Dziembowska M, Płoski R. Clin Genet. 2018 Dec;94(6):581-585. doi: 10.1111/cge.13450. Epub 2018 Oct 11. PMID: 30238967
4. Homozygous mutation in the Neurofascin gene affecting the glial isoform of Neurofascin causes severe neurodevelopment disorder with hypotonia, amimia and areflexia. Smigiel R, Sherman DL, Rydzanicz M, Walczak A, Mikolajkow D, Krolak-Olejnik B, Kosinska J, Gasperowicz P, Biernacka A, Stawinski P, Marciniak M, Andrzejewski W, Boczar M, Krajewski P, Sasiadek MM, Brophy PJ, Ploski R. Hum Mol Genet. 2018 Nov 1;27(21):3669-3674. doi: 10.1093/hmg/ddy277. PMID: 30124836
5. Evidence for HNRNPH1 being another gene for Bain type syndromic mental retardation. Pilch J, Koppolu AA, Walczak A, Murcia Pienkowski VA, Biernacka A, Skiba P, Machnik-Broncel J, Gasperowicz P, Kosińska J, Rydzanicz M, Emich-Widera E, Płoski R. Clin Genet. 2018 Oct;94(3-4):381-385. doi: 10.1111/cge.13410. Epub 2018 Aug 2.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Paulina Szadkowska	Year of studies:1st

Title of the project: Targeted next generation sequencing of circulating cell-free DNA from blood sample as a diagnostic tool for glioma patients

Name of the supervisor: Prof. Bożena Kamińska

Affiliation: Nencki Institute of Experimental Biology

AIMS of the project:

Malignant gliomas are the most common primary, diffuse brain tumors in adults. Numerous genetic alterations critical for glioma pathogenesis and affecting therapy response have been described. Genetic tumor profiling is used to facilitate a precise diagnosis of the disease. New methods allow to analyze genomic alterations occurring in circulating tumor DNA (ctDNA) which is more assessable and may provide useful, diagnostic information. A main goal of this project is to implement methods of genomic analysis of cfDNA obtained from serum of glioma patients to personalize treatment and achieve better diagnostics. A main objective of this study is to provide evidence that mutations can be detected cfDNA in blood enabling targeted therapy after surgical treatment or providing an earlier diagnosis. In addition studies of alterations within molecular characteristics and size profile of cfDNA will be done, comparing cfDNA obtained from a patient's blood before the surgical treatment to that taken after surgery. Analysis of ctDNA shows a great alternative to a traditional tumor biopsy or an analysis of a cerebrospinal fluid, as side effects of lumbar puncture often include central nervous system infection, leptomeningeal tumor spread, or increase of intracranial pressure. Tumor resection involves significant risks for a patient and correct timing of surgery during tumor progression would be more feasible with a non-invasive method. Liquid biopsy provides a great potential for routine diagnostics, prognostics, and progression monitoring.

Methods:

In the present study, fresh frozen patient's tumor tissues and blood samples were obtained from the cohort of 60 patients suffering from gliomas, 2 patients suffering from cancer metastasis into the brain, and one from aneurism. Blood was collected in EDTA-coated tubes in order to avoid clotting and tissue was be frozen in -80˚C until the DNA isolation step. Thus far tumor DNA was isolated from 37 tumor tissues so far using Trizol (Thermofisher Waltham, MA) - phenol chloroform based extraction to evaluate a spectrum of mutations. Additional blood was collected from 32 patients and whole blood DNA was isolated up to date from 20 samples so far using QiAmp DNA Blood Mini Kit (Qiagen, Germany), in order to provide healthy reference DNA to enable precise determination of somatic mutations for each patient. cfDNA was isolated from corresponding samples of blood using QIAvac 24 Plus pump with QIAamp Circulating Nucleic Acid Kit (Qiagen, Germany). Kappa Hyper Plus (Roche, Basel, Switzerland) library preparation kit was used for DNA samples from whole blood and tumours with larger panel, which involves target enrichment design of 600 gene mutations and 100 epigenetic modifications, both related to glioma. After DNA isolation, oligo-tagged libraries were prepared which was followed by a second generation DNA sequencing done using Illumina HiSeq1500 Genome Analyzer (Illumina, San Diego, CA) sequencing platform. Specific mutations and epigenetic modifications profile will be analysed and reviewed. Sure Select XT library preparation kit (Agilent, Santa Clara, CA) with a custom panel that targets 50 most commonly found somatic mutations in glial tumours [a panel has being prepared based on in house and published sequencing data, a manuscript in preparation] will be further used to process samples of cfDNA. Sequencing data have been analysed in collaboration with a group bioinformaticians from Department of biobanking and scientific research, Regional Science and Technology Centre in Krakow.

Results:

RNA and DNA have been isolated from blood and glioma samples matching cfDNA samples. Quantification cfDNA was performed for all samples. So far ten libraries were prepared from cfDNA and targeted NGS sequencing was done from matching glioma samples using a commercially available Comprehensive Cancer Panel Agilent. Mutations found in the resected tumours and within corresponding cfDNA were compared. Methods were developed to proceed with further cfDNA library preparation with the custom panel that target 50 most commonly found somatic mutations in glial tumors which selection was done based upon our most recent studies [data unpublished']. Up to date 29 gliomas and 16 whole blood DNA samples were molecularly characterized by targeted NGS sequencing using a custom panel comprising of 600 cancer related and 100 epigenetic genes.

Participation in conferences:

21-22 January 2019 COST Conference: CMI1406: Epigenetic Chemical Biology, Paris, France.

15-17 May 2019 EACR-ESMO Joint Conference Liquid Biopsies, Bergamo, Italy.

Publications, scientific presentations as a leading author:

Poster: "Targeted next generation sequencing of cell-free circulating tumor DNA from blood sample as a diagnostic tool for glioma patients"

Paulina Szadkowska, Jakub Mieczkowski, Kacper Zukowski, Tomasz Waller, Tomasz Gubala, Michał Karpata; Kinga Wilkus, Sergiusz Nawrocki, Wojciech Kaspera, Bozena Kaminska

15-17 May 2019 EACR-ESMO Joint Conference Liquid Biopsies, Bergamo, Italy.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Monika Pępek	Year of studies: 4th

Title of the project: Identification of new, clinically relevant genetic aberrations by next-generation sequencing in chronic myeloid leukemia patients with poor disease outcome

Name of the supervisor(s): Tomasz Stokłosa, MD, PhD, Prof. Rafał Płoski, MD, PhD

Affiliation: Medical University of Warsaw

AIMS of the project:

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell disorder, characterized by a reciprocal chromosomal translocation t(9;22)(9q34;q11), resulting in the formation of the Philadelphia chromosome encoding the *BCR-ABL1* gene. Despite effective therapy, drug resistance occurs in approximately 20-30% of patients. Next-generation sequencing (NGS) facilitates the searching for genetic aberrations leading to an unfavorable outcome of this disease.

Pseudogenes are generally nonfunctional copies of their parental genes. However, several of them may undergo transcription and affect the function of the parental genes as well as unrelated genes.

Previously, we showed that *SMAD4* pseudogene (Ψ) is not transcribed and thus, does not affect the regulation of parental gene expression. However, a significant increase in the incidence of the Ψ in CML, compared to other types of leukemia, may suggest its involvement in the development of this particular disease.

The main goal of this project is to identify and characterize functionally new genetic aberrations, including pseudogenes, which may lead to progression of the disease and resistance to targeted therapy in paired CML samples and functional analysis of these new findings. In addition, we want to assess whether the detected Ψ may be a genetic marker of CML and if its presence is associated with unique, coexisting mutations.

Methods:

We continued screening for the *SMAD4* pseudogene (Ψ) in CML samples. For this purpose, peripheral blood and/or bone marrow samples were obtained from CML patients after written informed consent obtained from each patient. We included genomic DNA isolated from further 27 Polish CML patients into the analysis. In addition, as part of scientific cooperation with the Institute of Hematology and Blood Transfusion in Prague, we analyzed 273 Czech CML samples. The incidence of the Ψ in the CML samples was examined using PCR method and specific, intron-spanning primers.

To determine whether the Ψ affects genes involved in leukemogenesis or cancerogenesis we analyzed NGS data obtained in the previous year. We also included newly diagnosed Czech CML samples with the Ψ into the analysis. Detected variants were filtered based on frequency in a population in public (1000 genomes, NHLBI ESP, gnomAD) and internal databases, as well as predictions of the consequences at the protein level using five bioinformatics predictors: CADD, PolyPhen2, SIFT, FATHMM and MutationTaster. All potentially pathogenic variants were viewed with Integrative Genomics Viewer (IGV).

Results:

In the previously reported results, we showed that the incidence of *SMAD4* pseudogene (Ψ) is almost three times higher in patients diagnosed with CML as compared to the observed frequency (approx. 0,5%) of this germline mutation in general Polish population (similarly to published data from other nationalities). This year, further samples from Polish CML patients were analyzed. We did not detect

new samples with the Ψ, however, the incidence of the Ψ in CML (9/671; 1,34%) is still significantly higher than the frequency in the control group (12/2242; 0,54%). In addition, we assessed the incidence of the Ψ in the Czech population, it was equal to 1.1% (3/273) and is also higher than observed in the general population.

Due to the germinal nature of the Ψ, using NGS data we decided to determine whether the presence of Ψ is associated with any other germline mutation in genes of known predisposition to cancer development. The analysis did not reveal enrichment of any mutated gene in CML samples with the Ψ as compared to all CML samples, but this was done on limited number of samples. However, we detected CML-typical mutations in genes such as *ASXL1, RUNX1*or *DNMT3A*. In addition, in one patient we found a pathogenic variant in the *CHEK2*, which is a known cancer- susceptibility gene. Future plans involve assessing copy number variation (CNV) in samples with Ψ compared with other CML samples and also testing hypothesis, whether the presence of the Ψ reincorporated near *ABL1* undergoing translocation t(9:22), may increase chromosomal fragility.

Participation in conferences:

1.Amyloidoza, II Interdyscyplinarna Konferencja, 2018 October 19, Warsaw

2. Dzień aplikacyjny Illumina, 2018 November 15, Warsaw

3. Dzień Chorób Rzadkich: diagnostyka, leczenie i opieka, 2019 February 28, Warsaw

4.Young Scientists Conference on Molecular and Cell Biology, 2019 April 11, Warsaw

Publications, scientific presentations as a leading author:

1. Szczepaniak A, Machnicki M, Gniot M, Pępek M, Rydzanicz M, Płoski R, Kaźmierczak M, Stokłosa T, Lewandowski K; "Germline missense NF1 mutation in an elderly patient with a blastic plasmacytoid dendritic cell neoplasm" Int J Hematol. 2019 Jul;110(1):102-106. doi: 10.1007/s12185-019-02642-w

2. XI spotkanie Zespołów Laboratoriów Sekcji Hematologii Molekularnej Polskiego Towarzystwa Genetyki Człowieka, 2019 May 24, Cracow, invited presentation: „Jak sekwencjonujemy w naszym laboratorium *TP53* na co dzień, czyli zalety i wady różnych technik NGS na tle sekwencjonowania Sangerowskiego"

3. XXVIII Zjazd Polskiego Towarzystwa Hematologów i Transfuzjologów, 2019 September 12-14, Lodz, poster (accepted): Pępek M, Machnicki M, Niesiobędzka-Krężel J, Drozd-Sokołowska J, Zawada M, Sacha T, Bogucka-Fedorczuk A, Czyż A, Rydzanicz M, Stawiński P, Płoski R, Stokłosa T; „Sekwencjonowanie następnej generacji jako pomocne narzędzie w diagnostyce rzadkich nowotworów mieloproliferacyjnych".

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Bartłomiej Tomasik	Year of studies: 2nd

Title of the project: Identification and utilization of circulating microRNAs in the monitoring of radiotherapy complications in patients with oropharyngeal cancer

Name of the supervisor: Wojciech Fendler, M.D., Ph.D., Associate Professor

Affiliation: Department of Biostatistics and Translational Medicine, Medical University of Lodz

Aims of the project:

The main purpose is to evaluate microRNA (miRNA) profile in OPSCC patients regarding acute radiation toxicity and to assess its utility as a marker in monitoring radiation therapy.

Methods:

This is a prospective cohort study which enrolled OPSCC patients treated with radiotherapy from June 2016. Serum miRNA profiling was performed on serum of 20 randomly selected patients (10 with high-grade toxicity and 10 without). Profiling was done in serum samples collected before radiotherapy, after 10 fractions of radiotherapy and within 24 hours after the last fraction of radiotherapy using qPCR arrays (miRCURY LNA, Human panels I & II, Exiqon, Copenhagen, Denmark). Data were normalized toward the average expression of microRNAs detectable in all samples. Acute side effects were prospectively assessed using EORTC QLQ-C30 and EORTC QLQ-H&N35 questionnaires. MiRNAs were shortlisted on the basis of univariate, Benjamini-Hochberg-adjusted, p values. The classifier for xerostomia was created using a stepwise, 5-fold cross-validated logistic regression model.

Results:

One hundred thirty-eight miRNAs were detected in more than half of the samples and used in the analysis. We observed that after receiving 20Gy, 14 miRNAs differed significantly between the compared groups and 3 of them (miR-425-5p, miR-18b-5p and miR-345-5p) maintained their differences after the final RT fraction. Interestingly, all three miRNAs presented varying temporal patterns depending on the presence of xerostomia. MiR-425-5p increased throughout RT in patients with severe xerostomia and decreased in those without it. MiR-345-5p showed no changes in the severe xerostomia group while a decline of expression levels was present in the remainder. MiR-18b-5p showed opposite changes in expression between 20Gy and 70Gy timepoints (p for interaction 0.007). The logistic regression model based on miR-425-5p and miR-345-5p expressions (after 20Gy), showed nearly perfect separation of the groups with an AUC=0.95 (95%CI: 0.91-1.00) and maintained its performance in 5-fold cross validation (AUC=0.88, 95%CI: 0.84-0.93).

Participation in conferences:

1. Annual Meeting of the American Society for Radiation Oncology (ASTRO) (21 – 24 October, 2018, San Antonio, Texas, United States of America)
2. ESTRO 38, the annual congress of the European Society for Radiotherapy & Oncology (ESTRO) (26-30 April 2019, Milan, Italy)
3. Polish Scientific Networks: Science & Medicine conference (21-23 June, 2018, Lodz, Poland).

Publications, scientific presentations as a leading author:

Publications:

1. "Recurrent Pineocytomalike Papillary Tumor of The Pineal Region: A Case Report and Literature Review" Braun M*, Tomasik B*, Bieńkowski M, Wiśniewski K, Kupnicka DJ, Jaskólski D, Papierz W, Fijuth J, Kordek R. *contributed equally. World Neurosurg. 2018 Dec;120:1-14.
2. "*GATA3* germline variant is associated with CRLF2 expression and predicts outcome in pediatric B-cell precursor acute lymphoblastic leukemia" Madzio J, Pastorczak A, Sedek L, Braun M, Taha J, Wypyszczak K, Trelinska J, Lejman M, Muszynska-Roslan K, Tomasik B, Derwich K, Koltan A, Kazanowska B, Irga-Jaworska N, Badowska W, Matysiak M, Kowalczyk J, Styczynski J, Fendler W, Szczepanski T, Mlynarski W. Genes Chromosomes Cancer. 2019 Sep;58(9):619-626. doi: 10.1002/gcc.22748. Epub 2019 Apr 3.
3. "Lipopolysaccharide-binding protein is an early biomarker of cardiac function after radiotherapy for breast cancer" Chalubinska-Fendler J, Graczyk L, Piotrowski G, Wyka K, Nowicka Z, Tomasik B, Fijuth J, Kozono D, Fendler W. Int J Radiat Oncol Biol Phys. 2019 Aug 1;104(5):1074-1083.
4. "Ossifying fasciitis at an extraordinary site – a case report and analysis of diagnostic pitfalls" Kowalczyk Ł, Braun M, Tomasik B, Piasecka D, Michno D, Fijuth J, Jesionek-Kupnicka D, Kordek R. Contemp Oncol (Pozn). 2019;23(2):121-125
5. "Predictors for ophthalmic segment aneurysms recanalization after coiling and flow diverter embolization in 6- and 12-month follow-up" Wiśniewski K, Tomasik B, Bobeff EJ, Stefańczyk L, Hupało M, Jaskólski DJ. J Clin Neurosci. 2019 Jul 12. pii: S0967 5868(18)31219-0.

Scientific presentations:

1. "Left Anterior Descending Coronary Artery Dose-Sparing Effects of Volumetric Modulated Arc Therapy in Patients Ineligible for Deep-Inspiration Breath-Hold in Left Breast Radiation Therapy" presented during Annual Meeting of the American Society for Radiation Oncology (ASTRO) (21 – 24 October, 2018, San Antonio, Texas, United States of America)
2. "Circulating MicroRNAs As Biomarkers Of Irradiation – A Systematic Review And Meta-Analysis" presented during Polish Scientific Networks: Science & Medicine conference (21-23 June, 2018, Lodz, Poland).

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Joanna Parada	Year of studies: 1st

Title of the project: Analysis of long non-coding RNA expression in ovarian carcinomas, in search for new diagnostic, prognostic and predictive markers.

Name of the supervisor: Prof. dr hab. Magdalena Chechlińska

Affiliation: Centrum Onkologii – Instytut im. Marii Skłodowskiej-Curie

AIMS of the project:

Long non-coding RNAs (lncRNAs) are a class of noncoding RNAs, involved in regulation of diverse physiological and pathological processes. LncRNA exhibit multiple biological functions in ovarian cancer development and progression.

The main goals of the project are to examine the level of expression, clinical significance and function of transcripts belonging to the lncRNA group and to assess their potential usefulness in the diagnostics and treatment of patients with ovarian cancer. The study may enable to identyfy new biomarkers (diagnostic, prognostic), and possibly also new potential therapeutic goals, which in the long term may lead to clinical applications. In addition, the study may reveal relationships between lncRNA expression and mRNA and microRNA transcripts, new lncRNA molecules may even be discovered.

Methods:

Our research team has collected 135 snap-frozen advanced ovarian tumors (32 subsequently treated with platinum/cyclophosphamide (PC) and 103 with the taxane/platinum (TP) regimen). The material was collected in the years 1995-2008, based on the approval of the relevant local bioethics board, and the patients were followed-up. A part from the carefully verified standard clinical and pathological characteristics, including the patient age, histological type, grade, and FIGO stages, all cases were characterized in terms of the size of residual disease, TP53 status, response to treatment and survival. Some of the tumors had also been tested for mRNA expression using
HGU133 Plus 2.0 Arrays (Affymetrix) (in collaboration with the Oncology Center in Gliwice). In some cases, the microRNA expression profile has also been determined.

LncRNA expression is to be examined by the next generation sequencing (NGS RNA-seq) in 135 tumor samples. Prior to the analysis, RNA extracted from all the samples (miRneasy ® Isolation Kit (QIAGEN) are tested on the Bioanalyzer 2100/TapeStation 4150 System (Agilent), and only those with the correct concentrations and the RNA integrity numbers (RIN) greater than 5 are regarded as suitable for the RNA-seq study. cDNA libraries are prepared with the use of Superscript II reverse transcriptase. NGS libraries are created with the TruSeq Stranded Total RNA Sample Prep Kit (Illumina), followed by assessment of their quality on the Bioanalyzer 2100/TapeStation 4150 System, and subsequent RNA-seq analysis by paired-end sequencing (2x100bp) conducted on the NGS HiSeq 1500 platform (Illumina).

Results:

During the first year doctoral studies RNA was isolated from 91 ovarian tumor. So far the quality was checked for 24 cases and very good quality of both classes RNA (RIN=8,10) was achived.

Reverse transcription was performed for all 24 samples and NGS libraries were prepared of the resulting cDNA. The quality of the libraries was assessed by the Agilent 2100 Bioanalyzer. The libraries obtained showed high quality with average size of 300 bp. Next, the libraries were

"pooled", and NGS analysis was performed. Preliminary quantitative and qualitative analyzes together with bioinformatics analyzes have shown that:
1. The collected frozen sections of primary ovarian tumors are a source of good quality RNA
2. The prepared libraries for NGS are of high quality
3. Sequencing of all the analyzed samples was performed correctly
4. The resulting transcript sequences map to the human reference genome with high efficiency (over 95%).

The bioinformatic analysis is still in progress and some preliminary results will be available to be reported at a PhD student session.

Participation in conferences:

- Fourth Congress of the Polish Society of Human Genetics, Bydgoszcz 13-15 September, 2018
- Fifth Warsaw Oncology Conference, Warszawa 6-7 March, 2019
- XXVIII Nobel Session, Warszawa 10 December, 2018

Publications, scientific presentations as a leading author:

Dansonka-Mieszkowska A., Szafron L.M, Moes- Sosnowska J., Kulinczak M., Balcerak A., Konopka B., Kulesza M., Budzilowska A., Lukasik M., Piekarska U., Rzepecka I., Parada J., Zub R., Pienkowska-Grela B., Radoslaw Madry, Siwicki J.K., Kupryjanczyk J.
Oncotarget, 3, 9:17735-17755, 2018
Clinical importance of the EMSY gene expression and polymorphisms in ovarian cancer.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Aleksandra Filipiak	Year of studies: 1st

Title of the project: The influence of hypoxia on the activity of genes related to drug resistance: studies with alternative methods.

Name of the supervisor: prof. dr hab. Claudine Kieda

Affiliation: Military Institute of Medicine

AIMS of the project:

In vivo murine syngeneic models are very reliable tools in recreating the full spectrum of the tumor in its microenvironment. Unfortunately, this type of models not always can be used in each phase of pre-clinical studies. Therefore most initial research depends on standard *in vitro* experiments, which mostly do not take in consideration real tumor micro-environment. In turn alternative *in vitro* methods such as spheroids/organoids cell cultures that aim taking into account those important aspects of cancer development.

The aim of the project is to:

- create and validate such *in vitro* model which will mimic the main aspects of tumor created micro-environment, such as: three-dimensional shape of the tumor and proper oxygen tension
- characterization of created model.

Methods:

- spheroid formation by combining hanging drop method and culture of single spheroids in polysaccharidic gel, using two murine cell lines B16 - murine melanoma and RenCa - murine renal cancer
- comparison of our model with standard two-dimensional *in vitro* cultures, carried out in normoxic and hypoxic (1% pO2) conditions
- viability of cells in various culture conditions measured by flow cytometry after dispersion of spheroids
- stem-like cells detection in spheroids by aldehyde dehydrogenase activity using flow cytometry method
- presence of ALDH1 protein quantification by Western Blot
- assessment of the sensitivity of cells in spheroids and two-dimensional culture to various drugs and of their viability by fluorescence microscopy after cell staining with propidium iodide.

Results:

We managed to obtain consistent spheroids from renal and melanoma murine cancer cells in their long term culture. Spheroids were cultured for seven days:

- three days in hanging drops
- four days in standard culture medium with methylcellulose.

After three days of culture in hanging drops irregular cell aggregates were observed for both cell lines: RenCa and B16. Next the single spheroids/aggregates were transferred to agarose-coated-bottom multiwell plate in culture medium with methylcellulose. Round and regular spheroids were obtained after seven days. We also performed standard two-dimensional *in vitro* culture in normoxia and hypoxia (1% pO_2) for five days to compare with our model.

First we compared cell viability in distinct culture conditions using flow cytometry method. Higher levels of viable cells were recovered from two-dimensional cultures from normoxic and hypoxic conditions, than from spheroids in both conditions. The cells from spheroids cultured in hypoxia showed very low viability. Cells growing in spheroids develop hypoxia in the center of the sphere. Consequently, spheroid culture in additional hypoxia causes cellular stress affecting the reproducibility of the data. This was confirmed by microscopic observation of spheres stained by calcein and propidium iodide showing the necrotic core of 3D structures in normoxia. We performed CD133 staining and Aldehyde Dehydrogenase activity assay to check for the presence of stem-like cells in all conditions. Higher levels of ALDH and CD133 were observed in three-dimensional cultures as compared to monolayers for both cell lines.
Moreover, Western Blot analyzes for the presence of ALDH in all conditions revealed a higher expression in 3D culture conditions. Spheres from B16 cells were also characterized by a higher expression of EMT proteins such as: ß-catenin and N-cadherin as compared to 2D cultures, both in normoxia and hypoxia.
To assess for the validity of the models for drug testing, cell viability was estimated after chemotherapeutic treatment. Sensitivity to various drugs such as: mTor, ALDH and MDR inhibitors and resistance to drugs was assessed by quantifying propidium iodide incorporation by fluorescence microscopy.
I am currently working on gathering results from first *in vivo* experiment using the RenCa murine model of renal carcinoma. We shall compare the data obtained in the mouse renal carcinoma tumors with three-dimensional *in vitro* model.
We have shown that in the three-dimensional models the cell populations are enriched in ALDH and CD133 positive cells indicative for stem-like cells in both cell lines. 3D cultured B16 concomitantly displayed higher expression of EMT markers. This coincided with resistance of spheroids to various drugs, showing that our 3D model can be a valuable tool for anticancer drug screening.

Participation in conferences:

- IV Zjazd Naukowy Polskiego Towarzystwa Biologii Medycznej „Biologia-Medycyna-Terapia" 2019 May 22-25, Szawnica, poster**:** Filipiak A., Brodaczewska K., Majewska A., Kieda C., "Development of an alternative drug-testing method in stem-like cells enriched melanoma model".
- 6th LIA Workshop report and evolution of the Polish-French cooperation in biotechnology "Biomarkers and mediators of Diseases - New approaches in repair/regenerative therapies" 2019 June 4-5, Warsaw, organization of conference and poster: Filipiak A., Brodaczewska K., Majewska A., Kieda C.," Enrichment in stem-like cells in melanoma provides an alternative drug-testing method".
- II Ogólnopolska Konferencja Naukowa "Wyzwania onkologii spersonalizowane i interdyscyplinarnej" 2019 May 10-11, passive participation.
- V Ogólnopolska Konferencja Genetyczna „Genomica" 2019 April 5-7, Kraków, passive participation

Publications, scientific presentations as a leading author:

- IV Zjazd Naukowy Polskiego Towarzystwa Biologii Medycznej „Biologia-Medycyna-Terapia" 2019 May 22-25, Szawnica, poster**:** Filipiak A., Brodaczewska K., Majewska A., Kieda C., "Development of an alternative drug-testing method in stem-like cells enriched melanoma model".
- 6th LIA Workshop report and evolution of the Polish-French cooperation in biotechnology 2019 June 4-5, Warsaw, poster: Filipiak A., Brodaczewska K., Majewska A., Kieda C.," Enrichment in stem-like cells in melanoma provides an alternative drug-testing method".

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Kinga Wilkus	Year of studies: 1st

Title of the project: Study of gene expression pattern of organospecific endothelial cells reflecting tumor microenvironment state

Name of the supervisor: Prof. dr hab. Claudine Kieda

Affiliation: Military Institute of Medicine, Laboratory of Molecular Oncology and Innovative Therapies (Warsaw)

Aims of the project:

The purpose of the project is to describe the global gene expression pattern characteristic for endothelial cells derived from tumour microenvironment and evaluate the effect of hypoxia as well as tissue-specific responses on the activity of pathological endothelium as compared to healthy one. Moreover, the project is focused on the discovery of new pathways involved in pathological angiogenesis which may serve as new therapeutic targets to control the disease by normalization of the vasculature.

During the first year I focused on the p53/Mdm2 and PTEN/PI3K pathways. I evaluated if they are modified in ECs originated from healthy and cancer tissue and if they are affected by the pO_2 values in the microenvironment.

Materials and culture conditions:

Methodology is based on the use of immortalized lines of endothelial cells isolated from healthy breast tissue (HBH.MEC) and primary tumour (HBCa.MEC) from a breast cancer patient. Cells were cultured in various oxygen tension conditions –normoxia (~19% pO_2) and hypoxia (~1% pO_2) for 48 hours. This allows a study of the influence of pO_2 on the cell biology and evidencing differences between normal and tumor tissues.

Methods:

- Western blots were used to measure PTEN, Mdm2, p53 in cell lysates
- Secretion of VEGF into the medium was detected by ELISA
- Cell growth and response to inhibitor of Mdm2-p53 interaction (Nutlin-3) was studied by Alamar blue assay
- Functional assays, like pseudo-tube formation were used to evaluate the effect of hypoxia on the activity of endothelial cells. This step was performed on 96-well plates covered by 100% Matrigel with OPTI 2% FBS + Nutlin-3 (15μM) for 5h.
- Using established model, global gene expression analysis with NGS will be performed to detect new pathways altered in pathological ECs (cancer derived and/or exposed to hypoxia).

Results:

Firstly, PTEN, Mdm2 and p53 protein levels were assessed by western blotting in lysates from ECs, both cultured in normoxia and hypoxia. I observed that p53, Mdm2 and PTEN proteins are expressed differently in healthy and pathological ECs and they responded variably to low pO_2. In healthy breast ECs, the level of p53 and Mdm2 proteins was reduced, whereas amount of PTEN protein was stable.

In the case of cancer derived ECs downregulation of Mdm2 in response to low pO_2 was not observed, but at the same time PTEN-tumor suppressor- was down regulated.

Then, we observed that in healthy ECs growth and activity are modulated by pO_2. HBH.MEC growth rate was reduced upon culture in hypoxia. Taking into account cancer-derived ECs, they responded differently to hypoxia as compared to healthy ECs. After a 48 hours incubation, HBCa.MEC show adaptation to low pO_2 and displayed increased cell density of the culture in hypoxia as compared to normoxia.

Next, functional assay- tube formation- was performed. In HBH.MEC, low pO_2 disturbed cell ability to form vessels *in vitro* as the numbers of nodes were lower in hypoxia. Next, Mdm2 inhibition by Nutlin-3 was investigated with regards to disturbance of ECs growth and function. The inhibitor reduced cell growth in a dose-dependent manner and down regulated tube formation *in vitro.* Moreover, this inhibition of Mdm2–p53 interaction did not influence PTEN level in HBH.MEC cultured in normoxia as it was observed in hypoxia. In cancer-derived ECs, Mdm2 was dysregulated. Hypoxic HBCa ECs in response to Nutlin-3 showed reduced angiogenic activity and PTEN was downregulated, as opposed to healthy ECs.

Conclusion:

- Mdm2 is down regulated because inhibition refers to its activity specifically in ECs in reaction to hypoxia in healthy breast derived cells only, it mediates the inhibition of cell angiogenic activity in low pO_2.
- In cancer-derived ECs Mdm2 is not downregulated by hypoxia; they proliferate in hypoxia, PTEN downregulation might be the target.

To sum up, these results suggest that Mdm2/p53 and PTEN/PI3K pathways are deregulated in cancer ECs and may control the pathological angiogenesis in hypoxic tumour microenvironment.

At present, experiments are pending for MCF-7 and MDA-MB-231 (human breast tumor) cell lines as model accordingly to ECs experiments described above. Moreover, MCF-7 and MDA-MB-231 spheroids are co-culture with Cytodex beads covered with ECs and HEPC.CB1/CB2 (endothelial progenitor cells) in order to observe and modulate the angiogenic response.

Participation in conferences:

Active participant:

- 44th FEBS Congress 2019 "From molecules to living systems", 2019 July 6-11, Cracow, poster presentation;
- 6th LIA Workshop report and evolution of the Polish-French cooperation in biotechnology "Biomarkers and mediators of Diseases - New approaches in repair/regenerative therapies", 2019 June 4-5, Warsaw, organization of conference and poster presentation;

Passive participant:

- 8th Intercollegiate Biotechnology Symposium "Symbioza", 2019 May 17-19, Warsaw.

Publications, scientific presentations as a leading author:

1. Poster presentation on 44th FEBS Congress 2019: "Potential role of p53/Mdm2 and PTEN/PI3K pathways in shaping the activity of ECs in hypoxia", *Wilkus Kinga, Brodaczewska Klaudia, Kieda Claudine*
2. Poster presentation during 6th LIA meeting Warsaw: "Modulation of endothelial cells activity in hypoxia by tumor suppressor / oncogene interactions", *Wilkus Kinga, Brodaczewska Klaudia, Kieda Claudine.*

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Magda Grynkieiwcz	Year of studies: 1st

Title of the project: Comprehensive computational approach to prediction of cancer therapy biomarkers, part 1: Synthetic Lethality inference using cancer cell line experiments (DRIVE and Achilles)

Name of the supervisor: Ewa Szczurek PhD

Affiliation: Faculty of Mathematics, Informatics and Mechanics, University of Warsaw

AIMS of the project:

Analysis of synthetic lethal interactions between genes in tumour genomes - finding new potential synthetic lethal gene pairs as possible targets of cancer therapy

Synthetic lethality is an epistatic genetic interaction with great potential for cancer therapy since inhibitors targeting synthetic lethal partners of genes already mutated in tumour can selectively kill cancerous but not healthy cells. Discovering synthetic lethal gene pairs (later denoted gene A and gene B) is an important step in developing new targeted cancer therapies. Large-scale gene knockout sensitivity screens performed on cancer cell lines (such as project DRIVE and Achilles) provide a rich data source for a range of computational methods which can be used for predicting synthetic dependencies.

The main goal of this part of the project was to compile a list of potential synthetic lethal gene pairs for experimental validation comprising scored results obtained using various experimental data sets. We hope that extensive comparison of results from different data sources will result in the discovery new potential targets for cancer therapy.

Methods:

Analysis of sensitivity scores for genes in a range of different cancer cell lines from projects DRIVE and Achilles. We use Wilcoxon rank-sum test test to check if cell lines with gene A already disabled (alteration such as loss of function mutation or deletion) are more dependent on gene B (higher sensitivity to gene B knock-out):

- a nonparametric test of the null hypothesis that it is equally likely that a randomly selected value from one sample will be less than or greater than a randomly selected value from a second sample,

- used to determine whether two independent samples were selected from populations having the same distribution,

- in our case used to determine if cell lines with alteration of gene A (first population) are significantly more sensitive to the knockout of the gene B than the cell lines without gene A alteration

Results:

Wilcoxon rank-sum test was performed for all genes A deleted or mutated (separately) in at least ten cell lines for each experimental setting. The genes B were all the genes targeted in each knock-out experiment. It was checked if population of cell lines with gene A altered has significantly different sensitivity for gene B knock out. The smaller the resulting p-value the higher the chance that the cell lines with gene A alteration were more sensitive to gene B knock-out which should confirm potential synthetic lethal interaction between gene A and gene B.

The most promising results (according to p-value histograms) were obtained for the tests checking gene B denpendency in gene A mutated cell lines. The pairs were ranked according to those results.

After that we filtered out results where gene B knock-out resulted in better cell proliferation because it is not an evidence for SL.

Since not all pairs were present in every data set and our goal was to obtain a list of pairs which have the most evidence of SL, at the end of this part of the project we left only pairs with p-values smaller than 0.05 for 3 most informative tests.

This resulted in the list of potential list of around 22 thousand SL gene pairs. This list was then compared with results obtained by colleague Magdalena Budzińska who analysed same pairs using test on TCGA patient data.

After comprehensive analysis under guidance of our supervisor Ewa Szczurek, 3 best pairs are going to be experimentally validated.

Participation in conferences:

- CONTRA Innovative Training Network Workshop: “Handling Single Cell Data”, Warsaw, 9-15 September 2018,
- Postgraduate School of Molecular Medicine Autumn Course: “High Throughput Genomic and Transcriptomic Technologies in Biomedicine”, Krakow, 12-14 November 2018,
- Postgraduate School of Molecular Medicine Spring Course: “ From Gene to Phenotype", Warsaw, 11-13 March 2019,
- CONTRA Innovative Training Network Workshop: “Models of Evolution”, Barcelona, 3-7 June 2019,
- Postgraduate School of Molecular Medicine Summer Course: “ Life science imaging – workshop on visualisation of molecules, interactions and biological processes", Warsaw, 10-12 June 2019

Publications, scientific presentations as a leading author: N/A

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Agata Borkowska	Year of studies: 1st

Title of the project: Role of autophagy and senescence in chemoresistance of renal cell carcinoma: *in vitro* and *in vivo* analysis

Name of the supervisor: dr Halina Waś

Affiliation: Military Institute of Medicine in Warsaw

AIMS of the project

One of the most challenging in treatment neoplasia is renal cell carcinoma. It is one of the most chemoresistant solid cancers. The poor outcome of conventional chemo- and radiotherapy may be related to therapy induced senescence (TIS). While naturally occurring senescence is a process related to telomere shortening, senescence in cancer is most likely related to cell damage caused by therapy. A senescent cell does not proliferate and exhibits characteristic morphological changes such as enlargement of the cell surface, high vacuolization and increased activity of Senescence Associated β Gal (SA-β-Gal). It is suspected that senescent cells, that are present in the tumor population, can abnormally divide, which may be an explanation for chemoresistance of the tumor as well as relapse of the disease after therapy has ended. Nowadays most of the researches focused on the senescent cancer cells are still performed in normoxic conditions (~19% O_2) whereas in solid tumors low oxygen level (hypoxia) is a naturally occurring phenomenon. That is why part of the experiments from this project has been performed in an environment where the oxygen level was decreased to 1%.

The main aim of this project is to investigate if inhibition of autophagy would trigger the activation of senescent renal cancer cells from quiescent to proliferative state and therefore make them sensitive to chemotherapy.

During this year I aimed to investigate if the most common drugs used for renal cancer chemotherapy would induce senescence of cancer cells *in vitro* in both normoxic (~19% O_2) and hypoxic (1% O_2) conditions.

Methods:

RCC4 and RenCa, respectively human and murine cell lines have been cultured in normoxic (~19% O_2) and hypoxic (1% O_2) conditions. The protocol of the experiment was designed to mimic one cycle of chemotherapy, where after 24H of treatment with chemotherapeutics, cells have been grown for an additional 7 days.

Three chemotherapeutic agents have been chosen to test: Gemcitabine, Vinblastine, and 5 - Fluorouracil. All the agents are used in the monotherapy of renal cell carcinoma in the clinic.

The first part of the experiment was to investigate the viability of the cells after treatment with chosen drugs. The viability of the cells was examined with the use of the MTT assay.

To confirm if tested chemotherapeutics induce TIS narrow range of non-toxic drug concentrations were chosen for proliferation tests and SA-β-Gal activity. Proliferation rate was established with use colorimetric assay, Cell Proliferation ELISA, BrdU.

Results:

Tested drugs decrease cell viability and metabolic activity in both cell lines in normoxic conditions. Hypoxia significantly decreases metabolic activity in both cell lines. Hypoxic condition does increase

the resistance of both cell lines to Vinblastine and 5-Fluorouracil. In the case of Gemcitabine only in RenCa cell line, some chemoresistance was observed.

BrdU assay showed that the chosen chemotherapeutics does decrease proliferation assay in both cell lines. After treatment with drugs in concentrations significantly decreasing cell proliferation senescent cancer cells have been observed. SA- β-Gal activity positive cells were stained in blue. RCC4 cell line shows lower SA- β-Gal activity than murine RenCa cell line. Hypoxia does decrease SA- β-Gal activity.

Conclusions: Treatment of renal cell carcinoma cell lines with clinically used chemotherapeutics induce TIS development in normoxia and hypoxia. Applied treatment may induce different phenotype changes in cells, including cell size and SA-β-activity. Decreased oxygen level does decrease cells' sensitivity to chemotherapy.

Future plans: Analysis of senescence and stemness markers on RNA and protein level in *in vitro* model. Investigation of effects of autophagy modulation on proliferative potential of senescent cancer cells in hypoxia.

Participation in conferences:

- 6th LIA Workshop report and evolution of the Polish-French cooperation in biotechnology 2019 June 4-5, Warsaw. Coorganization and active participation
- III Konferencja Doktorantów Nauk Przyrodniczych 2019 June 25-28, Gdańsk. Active participation.
- 8th Intercollegiate Biotechnology Symposium SYMBIOZA 2019 May 17-19. Passive participation
- II Ogólnopolska Konferencja Naukowa "Wyzwania onkologii spersonalizowanej i interdyscyplinarnej" 2019 May 10-11. Passive participation

Publications, scientific presentations as a leading author:

- 6th LIA Workshop report and evolution of the Polish-French cooperation in biotechnology 2019 June 4-5, Warsaw, poster: Borkowska A., Kieda C., Waś H. *Some chemotherapeutics used in clinic do induce senescence of human kidney cancer cells in normic and hypoxic conditions*
- III Konferencja Doktorantów Nauk Przyrodniczych 2019 June 25-28, Gdańsk, poster: Borkowska A., Kieda C., Waś H. *Komórki raka nerki ulegają starzeniu w warunkach normoksyjnych oraz hipoksyjnych po leczeniu chemoterapeutykami stosowanymi w klinice*
- Fit & Science 2019. Lecture about supplementation as a part of workships: *Vademecum żywienia i suplementacji*. In cooperation with Aleksandra Filipiak

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Michał Chojnacki	Year of studies: 4th

Title of the project: The role of HLA-G molecule in chronic lymphocytic leukemia

Name of the supervisor: prof. dr hab. Krzysztof Giannopoulos

Affiliation: Department of Experimental Hematooncology, Medical University of Lublin

AIMS of the project

Chronic lymphocytic leukemia (CLL) is a highly heterogeneous disease. Detection of certain biological markers might help determine the possible course of the disease. Increased expression of HLA-G and interactions with their receptors seems to be important handicapping mechanism of cellular immunity by reducing activity of cytotoxic cells, and by inhibiting CD4+ T cells proliferation and induction of the regulatory T cells. The activity of HLA-G in solid tumors has been associated with a poorer prognosis for the patient. In hematological malignancies adverse effect of HLA-G on the survival remains unclear because of the presence of HLA-G receptors on the surface of malignant cells. The aim of this project was to characterize the HLA-G expression and its interactions with specific ILT2 receptor in chronic lymphocytic leukemia (CLL). Furthermore, we want to demonstrate the effect of HLA-G molecules on CD4+ T lymphocytes as well as its ability to convert them into HLA-G-induced Treg cells.

Methods:

Peripheral blood mononuclear cells (PBMC) were obtained from 50 untreated CLL patients and isolated by density gradient centrifugation. Expression of the HLA-G molecule and ILT2 receptor on CD5+CD19+ CLL cells was determined using flow cytometry. sHLA-G was determined using ELISA. *NOTCH1* and *MYD88 L265P* mutations were investigated by ARMS PCR. *SF3B1* mutations were assessed by HRM method. The IGHV mutation status and *TP53* mutations were determined by Sanger sequencing. Phenotype of CD4+ T cells, converted into HLA-G-induced Treg cells, was evaluated by flow cytometry. Magnetically isolated CD4+ T cells were cultured for 24 to 72 hours in the presence of CLL CD19+/HLA-G+ cells. Trogocytosis between CD19+ CLL cells and CD4+ was analyzed by confocal microscopy.

Results:

The expression of HLA-G and ILT2 was detected by flow cytometry on the surface of CLL cells with the median percentage expression 16.5 (min-max: 0.6 – 45.3) and 91.3 (min-max: 60.7 – 99.6) respectively. Expressions of HLA-G and ILT2 in CLL patients were higher than in control group. Regarding Rai classification, expression of HLA-G and ILT2 did not show any statistically significant differences (p=0.75 and 0.77; respectively). The analyses of molecular factors such as NOTCH1, MYD88, SF3B1 mutations and IGVH mutational status and prognostic factors CD38>30% and ZAP70>20% demonstrated no significant differences between surface expression of HLA-G as well as ILT2 receptor in CLL. Higher levels of sHLA-G in CLL patients than in healthy volunteers were assessed by ELISA. The analyses of molecular and prognostic factors did not show statistically significant differences according to sHLA-G concentrations. Co-culture of CD4+ cells and CD19+ HLA-G+ affected the induction of population of CD4+HLA-G+ cells. Expression of HLA-G on CD4+ cells significantly increased according to the time of culture (medians: 24h=0.9; 48h=1.75; 72h=2.15, p=0.0017). Percentage of induced Tregs was associated with the stage of disease

progression. Confocal microscopy provided visual demonstration that CD4+ cells acquired biotinylated fragments of cell membrane from CD19+/HLA-G+ CLL cells. Functional studies suggested that co-culture of CD4+ cells with CD19+/HLA-G+ cells may induce a novel generation of regulatory -CD4+ HLA-G+ T cells in CLL. The HLA-G transfer from CD19+ to CD4+ cells might be novel mechanism of immunosuppression in CLL.

Participation in conferences:

Ocena znaczenia cząsteczki HLA-G w patomechanizmie przewlekłej białaczki limfocytowej

M. Chojnacki, M. Paziewska, A. Karczmarczyk, K. Skórka, W. Tomczak, K. Giannopoulos; XXVIII Zjazd Polskiego Towarzystwa Hematologów i Transfuzjologów, Łódź, 12-14.09.2019

Przewaga metody emulsyjnego PCR nad ARMS-PCR w ocenie rokowniczego znaczenia mutacji NOTCH1 u chorych na przewlekłą białaczkę limfocytową

M. Chojnacki, K. Skórka, J.Purkot, M. Podgórniak, E. Subocz, J. Hałka, K. Giannopoulos; XXVIII Zjazd Polskiego Towarzystwa Hematologów i Transfuzjologów, Łódź, 12-14.09.2019

Publications, scientific presentations as a leading author:

The predominant prognostic significance of NOTCH1 mutation defined by emulsion PCR over

ARMS-PCR in chronic lymphocytic leukemia.

K. Skórka, M. Chojnacki, E. Subocz, J. Hałka, K. Giannopoulos (submitted)

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Olga Sokołowska	Year of studies: 4th

Title of the project: Investigation of the antitumor potential of stimulator of interferon genes (STING) - an adaptor protein in innate immune signaling

Name of the supervisor: Prof. dr hab. Dominika Nowis

Affiliation: Centre of New Technologies

AIMS of the project:

Stimulator of interferon genes (STING) is an adaptor protein that plays an important role in the induction of innate immune response upon the recognition of cytosolic nucleic acids. Activation of STING-dependent signaling leads to production of type I interferons (IFNs), tumor necrosis factor alpha (TNFα) as well as other proinflammatory cytokines. Recent evidence indicates involvement of STING pathway in the induction of adaptive response against tumor. STING agonists were shown to potentiate the therapeutic effects of radiation and anticancer vaccination. Here we propose to combine STING agonist with bortezomib – a clinically used proteasome inhibitor – treatment for the therapy of plasma cell myeloma (PCM). The aim of the project is to evaluate antitumor activity of proteasome inhibitor in combination with STING agonist *in vivo* in mouse model of PCM and investigate the mechanism of the antimyeloma activity of combination treatment.

Methods:

To investigate the influence of immunogenicity of PCM cell death induced by bortezomib and proposed combination treatment we studied the surface expression of heat shock protein 90 (HSP90) on murine PCM cell line 5TGM1 after different protocols of treatment. Furthermore, we performed coculture experiments of 5TGM1 and murine dendritic cell line JAWSII with various treatment regiments. We assessed expression of activation markers: cluster of differentiation 86 (CD86), major histocompatibility complex II (MHCII) and induction of 5TGM1 uptake by JAWSII with the use of flow cytometry. Cells for the coculture experiments were stained with CellTrace dyes.

Results:

Bortezomib treatment of 5TGM1 cells results in increased cell death joined with increased surface expression of HSP90 and induction of cell uptake by JAWSII cells. cGAMP treatment of JAWSII did not impair the ability of cells to uptake the PCM cells, however it decreased to a small extent the percentage of cells that performed phagocytosis. What is important, cGAMP treatment of JAWSII lead to higher amount of activated cells that performed phagocytosis. Acquired results further support our hypothesis of double axis of STING agonist and bortezomib-mediated antimyeloma effect with direct cytostatic and/or cytotoxic effect of STING-induced cytokines on the one hand and stimulation of adaptive immune response by immunomodulatory activity of combination therapy on the other.

Participation in conferences:

Adoptive immunotherapy for cancer, STREAM workshop, Warsaw

Publications, scientific presentations as a leading author:

Marusiak AA, Prelowska MK, Mehlich D, Lazniewski M, Kaminska K, Gorczynski A, Korwat A, Sokolowska O, Kedzierska H, Golab J, Biernat W, Plewczynski D, Brognard J, Nowis D. Upregulation of MLK4 promotes migratory and invasive potential of breast cancer cells. Oncogene 2019, 38 (15): 2860-2875.

Szczałuba K, Chmielewska JJ, Sokolowska O, Rydzanicz M, Szymańska K, Feleszko W, Włodarski P, Biernacka A, Murcia Pienkowski V, Walczak A, Bargeł E, Królewczyk K, Nowacka A, Stawiński P, Nowis D, Dziembowska M, Płoski R. Neurodevelopmental phenotype caused by a de novo PTPN4 point mutation disrupting protein localization in neuronal dendritic spines. Clinical Genetics 2018, 94(6): 581-585.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Mateusz Gielata	Year of studies: 2nd

Title of the project: Alpha-catulin as a marker of a specific population of invasive breast cancer cells

Name of the supervisor: Agnieszka Kobielak

Affiliation: Centre of New Technologies/Medical University of Warsaw

AIMS of the project :

Test the role of EMT in breast cancer initiating cell potential; Test the role of alpha catulin in breast cancer metastasis and cancer initiating potential; Characterize new invasion markers and possible therapeutic targets crucial for cancer metastasis.

Methods:

2 different triple negative breast cancer cell lines MDA-231 and HCC1806 were transfected with alpha catulin- GFP promoter reporter system and separately transduced with lentiviral shRNA to knockdown alpha catulin. All of the cell lines (reporter and KD) were injected into fat pad of mammary gland in Nod.Scid mice. Tumours were formed after 8 weeks. Cells from the tumours (GFP+/CD49f+ and GFP-/CD49f+) were collected and RNA isolation was performed. All of the samples were subjected to RNAseq analysis.

All of the generated cell lines were used in mammosphere experiments. KD mammospheres vs control were used to measure distance of invasion. Mammosphere serial replating assay was performed on KD and control spheres. Reporter Catulin-GFP spheres were tested in immunochemistry stainings to see if GFP signal overlaps with other known EMT markers.

Results:

MDA-231-Cat-GFP mammospheres were seeded on matrigel-collagen matrix and allowed to invade for 0 and 72 hours. Immunostaining for GFP (catulin) and DAPI revealed that the highest catulin expression level is located outermostly suggesting that invading cells have higher catulin expression level. MDA-231-Cat-GFP mammospheres were immunostained for Vimentin – well known EMT marker, GFP (catulin), DAPI. Noticible is high collocalization of signal coming from Vimentin and GFP (catulin). Other EMT markers are being currently being checked on IF.

MDA-231 KD and control mammospheres were stained for actin, GFP. Distance of invasion was measured starting from the center of the spheres and ending on the furthest invading cells. Catulin KD cells have impaired motility and migrate more than 2 times less efficient.

MDA-231 KD and control mammospheres serial replating assay (3 replicates) was performed. Cells (20000) were plated in sphere medium to allow to form spheres in 6 well plate. After 7 days spheres were counted, disrupted mechanically and seeded again. After 28 days KD cells showed almost zero sphere forming potential whereas control cells showed no difference over time. Almost the same results were obtained from HCC1806 cell line.

Flow cytometry analysis of the cells (MDA-231-Cat-GFP) from mammospheres. Cells were stained for CD44 and CD24 – well known breast cancer stem cell markers. Cells were at first gated as FITC (GFP) positive and then analyzed for CD44+/CD24-. It showed that there is a population comprising around 7% of all GFP+ cells that is highly CD44 positive and CD24 negative showing correlation between catulin expression and well known breast cancer stemness markers.

Flow cytometry analysis of the cells (MDA-231-KD and control) from mammospheres. Cells were immunostained for CD44 and CD24. Control cells showed around 6% of CD44+/CD24- population whereas KD cells showed a striking change in CD44 signal pattern shifting all of the cells into CD44 negative.

Immunohistochemistry staining of tumours formed from injection of a MDA-231-Cat-GFP cell line into fat pad of Nod.Scid mice showed a distribution of CD44 and GFP across tumour burden. I also showed that N-cadherin collocalizes with GFP signal on the outermost part of the tumour. GFP positive cells were seen as the most invasive on the tumour burden together with signal from tenascin.

RNAseq results from MDA-231 cells KD vs control showed more than 270 gene being downregulated and around 200 genes upregulated in the absence of catulin. Many of downregulated genes are involved in Wnt signaling pathway and in the process of motility. More RNAseq results, from tumours formed after injections of reporter cell lines, will be obtained and analysed very soon.

Participation in conferences:

FEBS congress 2019 - poster

Publications, scientific presentations as a leading author:

"Alpha-catulin as a marker of a specific population of invasive breast cancer cells." – poster, 1st author, FEBS congress 2019.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Katsiaryna Marhelava	Year of studies: 2nd

Title of the project: Evaluation of the anticancer efficacy of a new chimeric antigen receptor targeting PD-L1 molecule

Name of the supervisor: Radoslaw Zagozdzon, MD, PhD

Affiliation: Medical University of Warsaw

AIMS of the project :

Cancer cells have the ability to increase the expression of immune checkpoint molecules on their surface and thus escape immune response. For example, interaction between programmed death-ligand 1 (PD-L1) on cancer cells and programmed death-1 receptor (PD-1) on the effector cells has strong immunosuppressive effect. In this project, we propose a new approach of targeting cancer cells expressing PD-L1 by chimeric antigen receptor (CAR)-based technology. The main aim of the study is to provide the "proof-of-concept" results for the therapy of tumors with PD-L1 overexpression and to make it safer. We optimize the technology of modifying either human T or NK cells with anti-PD-L1-CAR and target them against cancer cells expressing PD-L1 molecule.

Methods:

- primary T/NK cells isolation and cultivation;
- sgRNA design for *in vitro* synthesis and lentiviral transduction;
- mRNA/sgRNA *in vitro* synthesis;
- mRNA/sgRNA electroporation of primary cells;
- molecular cloning (anti-PD-L1-CAR-encoding cDNA was cloned from a lentiviral plasmid to SFFV promoter-based vector gifted by professor Tonn from Technical University in Dresden);
- lentivirus-mediated genetic modification of both effector and target cancer cell lines;
- Western Blotting was used to confirm lentiviral modification of the cells;
- immunofluorescent staining and flow cytometry analysis were performed both to characterize modified cells and evaluate cytotoxicity of modified effector cells;
- cell sorting;
- luciferase-based assays were performed to evaluate cytotoxicity of modified effector cells.

Results:

Within the second year of the PhD project, the effectiveness of genetic modification of primary T cells and NK92 cell line was successfully improved – populations with 90-100% of cells expressing anti-PD-L1-CAR on their surface were established.

Primary T cells were modified by electroporation. The mRNA encoding anti-PD-L1-CAR construct was obtained by *in vitro* mRNA synthesis and the process was optimized by using different reagents and protocols. The process of mRNA electroporation was also optimized, and its effectivity was improved by testing various conditions and confirmed by flow cytometry. Moreover, the method of gene knock out by electroporation of Cas9-sgRNA ribonucleoproteins (RNPs) was elaborated on primary T cells and 80% efficacy was achieved. NK92 cells were modified by lentiviral transduction and sorted.

Additional tools, such as modified target cells, were generated. PD-L1 knock out was performed on human breast cancer MDA-MB-231 cell line by lentiviral transduction and the result was confirmed

both by flow cytometry and Western Blotting. Cell lines with previously created knock out or overexpression of PD-L1 molecule were also modified by lentiviral transduction to express luciferase.

The cytotoxicity of modified effector cells against target cells with different levels of PD-L1 expression was evaluated. Both flow cytometry and luciferase-based assays were optimized and confirmed, that CAR expressing effector cells have improved ability to kill cancer cells as compared to unmodified. The specificity of CAR construct was confirmed by use of PD-L1 blocking antibody.

Participation in conferences:

Malgorzata Bajor, Agnieszka Graczyk-Jarzynka, Katsiaryna Marhelava, Marta Kłopotowska, Karl-Johan Malmberg, Jakub Golab, Magdalena Winiarska and Radoslaw Zagozdzon: "*Generation of a new human/mouse cross-reactive anti-PD-L1 chimeric antigen receptor for preclinical studies*", Defence is the Best Attack: Immuno-Oncology Breakthroughs (03/2019), Barcelona, Spain.

Publications, scientific presentations as a leading author: None yet

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Victor Abel Murcia Pienkowski	Year of studies: 4th

Title of the project: Mate pair sequencing for precise breakpoint detection in chromosomal aberrations

Name of the supervisor: prof. dr hab. n. med. Rafał Płoski, dr n. med. Małgorzata Rydzanicz, dr n. med. Agnieszka Pollak.

Affiliation: Medical University of Warsaw, Department of Medical Genetics

AIMS of the project:

The aim of the study is the discovery of novel genes followed by verification of their pathogenic function status in symptomatic balanced translocations (BCTs).

Methods:

I. DNA from affected patients with previously detected BCTs derived from The Institute „Pomnik – Centrum Zdrowia Dziecka" are selected. Usually patients are sequenced using nextera mate pair library, while patients with complex translocations are sequenced using Oxford Nanopore technology.
II. Preparation of next generation sequencing (NGS) mate pair libraries is conducted with a standard manufacturer protocol. The libraries are sequenced on the Illumina Hiseq 1500 platform or MinION. For analysis of the results a custom R package is used.
III. Mapped chromosomal break points are verified by direct Sanger sequencing.

IV. In cases where a break point ends up being outside of genes it will be studied in cellular model (fibroblast culture) to verify the expression of chosen genes. For expression verification qPCR was conducted.

Results:

12 patients with symptomatic BCTs have been analyzed so far. In 1 patient the BCT created a fusion gene. In 9 patients a shorter gene was created due to the translocation. In 1 patient the identified translocation was found between noncoding regions. The genes that ended being damaged are: SLC6A1, NAA15, EFNA5, EBF3, LARGE, PPP2R5E, BLOC1S5-TXNDC5, ZNF423, VANGL1, FBRSL1, NIPBL, BAHD1, RET, TRPS1, SLC4A10. In the patient where the BCT was in noncoding DNA, qPCR proved the downregulation of gene DKK2 that is present in the disrupted topologically associating domain. In conclusion mate pair sequencing gives the unique opportunity to search for a precise location of break points boundaries in samples with BCTs.

Publications, scientific presentations as a leading author:

1. Murcia Pienkowski V[#], Kucharczyk M[#], Młynek M[#], Szczałuba K, Rydzanicz M, Poszewiecka B, Skórka A, Sykulski M, Biernacka A, Koppolu AA, Posmyk R, Walczak A, Kosińska J, Krajewski P, Castaneda J, Obersztyn E, Jurkiewicz E, Śmigiel R, Gambin A, Chrzanowska K, Krajewska-Walasek M, Płoski R. Mapping of breakpoints in balanced chromosomal translocations by shallow whole-genome sequencing points to *EFNA5*, *BAHD1* and *PPP2R5E* as novel candidates for genes causing human Mendelian disorders. J Med Genet. 2019 Feb;56(2):104-112. doi: 10.1136/jmedgenet-2018-105527. [#] equal cintribution

2. Jezela-Stanek A, Walczak A, Łaźniewski M, Kosińska J, Stawiński P, Murcia Pienkowski V, Biernacka A, Rydzanicz M, Kostrzewa G, Krajewski P, Plewczyński D, Płoski R. Novel COL12A1 variant as a cause of mild familial extracellular matrix-related myopathy. Clin Genet. 2019 Jun;95(6):736-738. doi: 10.1111/cge.13534.

3. Jakubiuk-Tomaszuk A[#], Murcia Pienkowski V[#], Zietkiewicz S, Rydzanicz M, Kosińska J, Stawiński P, Szumiński M, Płoski R. Syndromic chorioretinal coloboma associated with heterozygous de novo RARA mutation affecting an amino acid critical for retinoic acid interaction. Clin Genet. 2019 Jul 25. doi: 10.1111/cge.13611. [#] equal cintribution

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Monika Prełowska	Year of studies: 3rd

Title of the project: The evaluation of the role of MLK4 kinase in triple negative breast cancer development and progression

Name of the supervisor: prof. dr hab. Dominika Nowis (main supervisor), dr Anna Marusiak (cosupervisor)

Affiliation: Warsaw Medical University and University of Warsaw

AIMS of the project:

The main aim: To investigate the role of MLK4 in breast cancer progression.

Detailed aims:

1. To establish the influence of selective MLK4 silencing on growth, proliferation, migration and invasion of triple negative breast cancer in 2D and 3D models *in vitro*
2. To investigate the effects of MLK4 silencing on the growth of the tumors in xenograft mice model of triple negative breast cancer
3. To evaluate of the efficacy of MLK4 inhibitors in 2D and 3D models *in vitro*
4. To assess the level MLK4 expression in the samples of triple negative breast cancer patients.

Methods:

The influence of small molecule MLK4 inhibitors on growth of spheroids in Matrigel. To assess the efficiency of MLK4 inhibition of spheroids' growth, 12 well plates were coated with 100 µl of Matrigel using method from the middle to the walls of well, in cooled conditions (on ice, tubes and tips were prefrozen). Plates coated with Matrigel were then incubated in 37°C for 30 minutes. After bottom layer of Matrigel polymerized, 5000 of HCC1806 cells were suspended in 2. 5% Matrigel and plated on the bottom layer of Matrigel. 2 days later, URMC-099 and CEP-1347 (2 and 4 µM) were added to the spheroids and left to culture up to 5 days. Pictures were taken.

Preparations for *in vivo* xenograft mouse model of triple negative breast cancer and for the doxycycline effect control. To assess the growth potential of tumors lacking MLK4, the *in vivo* xenograft experiments were performed. Cell line with doxycycline-inducible *MLK4* knockdown - HCC1806 sh6 - was prepared, which included testing for mycoplasma contamination, authentication of cells by ATCC service, and growing them before injection into mammary pads of immunedeficient mice RAG-/-. Two experiments were performed. Pilot and larger scale experiment. The doxycycline (1mg/ml and 2 mg/ml) was administered with the water.

To exclude the effect of doxycycline itself on growth potential of tumors, the *in vivo* xenograft experiment was performed. HCC1806 cell line was prepared, which included testing for mycoplasma contamination, authentication of cells by ATCC service, and growing them before injection into mammary pads of immune-deficient mice RAG-/-. The doxycycline (2 mg/ml) was administered with the water.

The epithelial to mesenchymal transition. Previously generated HCC1806_sh6 cells, with doxycycline induced *MLK4* silencing system, were treated with doxycycline (1 µg / ml) for 6 days. After this time, cells were lysed and the RNA was isolated. Next, the reverse transcription was performed to obtain the cDNA. Then, the levels of Snail and TWIST mRNA was quantified with the method of RT-qPCR.

Doctoral thesis. Based on all results obtained during this project, the doctoral thesis has been prepared.

Results:

The influence of small molecule MLK4 inhibitors on growth of spheroids. To evaluate the influence of small molecule MLK4 inhibitors on growth of spheroids we performed the protocol as described above. The results indicate that MLK4 inhibition affects growth and number of spheroids in a dose dependent manner.

Preparations for in vivo xenograft mice model of triple negative breast cancer and the doxycycline effect control. The data obtained from xenograft mice model described above show that silencing the expression of *MLK4* leads to decrease in the size of tumor and that doxycycline treatment itself in dose used for does not affect the tumor growth.

The epithelial to mesenchymal transition. The silencing of *MLK4* expression in the HCC1806_sh6 cell line led to decrease in the mRNA levels of the mesenchymal markers Snail and TWIST.

Participation in conferences:

San Antonio Breast Cancer Symposium USA, San Antonio (4.12.18- 8.12.18) - poster "The role of MLK4 amplification in progression of triple negative breast cancer " .

Publications, scientific presentations as a leading author:

Marusiak AA,, Prelowska MK, Mehlich D, Lazniewski M, Kaminska K, Gorczynski A, Korwat A, Sokolowska O, Kedzierska H, Golab J, Biernat W, Plewczynski D, Brognard J, Nowis D. Upregulation of MLK4 promotes migratory and invasive potential of breast cancer cells. Oncogene. 2019 Apr;38(15):2860-2875. doi: 10.1038/s41388-018-0618-0.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Sonia Dębek	Year of studies: 1st

Title of the project: Discovery and Characterisation of Non-Canonical PIM Kinase Functions in Diffuse Large B Cell Lymphoma

Name of the supervisor: prof. Przemysław Juszczyński

Affiliation: Institute of Haematology and Transfusion Medicine, Warsaw

Aims of the project:

The aim of this study is to characterise chromatin association and super-enhancer-specific functions of PIM kinases, as well as their role in epigenetic regulation of transcription.

Methods:

Analysis of changes in the epigenetic landscape in response to PIM kinase inhibition: Three DLBCL cell lines were treated with PIM inhibitor, or diluent as a control. Samples were collected from the experimental, and the control cultures at five time points. Efficacy of inhibition was confirmed by immunoblot analysis with antibodies against PIM substrates. Histones were isolated from each sample, and levels of selected histone modifications were quantified through western blot.

Preparation of histone 3 (H3) plasmids: In order to investigate the role of H3S10 phosphorylation, I constructed a plasmid with cDNA of H3 with serine 10 substituted with non-phosphorylable alanine (S10A mutation). Site-directed mutagenesis and cloning to the expression plasmid was conducted with classical methods of genetic engineering: PCR, mini- and maxi-prep, restriction digestion, ligation, and Sanger sequencing.

Cellular fractionation: To establish subcellular localisation of PIM kinases, cytoplasmic, nuclear, and cellular fractions from DLBCL cell lines were isolated, and investigated by western blot for the presence of PIM1, PIM2 and PIM3.

Chromatin fractionation: Affinity of PIM1 to the chromatin is being assessed by chromatin fractionation following a modified protocol by Herrmann *et al*., 2017. Isolated nuclei are treated with MNase, and sequentially washed in buffers with increasing concentration of NaCl. Obtained fractions of chromatin-bound proteins are analysed via western blot.

Flow cytometry: Activity of PIM kinases in different phases of cell cycle is being analysed by flow cytometry. DLBCL cells are stained with H3S10ph primary antibody, and Alexa Fluor 488 secondary antibody, as well as PI (the method is currently being optimised).

Results:

An early step in super-enhancer formation is acetylation of H3 and H4 lysines. We assume that phosphorylation of H3S10 by PIM1 plays a critical role in this process. The results to-date show that inhibition of PIM kinases leads to the decrease in phosphorylation of H3S10, and concomitant decrease in acetylation of histone H3 and H4, as well as H3K4me3, which is a marker of active transcription. This observation was confirmed in three independent DLBCL cell lines.

PIM family consists of three kinases, PIM1, PIM2 and PIM3. While it is known that PIM1 is an epigenetic writer, (e.g. Zippo *et al.*, 2007), the role of PIM2 and PIM3 in epigenetics has not been studied. In my project, I confirmed that in the studied DLBCL cell lines, PIM1 is located in both the nucleus and the cytoplasm, while PIM2 and PIM3 occupy cytoplasm only – at least in stress-free

conditions. Thus, PIM1 is likely the only kinase of the PIM family capable of changing epigenetic landscape, and, presumably, inducing super-enhancers.

In order to characterise how strong the interaction between PIM1 and chromatin is, I plan to conduct chromatin fractionation. So far, the protocol has been optimised, which included establishing the best method of nuclear isolation, and optimal conditions for MNase digestion, as well as conditions for visualising PIM kinases in western blot.

To investigate the role of H3S10ph modification, I have also successfully constructed a plasmid with cDNA of H3 with serine 10 substituted with non-phosphorylable alanine (S10A mutation). The mutated histone will be expressed in a doxycycline-dependent setup, and changes in the epigenome will be analysed similarly to the chemical inhibition of PIM.

Further plans include confirmation of the role of the H3S10ph modification, and the role of PIM kinases by conducting experiments with, respectively, H3_S10A plasmid, or transcriptional inhibition of PIM kinases; characterisation of PIM1 association with the chromatin; characterisation of the activity of PIM kinases in different phases of cell cycle; and description of the role of PIM kinases in the transcription of super-enhancer-regulated genes through RNA-Seq.

Participation in conferences (as a listener):

1. XVIII Zjazd Polskiego Towarzystwa Hematologów i Transfuzjologów. Łódź, 12-14.09.2019.
2. Hematologia 2019. Od diagnostyki do leczenia. Warsaw, 31.05-1.06.2019.
3. Zastosowania NGS w praktyce onkohematologicznej. Institute of Haematology and Transfusion Medicine, Warsaw, 20.05.2019,
4. Young Scientists Conference on Molecular and Cell Biology. International Institute of Molecular and Cell Biology, Warsaw, 11.04.2019.

Publications, scientific presentations as a leading author:

Dębek S. 'Super-enhancers in deregulation of transcription in haematopoietic and lymphatic tumours, and their targeted inhibition as a therapeutic strategy' – in preparation.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Bartłomiej Pawlik	Year of studies: 1st

Title of the project: mTOR modulation in KMT2A (MLL) acute lymphoblastic leukemia and its impact on immune checkpoints activation.

Name of the supervisor: prof. dr hab. n. med. Wojciech Młynarski

Affiliation: Medical University of Lodz

AIMS of the project:

One of the main hematopoietic malignances in pediatric patients is acute lymphoblastic leukemia, specifically its subset from immature B lymphocytes (BCP-ALL). This leukemia can be classified based on the presence of specific chromosomal rearrangements and other genetic disorders, that lead to changes on cellular metabolism. Leukemias with rearrangements of the *KMT2A (MLL)* gene occur in over 80% of infant B-ALLs and are directly associated with poor prognosis. Patients with *KMT2A*-gene mutations are classified in high-risk group. Our previous studies have shown that mTOR activity impacts the clinical outcome of acute lymphoblastic leukemia, thus we would like to evidence that mTOR activation is directly involved in the pathogenesis of KMT2A-ALL. At the same time, we showed that one of immune checkpoint protein – galectin 1 (Gal1) is a selective marker of KMT2A-ALL, but the classical activation pathway of this protein is not found in the case of this leukemia subtype. Therefore, we would also like to find the pattern of Gal1 activation in KMT2A-ALL.

Methods:

In silico analysis of activation status of 44 genes involved in mTOR pathway (KEGG) was performed in mRNA expression arrays from various leukemia subtypes (GEO: gse26366 and gse13425). SEM-K2 (KMT2A-ALL), REH (ETV6-RUNX1 ALL) and SUP-B15 (Philadelphia chromosome positive ALL) cell lines were cultured for 72h with increased dosage of everolimus (1nM – 10uM). Proliferation status was measured by Cell Countess Invitrogen. Cytotoxicity level was achieved by tripan blue and propidium iodide staining. RNA isolation was performed by RNeasy Mini Kit (Qiagen). Level of mRNA transcripts of *PDCD1*, *CD274* and *LGALS1* genes was achieved by Real-Time PCR. Total protein was isolated form cell lines and its concentration was measured by RC-DC Protein Assay Kit (BioRad). Level of proteins was obtain by Western Blot analysis.

Results:

Results from *in silico* analysis indicated a stimulation of mTOR pathway related genes in ALL subtype with *KMT2A* gene rearrangement. mTOR selective inhibitor – everolimus (EVO), inhibited cell growth and proliferation exclusively in SEM-K2 (KMT2A positive) cell line in dose–dependent manner, whilst not affected other leukemia subtypes. EVO was not cytotoxic against all tested cell lines. mTOR pathway was successfully inhibited by EVO in all tested cell lines, which was confirmed by level of phosphorylated S6K1 kinase level. Culture of KMT2A positive cells (SEM-K2) with EVO resulted in increased mRNA level of some of the PD-1/PD-L1 immune checkpoint related genes, e.g. *PDCD1* and *CD274*, while level of another KMT2A-subtype specific checkpoint – Gal1 (*LGALS1* gene), was decreased after treatment with EVO. That observations were exclusive to KMT2A subtype and in the case of PD-1/PD-L1 not observed on protein level.

Participation in conferences:

Poster presentation at the IX Congress of the Polish Society of Pediatric Oncology and Hematology (Poznań) - abstract in Pediatric Review (2018/Vol. 47/ No. 2/Suplement /p. 5-6, ISSN 0137-723X).

Publications, scientific presentations as a leading author:

Kielbik M., Krzyzanowski D., Pawlik B., Klink M. Cisplatin-induced ERK1/2 activity promotes G1 to S phase progression which leads to chemoresistance of ovarian cancer cells. Oncotarget, 9(28) 2018, 19847–19860. IF = 5,168

Wrona E., Jakubowska J., Pawlik B., Pastorczak A., Madzio J., Lejman M., Sędek Ł., Kowalczyk J., Szczepański T., Młynarski W. Gene expression of *ASNS*, *LGMN* and *CTSB* is higher in a subgroup of childhood BCP-ALL with *PAX5* deletion. 2019. Manuscript accepted for publication in Oncology Letters. IF = 1,871.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Jakub Czapiński	Year of studies: 3rd

Title of the project: Regulation of gene expression in tumor cells in the epithelial-mesenchymal transition

Name of the supervisor: Professor Adolfo Rivero-Müller

Affiliation: Medical University of Lublin

AIMS of the project :

The epithelial-mesenchymal transition (EMT) is a critical developmental process in which a polarized epithelial cell reversibly undergoes gene transcription reprogramming that enables it to assume a mesenchymal cell phenotype. The temporal nature of this process, transition, means that the process can be reversed; such reverse effect is called mesenchymal-epithelial transition (MET). While EMT seems to be essential for cancer cell metastasis, MET is also important during the establishment of metastatic cells to distant organs. The objective of our work is:

1. To create tools for EMT/MET study including reporter cell lines - cancer cell lines that differently fluoresce during epithelial or mesenchymal stages;

2. To repress or activate specific endogenous epithelial or mesenchymal genes in order to check their roles during EMT and MET.

Methods:

Plasmids construction:

Cas9 vector (PX459, #48139) was purchased from AddGene repository. The dCas9-VP64-T2A-Puro plasmid (CRISPRa) has been prepared in our lab. Gene encoding Cas9 has been mutated (D10A & H840A) with the REPLACR method. Next, downstream of dCas9, the VP64 gene has been cloned, using the Gibson Assembly method according to the manufacturer's protocol. The resulting DNA sequences were verified by sequencing.

Guide RNAs (gRNAs) were cloned into this vector using annealed oligos according to Zhang Labs protocol (http://www.genome-engineering.org/crispr/?page_id=23), using T7 ligase and BbsI (both from Thermo Fisher).

Cell culture and nucleofection:

H2170 cell line and HEK293 bought from ATCC. Cells were nucleofected using Lonza nucleofector Kit V reagents. Screening of the KI single cell clones: The genotyping were done using Mouse Direct PCR KIT (Biotools). Then, DNA from the positive clones were isolated, the transgene was amplified and sequenced.

Confocal microscopy:

The cells were seeded onto 24-well glass-bottom plates (MoBiTec) one day before transfection. Transfection was conducted as described above. Transfected cells were then Hoechst stained and visualized under Nikon Ti Confocal microscope within 24h after transfection using a 563 nm laser.

qPCR

Total RNAs was isolated using ExtractMe total RNA kit (Blirt). Next, cDNAs were synthesized using the High-Capacity cDNA Reverse Transcription Kit with addition of a RNase Inhibitor (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene

that is stably and consistently expressed in most cell lines analyzed. Prior to use, all primer sets were tested for specificity and sensitivity, resulting in a single amplicon of the expected size. PCR reactions were then performed with the LightCycler® 480 Instrument (Roche) in triplicates. The number of cycles needed to reach specific threshold of detection (CT) was used to calculate relative quantification (RQ). Relative mRNA expression was calculated using the delta CT subtraction and normalized to the expression of GAPDH.

Results:

We have knocked in (KI) mCardinal in fusion to Vimentin (VIM) gene in H2170 and HEK293 cells, which was verified via sequencing, confocal microscopy visualization and functional studies. KIs of cadherin 1 (CDH1) were done and sequence-verified but the KIs are not functional. gRNAs were designed, cloned and have been validated for CRISPRa and CRISPRi, and first tests have been performed.

We created a method called PSSC (Promoter Specific Split Cas9) which is based on mesenchymal specific promoters and a split Cas9. The system is being used to obtain extreme phenotypes, mesenchymal or epithelial, from the cell lines used for this project.

Additionally we created Cell-based TWIST1 bioassay which is composed by TWIST1 binding domain and Gaussia Luciferase (GL). The assay shows a clear correlation between the activity of TWIST1 and the reporter. Likewise, the reporter was used to test the activity of TWIST inhibitors, demonstrating its potential for high throughput screening of such molecules. That system is being used to monitor TWIST activity when particular genes are down or upregulated without need of cell destruction – the GL is secreted outside the cell.

Participation in conferences:

A comparison of two methods for gene inactivation using CRISPR. Kałafut J., Przybyszewska A., Czapiński J., Okoń E., Stepulak A., Rivero-Müller A., 5th International Conference of Cell Biology. Kraków, 10-12 May 2019.

Publications, scientific presentations as a leading author:

Histone deacetylase inhibitors and phenotypical transformation of cancer cells, Wawruszak A., Kałafut J., Okoń E., Czapiński J., et all , Cancers, 2019, vol. 11, nr 2.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Iga Kołodziejczak	**Year of studies:** 1 st

Title of the project: Development of gene expression-based and protein expression-based Stemness Indices using machine learning and molecular profiling

Name of the supervisor: Prof. dr hab. Maciej Wiznerowicz

Affiliation: Artificial Intelligence Laboratory at the International Institute for Molecular Oncology in Poznań

AIMS of the project:

The main goal of the project is to develop Stemness Indices based on gene expression, DNA methylation, expression of proteins as well as modifications of proteins by means of machine learning and molecular profiles from embryonic stem cells and pluripotent stem cells: mRNAsi, miRNAsi, lncRNAsi, mDNAsi, PROTsi and phospho-PROTsi. Obtained Stemness Indices are correlated with gene expression, expression of proteins and modifications in proteins in such cancers as lung cancer, breast cancer, glioblastoma, ovarian cancer, uterine cancer and more. Processing and analysis of the proteogenomic datasets are powered by the Human Induced Pluripotent Stem Cells Initiative (HipSci), which generates a large, high-quality reference panel of human iPS cell lines comprising hundreds of cell lines derived from phenotypically healthy donors and several cohorts of donors carrying inherited genetic diseases. In addition, CPTAC (Clinical Proteomic Tumor Analysis Consortium formed by The National Cancer Institute to facilitate the discovery of cancer-specific protein biomarkers) data resources are used. CPTAC collects proteomics data generated by the mass spectrometric interrogation of tumour samples, which have been already analyzed by The Cancer Genome Atlas program.

Subsequently, deriving a suitable protein target based on bioinformatic analysis will be validated using sphere formation assay in soft agar to evaluate the stemness of cancer cells *in vitro*. Transcriptome sequencing followed by the bioinformatic analyzes and investigation of the publicly available datasets will expand the understanding of specific molecular mechanisms regulating cancer stemness.

Methods:

- Location, downloading and bioinformatic analysis of publicly available data deposited into Gene Expression Omnibus (GEO) data repository. Raw files were downloaded with NCBI SRA Toolkit and converted into FASTQ format.
- Utilization of HipSci data repository containing RNA-seq and proteomics mass spectrometry data. HipSci datasets are characterized by systematic derivation of iPSC from hundreds of healthy volunteers using a well-defined and standardized experimental pipeline. The HipSci cell lines are widely characterized and available to the research community along with the accompanying phenotypic and genetic data.
- All bioinformatic analyzes including for example writing and applying scripts as well as plots creation were performed using R software, which is a freely available language and environment for statistical computing and graphics providing a large variety of graphical and statistical techniques like statistical tests, classification, clustering and linear and nonlinear modelling.

- Generation of Stemness Indices was performed using One-Class Logistic Regression (OCLR), which is a machine learning algorithm learned on stem cell types (ESC, iPSC) and their differentiated ecto-, meso- and endodermal precursors. The OCLR algorithm was used because it does not penalize the incorrect classification of precursors derived from the stem cell at various stages of differentiation, which still have some features of undifferentiation in their molecular profiles.

Results:

- Generation of gene expression-based index using 532 HipSci samples computed using OCLR (One-Class Logistic Regression) method and validating by external datasets to confirm that stem cells have higher Stemness Index than differentiated samples. The Stemness Index provides information on how well cancer cells resemble stem cells and is based on the assumption that there is a similarity between cancer cells and stem cells. The Stemness Index is based on similarity due to gene expression, and the index ranges from 0 to 1, with 0 indicating low similarity to stem cells and 1 high similarity. Previously generated Stemness Index was also based on gene expression, but it utilized only PCBC datasets of 77 samples. Our lab expanded the dataset by using HipSci data repository consisting of 532 RNAseq datasets obtained from human iPS cells. The use of HipSci data sets is not only associated with a much larger number of samples, but also with greater accuracy resulting from the use of newer and more advanced RNAseq technology. Validation of the obtained HipSci-532 gene-expression-based stemness index using external datasets confirmed its ability to separate ES/iPSCs from the differentiated cells.

- Generation of gene expression-based indexes using stemness score computed using 532 RNAseq dataset of human iPS cells and PanCancer TCGA RNAseq dataset.

- Processing and analysis of the pilot protein datasets from HipSci of 18 HipSci iPSC samples to obtain protein expression matrix using Common Data Analysis Pipeline (CDAP).

- Generation of novel pilot protein expression-based index using 17 HipSci samples computed using OCLR method and validating by external datasets. This analysis as well as the following ones are actually at the final stage of work.

- Processing and analysis of the proteogenomic datasets from HipSci – 217 HipSci iPSC lines obtained from 163 donors and selection of 217 samples to obtain protein expression matrix using Common Data Analysis Pipeline (CDAP).

Participation in conferences:

Publications, scientific presentations as a leading author:

Czerwińska P., Mazurek S., Kołodziejczak I., Wiznerowicz M. *Gene delivery methods and genome editing of human pluripotent stem cells*. Rep Pract Oncol Radiother. 2019 Mar-Apr;24(2):180-187.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Paulina Misiukiewicz-Stępień	Year of studies: 1st

Title of the project: The comparison of mRNA expression profile of respiratory epithelium in the interactions between epithelium, dendritic cells and macrophages in obstructive lung diseases.

Name of the supervisor: dr hab. n. med. Magdalena Paplińska-Goryca

Affiliation: Department of Internal Medicine, Pulmonary Diseases and Allergy, Medical University of Warsaw

AIMS of the project:

The main goal of the study is to evaluate the mRNA expression profile of the respiratory epithelium in the interactions between epithelium, macrophages and dendritic cells in asthma and COPD.

The secondary aim of the project is to explore the possibilities of NGS to personalized diagnostic via differences in mRNA expression profiles of epithelium of asthma and COPD patients.

Methods:

- Primary human epithelial cells, obtained from nasal brushing specimen, was cultured in the air–liquid interface (ALI) for 21 days in order to obtain a specialized types of respiratory epithelium which contain secretory, ciliated and basal epithelial cells. The respiratory epithelium for ALI culture was obtained from nasal brushing specimen. Macrophages and dendritic cells were derived from peripheral blood mononuclear cells (PBMCs). Monocyte derived macrophages (MDM) were placed on top and monocyte derived dendritic cells (MDDC) were placed underneath the epithelial monolayer.
- Cells were cultured in different combinations: epithelial cells alone, epithelial cells+dendritic cells, epithelial cells+macrophages and epithelial cells+dendritic cells+macrophages. All cell subpopulations were obtained from different single one patient (asthma, COPD and control subjects).
- Additional stimulation of epithelial cells with IL-13 and polyinosinic:polycytidylic acid (poly I:C) was used in all culture types.
- The expression of TSLP was evaluated by real time PCR by $2^{-\Delta\Delta CT}$ method, where unstimulated cells were controls and 18S rRNA was used for endogenous control.

Results:

The studies performed this year allowed to determine *in vitro* conditions of experiments. The methodology of nasal epithelial cells isolation, maintenance and cultivation of epithelial cells on air liquid interference (ALI) as well as macrophages and dendritic cells specialization from PBMCs was adapted. The optimal experiment conditions like concentration of selected stimulators was established but most importantly the technique of triple-cell co-culture was successfully determined.

The presented results came from experiments performed in three groups: controls (n=6), asthma patients (n=7) and COPD patients (n=5). In the preliminary study we evaluated the changes in TSLP mRNA expression as important epithelium derived cytokine for obstructive lung diseases pathobiology. The effect of IL-13 and poly I:C stimulation on TSLP mRNA expression in air liquid interference (ALI) nasal epithelial cells cultured alone or co-cultured with macrophages or dendritic cells or in triple co-culture scheme of controls, asthma or COPD patients was evaluated. The PCR

results showed lower expression of TSLP mRNA in epithelial- dendritic cells co-culture stimulated with poly I:C compared to epithelial- dendritic cells co-culture without additional stimulation. Application of macrophages without dendritic cells with simultaneous stimulation with poly I:C revealed increased TSLP mRNA expression in nasal epithelium. Stimulation of epithelial monocultures from COPD patients with IL-13 resulted in elevated level of TSLP mRNA in comparison to control. We observed various regulation of TSLP mRNA expression in co-culture model from asthma patients compared to control. In co-culture of epithelial and dendritic cells from asthma patients stimulated with IL-13 with elevated TSLP expression compared to the control cultures was observed. In contrast, triple co-cultures of control group showed higher expression of TSLP than co-cultures from asthmatics after stimulation with poly I:C. The TSLP expression after IL-13 stimulation was upregulated in co-cultures of epithelium and macrophages in COPD compared to the asthma group.

The results of this study indicate that co-stimulation of airway epithelial cells with macrophages and dendritic cells as well as IL-13 and poly I:C 1) may change the cytokine expression in epithelium 2) the airway epithelium from asthma, COPD or control groups differs in response from macrophages and DCs co-stimulation. We suggest that mRNA profile of respiratory epithelium may depend on cell interactions and be different in various lung biochemical background associated with asthma or COPD.

Participation in conferences:

- Molecular methods of RNA analysis, series of lectures on University of Warsaw
- XII 2018: Exome sequencing as a method of genome analysis, Centre of Postgraduate Medical Education, Warsaw
- II 2019: ERS Satellites: Advances in precision medicine in COPD & ILD, Warsaw
- IV 2019: Young Scientists Conference on Molecular and Cell Biology, IIMCB, Warsaw.

Publications, scientific presentations as a leading author:

Paplińska-Goryca M, Goryca K, Misiukiewicz-Stępień P, et al (2019) mRNA expression profile of bronchoalveolar lavage fluid cells from patients with idiopathic pulmonary fibrosis and sarcoidosis. Eur J Clin Invest e13153. https://doi.org/10.1111/eci.13153

Misiukiewicz-Stępień P, Paplińska-Goryca M, Górska K, Krenke R: Cilia associated proteins level in induced sputum of asthma and COPD patients, poster presentation on Young Scientists Conference on Molecular and Cell Biology, IIMCB, 11.04.2019, Warsaw.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Aneta Moskalik	Year of studies: 2nd

Title of the project: Evaluation of inflammatory cell profile, including macrophages that modulate cardiac lymphatic vessel remodeling in lepr$^{db/db}$ mice mimicking metabolic syndrome

Name of the supervisor: Prof. Anna Ratajska

Affiliation: Medical University of Warsaw

AIMS of the project:

Macrophages as well as lymphatic vessels are recognized as crucial players in cardiovascular complications and myocardial remodeling in metabolic syndrome (MetS). In obesity, type 2 diabetes and hypertension, associated with MetS, lymphatic vessels display disturbed function, abnormal remodeling, and impaired lymphangiogenesis. In the 2nd year of the Ph.D. project the main aim was to assess cardiac tissue macrophage (cTM) phenotypes in a confocal microscope and verify hypothesis that these extremely plastic and multifunctional cells could contribute to the adverse cardiac lymphatic vessel remodeling in MetS. This cTM detrimental role could be mediated by miRNAs - small molecules that regulate functions of specific groups of genes.

Methods:

Studies were performed on C57BL/6 male mice – control and Lepr$^{db/db}$ which mimic MetS. Some mice were treated with angiotensin II to evoke hypertention. Macrophage density, phenotypes and location were evaluated on frozen sections of mouse hearts by confocal microscopy. Macrophages from single cell suspension from cardiac tissue were sorted into pro-inflammatory and anti-inflammatory populations, respectively CD45+CD11b+CD64+Ly6C+ and CD45+CD11b+CD64+Ly6C-, using flow cytometer (BD FACSAria III). To analyze macrophage miRNA in these populations Next-Generation Sequencing (NGS) and bioinformatic analysis were performed. miRNA selection was made on the basis of PubMed as well as TargetScanVert and miRDB databases. Next step of experiments was assessment of the level of chosen miRNAs in RAW 264.7 macrophage by PCR. Then RAW 264.7 were cultured and transfected with miRNAs (mimics or inhibitors). Morphology of transfected RAW 264.7 macrophages was assessed in an inverted microscope and macrophage supernatants were collected for PCR analysis and ELISA assay (pending).

Results:

The vast majority of cTM in control and db/db mice were CD68+CD206+ (M2 macrophages). However, the density of cTM was significantly reduced in db/db mice. Db/db mice treated with angiotensin II were characterized by presence of both CD68+CD206+ and CD68+CD206- cTM populations. These populations were observed in large clusters in areas of wound healing/scar tissue. Specific miRNAs of key importance in gene regulation involved in modulation of vascular remodeling, especially lymphatic vessel remodeling were selected. These miRNAs were up- or downregulated vs. miRNA levels in the reference cTM population (from myocardium of healthy animals bearing CD45+CD11b+CD64+Ly6C- phenotype. Three of four chosen miRNAs were expressed in RAW 264.7 macrophages, as assessed by RT-PCR technique. Culture of RAW 264.7 transfected with selected miRNA/antago-miRNA showed significant differences in cellular shapes. Future analysis of macrophage secretome will give answers what changes occur at mRNA level.

Molecules, secreted by transfected macrophages, that could potentially alter lymphatic endothelial cell (LEC) behavior in vitro will be selected.

Participation in conferences:

1. Moskalik A, Gondek A, Żera T, Niderla-Bielińska J, Jankowska-Steifer E, Tomczyk M, Zabost A, Bartkowiak K, Bartkowiak M, Ratajska A. Leprdb/db mice as an experimental model in study on the metabolic syndrome. IX National Conference on Advances in Biomedical Research, Warsaw, Poland, December 2018
2. Bartkowiak K, Bartkowiak M, Niderla – Bielińska J, Jankowska-Steifer E, Moskalik A, Flaht-Zabost A, Ratajska A. Preliminary results of vascular wall thicknesses assessment in the heart of db/db mice with angiotensin II pump. IX National Conference Advances in Biomedical Research, Warsaw, Poland, December 2018
3. Bartkowiak K, Moskalik A, Flaht-Zabost A, Kiernozek E, Bartkowiak M, Ratajska A, Niderla-Bielińska J. The vascular endothelium of the heart in the metabolic syndrome in db/db mice - preliminary study. 52 Scientific Congress of the Polish Society of Histochemists and Cytochemists 'Immunohistochemistry and molecular biology in morphology'. Bialystok, Poland, September 2018.

Publications, scientific presentations as a leading author:

The manuscript 'Dysfunctional epicardial adipose tissue and its cardiovascular consequences' submission is processed.

Oral presentation: Moskalik A, Gondek A, Żera T, Niderla-Bielińska J, Jankowska-Steifer E, Tomczyk M, Zabost A, Bartkowiak K, Bartkowiak M, Ratajska A. Leprdb/db mice as an experimental model in study on the metabolic syndrome. IX National Conference on Advances in Biomedical Research. Warsaw, Poland, December 2018.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Aleksandra Olszewska	Year of studies: 1st

Title of the project: Role of autophagy and senescence in lung cancer chemoresistance: in vitro studies, in vivo studies, and analysis of clinical samples.

Name of the supervisor: dr Halina Waś

Affiliation: Laboratory of Molecular Oncology, Military Institute of Medicine, Warsaw, Poland

AIMS of the project:

Lung cancer is the leading cause of deaths from cancer. Low survival rate in this cancer is mainly caused by the late diagnosis and the rapid development of a disease that is resistant to the treatment. Chemotherapeutics kill tumor cells, but they can also lead to therapy induced senescence (TIS). Although, senescence is believed to be related to irreversible growth arrest, TIS cells may divide in an unusual way and contribute to the recurrence of cancer. A characteristic feature of solid tumors is hypoxia, which can be crucial for development of chemoresistance. Studies indicate the key role of hypoxia in the selection of cancer stem cells, considered to be responsible for tumor initiation after therapies. Therefore, to reproduce the environmental condition of tumour growth, our experiments are conducted under hypoxic conditions. The aim of the study is to determine, whether chemotherapeutics used in clinic can cause the senescence of lung cancer cells with characteristic of tumor-initiating cells under hypoxic conditions.

Methods:

The studies are conducted on A549 human non-small cell lung cancer cells and LLC1 murine Lewis lung carcinoma cell line. For treatment chemotherapeutics used to treat patients with lung cancer: cisplatin, vinorelbine and docetaxel were applied. Cells were first cultured with appropriate concentrations of drugs and then for several days in a drug-free medium. Changes in metabolic activity, were detected by the MTT test. The proliferation rate was estimated by bromodeoxyuridine incorporation into DNA. In order to measure the activity of SA-β-gal, a lysosomal enzyme characteristic for senescent cells, cytochemical staining was carried out. All experiments were performed in normoxia (~19 % O_2) and hypoxia (1% O_2).

Results:

Metabolic activity showed that human A549 cells were sensititive to cisplatin and vinorelbine in a dose-dependent manner, while the viability of murine LLC1 cells rapidly decreased over a threshold dose. In addition, LLC1 cell viability was significantly reduced in hypoxia. The sub-toxic doses were selected for further research on the basis of the MTT test. The proliferation of human lung cancer cells A549 was reduced after all chemotherapeutic treatments. There was no hypoxia effect. Murine LLC1 cell line proliferation decreased after using docetaxel and cisplatin and effect of cisplatin was enhanced in hypoxia. Inhibition of proliferation in human A549 cells correlated with the development of a phenotype characteristic for senescent cells, e.g. morphological changes and increased SA-β-gal activity. The SA-β-gal staining intensity was weaker in hypoxia than in normoxia. In the case of the murine LLC1 cell line, SA-β-gal-positive cells also occurred, however, difference in staining intensity between normoxia and hypoxia was slight. Those results suggest that chemotherapeutics induce morphological changes characteristic for cellular senescence both in normoxia and hypoxia. However, intensity of SA-β-gal staining might be dependent on cell line and oxygen conditions. These

results will be verified by examining other senescence markers than SA-β-gal activity (e.g. cell size and granularity, polyploidy, expression of senescent proteins and the senescence-associated secretory phenotype- SASP). In the next step we plan to search for the stem cell-related features in senescent cells and determine the role of autophagy in the recovery from chemotherapy induced-senescence.

Participation in conferences:

1. LIA Meeting Warsaw 2019 "Biomarkers and mediators of diseases-new approaches in repair/regenerative therapies"- active participation and aid in organization
2. III Konferencja Doktorantów Nauk Przyrodniczych- active participation
3. **„**Biologia molekularna w diagnostyce chorób zakaźnych i biotechnologii– DIAGMOL 2018- passive participation
4. „Nowości w leczeniu nowotworów klatki piersiowej" po European Lung Cancer Conference- passive participation
5. II Ogólnopolska Konferencja Naukowa „Wyzwania onkologii spersonalizowanej i interdyscyplinarnej"- passive participation
6. VII Intercollegiate Biotechnology Symposium "Symbioza"- passive participation

Publications, scientific presentations as a leading author:

1. Senescence of human lung cancer cells is induced by treatment with cisplatin and vinorelbine in hypoxic and normoxic conditions. Aleksandra Olszewska, Claudine Kieda, Halina Waś- poster session- LIA Meeting Warsaw 2019 "Biomarkers and mediators of diseases-new approaches in repair/regenerative therapies"
2. Starzenie ludzkich oraz mysich komórek nowotworowych indukowane cisplatyną, winorelbiną I docetakselem w warunkach hipoksji I normoksji. Aleksandra Olszewska, Claudine Kieda, Halina Waś- poster session- III Konferencja Doktorantów Nauk Przyrodniczych
3. Fit & science- Profilaktyka raka szyjki macicy- presentation of the lecture as part of the workshop: „Poradnictwo genetyczne w raku piersi, jajnika i szyjki macicy" in cooperation with dr Agnieszka Synowiec.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Martyna Smolińska	Year of studies:3rd

Title of the project: EVER1 and EVER2 expression in psoriasis

Name of the supervisor: Prof. dr hab. n. med Jacek Malejczyk

Affiliation: Katedra i Zakład Histologii I Embriologii, Medical University of Warsaw

AIMS of the project :

To examine potential differences in the expression of EVER1 and EVER2 genes between psoriatic skin and healthy skin of patients with psoriasis.

To study if psoriasis associated cytokines influence the expression of EVER genes in human keratinocytes.

Methods:

Cell lines: Human immortal keratinocyte line HaCaT was cultured under standard conditions until 60-80% confluence. Then, the cells were treated with one of the following cytokines: IFNγ,,TNFα, IL-1, IL-22, IL-17, IL-23, IL-4, IL-6, TGF, EGF; after 24 hours, we isolated mRNA.

PBMC isolation and culture: Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation from blood samples collected from patients with psoriasis and from healthy controls. The cells were cultured in complete RPMI-1640 medium containing either phytohemagglutinin (PHA) or superparamgnetic beads coupled with an anti-human CD3 and CD28 antibodies, or in culture medium alone, and harvested after 24 hours for RNA isolation.

Skin samples: Four mm-punch skin biopsies were taken for gene expression analysis from affected and unaffected skin of eight patients with psoriasis.

QRT-PCR: RNA from the HaCaT cell line, PBMCs and skin samples was isolated, and reverse transcription was performed. The obtained cDNA was used for assessing EVER1 and EVER2 expression with quantitative RT-PCR.

Results:

EVER1 and EVER2 genes were both expressed in human PBMCs and spontaneous EVER1 gene expression was greater than EVER2 gene spontaneous expression. There was no significant difference in EVER1 or EVER2 expression in PBMC between healthy volunteers and patients with psoriasis. Stimulation of PBMC with anti-CD3/CD28 beads and PHA downregulated the expression of EVER1 and EVER2 genes. This inhibiting effect was comparable in cells isolated from controls and patients.

We also found that the EVER1 and EVER2 genes were expressed in the skin of patients with psoriasis. We observed that expression of EVER genes was greater in the healthy skin than in lesional skin.

We also determined that HaCaT cells expressed the EVER1 and EVER2 genes and this expression is upregulated by TGF and TNF stimulation, and downregulated by IL-17 stimulation.

Participation in conferences: -

Publications, scientific presentations as a leading author: -

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Karolina Janyst	Year of studies: 2nd

Title of the project: Optimization of ovarian cancer therapy: study of the antitumor effect of histone deacetylase (HDAC) inhibitor scriptaid, proteasome inhibitor bortezomib, and deubiquitinating enzymes (DUBs) inhibitors on human ovarian cancer cells in the in vitro model

Name of the supervisor: Prof. dr hab. Witold Lasek

Affiliation: Medical University of Warsaw, Department of Immunology

AIMS of the project:

The aim of the study is to find new therapeutic options and improve ovarian cancer treatment. For this purpose, several-step investigation will be performed.

1. a) investigation of antitumor effects of histone deacetylase (HDAC) inhibitor - scriptaid combined with proteasome inhibitor - bortezomib and currently used chemotherapeutics in ovarian cancer therapy on ovarian cancer cell lines. Later on, checking if agents belonging to DUBs inhibitors can further improve cytotoxic/cytostatic effects against ovarian cancer cells.

b) checking of alterations in signaling pathways involved in antitumor effects of presented combinations (expression of apoptosis-related proteins: caspase 3, caspase 9, PARP, bid, bim, mcl-1 and the key marker of cell cycle arrest protein p21) and mechanisms responsible for synergistic antitumor effect (expression of USP7 and USP14, MDM2 and p53; to evaluate autophagy activation or ER stress responses followed by inhibition of proteasome-associated DUBs in the cells, measuring protein expression levels of the LC3 isoforms and GRP-78 protein)

2. Confirmation whether the most effective combinations of agents, selected in the previous part of the study, will be effective on primary cancer cell cultures, established from ascitic fluids of patients undergoing primary surgery in Gynecological Hospital for ovarian cancer.

Methods:

In the continuation of research, MTT assay was performed to determine cytotoxic/cytostatic effects of scriptaid and doxorubicin, used either alone or in combination on ovarian cancer cell lines (SKOV-3, OVP-10, MDAH2774) and HEL-299 normal human fibroblast lung cells. Synergism was tested using Compusyn Software. Apoptotic and necrotic cells was determined in Annexin/PI assay using flow cytometry method. Pro-apoptotic (caspase 3, caspase 9, PARP) and p21 proteins were determined using Western blotting technique.

Results:

Conducted experiments show synergistic inhibitory effects of scriptaid and doxorubicin on viability, firstly in MTT test. Flow cytometry analyses confirmed significant increase in cell apoptosis of cells exposed to combined treatment in compared to single agents and control. Mechanistically, treatment with scriptaid and doxorubicin, either alone or in combination, does not seem to change expression of caspase 3, caspase 9 or p21 in 72-hour cultures of SKOV-3 cells.

Scriptaid seems to be promising HDAC inhibitor for further investigation in animal models in the combined treatment of ovarian cancer. This agent may enhance effectiveness of conventional chemotherapy of ovarian cancer. New combination: scriptaid and bortezomib or doxorubicin could be

used as a treatment option of heavily pretreated patients. This combination may be favorable in selected group of patients, for example, as intraperitoneal infusions in women with recurrent disease.

Participation in conferences:

Karolina Janyst, Michał Janyst, Marta Siernicka, Witold Lasek. Synergistic antitumor effect of histone deacetylase inhibitor scriptaid and proteasome inhibitor bortezomib in vitro against ovarian cancer cells, Molecular Biology and Immunology of Cancer – R&D perspectives: ScanBalt Forum 2019, Gdańsk, 24-25 of September 2019 - poster, in review.

Publications, scientific presentations as a leading author:

1. Michał Janyst, Beata Kaleta, Karolina Janyst, Radosław Zagożdżon, Witold Lasek. Comparative study of immunomodulatory agents to induce human T regulatory (Treg) cells: preferential Treg-stimulatory effect of prednisolone and rapamycin, Archivum Immunologiae et Therapiae Experimentalis (AITE) - in review
2. Joanna Litwińczuk-Hajduk, Tomasz Bednarczuk, Gabriela Bluszcz, Magdalena Hołówko, Karolina Janyst, Michał Popow. Zatrucie witaminą D – opis przypadku, Medycyna po Dyplomie – accepted.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Filip Garbicz	Year of studies: 2nd

Title of the project: Pan-PIM inhibition as a rational strategy of targeted therapy in multiple myeloma abrogating its interactions with the microenvironment

Name of the supervisor: Maciej Szydłowski PhD

Affiliation: Institute of Hematology and Transfusion Medicine, Warsaw, Poland

AIMS of the project:

Multiple myeloma (MM) is characterized by a unique network of interactions between the malignant plasma cells and the bone marrow vascular niche. MM cells secrete multiple proangiogenic proteins, such as VEGFA or Galectin-1 (Gal-1), that enhance the recruitement of multiple myeloma-associated endothelial cells (MMECs), promote their proliferation and vessel formation. Recent in vivo studies have indicated that increased bone marrow vasculogenesis is critical for early stages of MM progression. Therefore, defining pathways involved in the interactions between MM and MMECs could lead to development of novel therapeutic strategies against MM. The expression of VEGFA and Gal-1 can be modulated by myeloma-specific constitutively active NFκB, JAK/STAT and mTOR signaling. Since PIM kinases modulate activity of these pathways, we hypothesized that their inhibition would attenuate MM-MMEC cross-talk, and thus affect MM survival also in an indirect manner. For these reasons, we investigated PIM1/2/3 expression in MM and MMECs, evaluated VEGFA and Gal-1 expression, and assessed activity of pathways regulating expression of these proangiogenic proteins in PIM inhibitor-treated MM cells. Furthermore, we studied the effects of PIM blockade on endothelial cell activation and vessel formation. Finally, we explored the role of PIM kinases in mediating the microenvironmental crosstalk between MM and MMECs in a transwell coculture system.

Methods:

The expression of PIM kinases in MM bone marrow samples was assessed using immunohistochemical and immunofluorescent staining. *Ex vivo* toxicity testing on primary plasma cells from MM patients was performed following CD138+ MACS separation. CFSE-stained plasma cells were incubated alone or in the presence of bone marrow microenvironment with increasing PIM inhibitor concentrations. After 24h of incubation the cells were counterstained with AnnexinV-PE and analyzed using FACS. The changes in intracellular cell signaling were detected using Western blot. The impact of PIM inihibition on angiogenesis was detected using *in vitro* Matrigel tube formation assay, using HUVECs as a model of endothelial physiology. Migration was assessed using wound healing assay. In order to simulate the microenvironmental paracrine interactions between MM and endothelial cells, we cocultured both cell populations, separating them with a permeable transwell membrane. In this setting, we measured the proliferation rate of both cell types by manual counting.

Results:

Immunohistochemical analysis of MM bone marrow biopsies (n=7) revealed abundant PIM3 kinase expression in MM cells. Surprisingly, we also detected the presence of PIM3 in multiple myeloma-associated endothelial cells (MMECs), which was confirmed using immunofluorescent staining. For

subsequent experiments we used a recently developed pan-PIM inhibitor, SEL24/MEN1703. The compound was toxic to all MM cell lines with IC50 in the submicromolar range, induced G2 cell cycle arrest and apoptosis. Moreover, MEN1703 was active ex vivo against primary MM cells, even in the presence of bone marrow microenvironment. MEN1703 treatment inhibited the phosphorylation of mTOR substrates S6 and 4EBP1, STAT3/5, as well as RelA/p65. In addition, it also decreased the expression of MYC and IRF4. Consequently, we observed markedly decreased VEGFA and Gal-1 levels in MEN1703 treated cells. Using HUVECs as an in vitro endothelial cell (EC) model, we found that exposure of HUVECs to recombinant VEGFA or coculture with VEGFA-expressing MM cells elevated their proliferation and induced PIM3 levels in endothelial cells (ECs). PIM kinase inhibtion in ECs stimulated with VEGFA or MM-conditioned medium impaired their migration. In addition, PIM inhibition blocked the formation of new vessel-like structures in Matrigel. In order to simulate the microenvironmental paracrine interactions between MM and ECs, we cocultured both cell populations, separating them with a permeable transwell membrane. When cultured together, both MM cells as well as HUVECs exhibited increased proliferation, an effect that was completely blocked by MEN1703 treatment.

Participation in conferences:

- Garbicz F. (2019). PIM inhibition as a rational therapeutic strategy against multiple myeloma. *15th Warsaw International Medical Congress*, Warsaw, Poland. (1. Place, PhD Basic & Preclinical Science Session).

Publications, scientific presentations as a leading author:

- Mehlich, D., Garbicz, F., & Włodarski, P. K. (2018). The emerging roles of the polycistronic miR-106b ~ 25 cluster in cancer – A comprehensive review. Biomedicine & Pharmacotherapy, 107(June), 1183–1195. https://doi.org/10.1016/j.biopha.2018.08.097
- Grzywa, T. M.*, Klicka, K.*, Rak, B., Mehlich, D., Garbicz, F., Zieliński, G., Włodarski, P. K. (2019). Lineage-dependent role of miR-410-3p as oncomiR in gonadotroph and corticotroph pituitary adenomas or tumor suppressor miR in somatotroph adenomas via MAPK, PTEN/AKT, and STAT3 signaling pathways. Endocrine. https://doi.org/10.1007/s12020-019-01960-7.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Sylwia Osuch	Year of studies: 1st

Title of the project: Analysis of relationships between T cell exhaustion, genetic heterogeneity of hepatitis C virus (HCV) epitopes recognized by T cells and anti-HCV treatment

Name of the supervisor: prof. dr hab. n. med. Marek Radkowski, dr n. med. Kamila Caraballo Cortés

Affiliation: Department of Immunopathology of Infectious and Parisitic Diseases , Medical University of Warsaw

AIMS of the project:

Adaptive immune responses play a critical role in the course of infection with hepatitis C virus (HCV). HCV-specific T cell responses in chronic hepatitis C are exhausted which manifests as decline in effector cytokines release, impaired elimination of infected cells, and decreased proliferative potential. These disturbances are mediated by continuous antigen stimulation, progress along with time of infection and are accompanied by expression of "inhibitory" receptors: PD-1 (programmed cell death protein 1) and Tim-3 (T cell immunoglobulin and mucin domain-containing molecule-3) on total and HCV-specific T cells, which have been shown to inhibit T cell activation after antigen recognition, and increase in IL-10 secretion. To date, studies of T cell exhaustion in HCV infection were focused on the markers of this phenomenon, whereas the relationship between T cell exhaustion and heterogeneity of HCV genes encoding immune epitopes, is unraveled. It is also uncertain how anti-HCV treatment modifies T cell exhaustion.

The aims of the present study are:

1) to analyze the relationship between the T cell exhaustion and genetic heterogeneity of HCV epitopes recognized by T cells;

2) to assess the effect of antiviral therapy on T cell exhaustion.

Methods:

The study material included whole blood samples from 105 patients with chronic hepatitis C, who were qualified for antiviral therapy with direct-acting antivirals drugs (IFN-free) in Warsaw Hospital for Infectious Diseases and 18 healthy controls (anti-HCV negative as assessed by ELISA). Exhaustion markers were assessed before treatment and six months after completion of the therapy (evaluation of response to treatment) using:

1) flow cytometry – analysis of expression of inhibitory receptors PD-1 and Tim-3 on total CD4+ , CD8+ and HCV-specific CD8+ T cells from peripheral blood;

2) ELISA, by measuring IL-10 levels in plasma.

HCV genetic heterogeneity (number of viral variants, nucleotide diversity π, genetic distance, number of substitutions, amino acid sequence) of genes encoding immunodominant T cell epitopes are evaluated by next-generation sequencing (NGS), using MiSeq (Illumina).

Results:

Samples, clinical and epidemiological data from 105 patients involved in study were collected. A set of samples (i.e. before and after the therapy) was obtained from 99 patients infected exclusively with genotype 1, of whom 98 patients achieved sustained virologic response (SVR). Additionally, samples

from healthy controls (HC) were collected. For all the involved subjects, analysis of expression of inhibitory receptors PD-1 and Tim-3 on total CD4+, CD8+ T-cells from peripheral blood and IL-10 levels in plasma has been performed.

Before the therapy, the proportions of CD4+ and CD8+ T-cells with expression of exhaustion markers were significantly higher in patients than in HC. When investigating the impact of treatment on exhaustion status of T-cells, a significant decrease in frequencies of Tim-3+ and PD-1+Tim-3+ T-cells was observed in patients after the therapy (both analyzed subpopulations). Normalization of frequency of CD4+ and CD8+ T-cells with expression of exhaustion markers (Tim-3 and PD-1/Tim-3 co-expression) could be observed in patients after the therapy. The levels of IL-10 also significantly decreased in post-treatment follow-up. However, the percentages of PD-1+CD8+ T-cells have significantly increased in patients after treatment.
In conclusion, the results indicate that the exhaustion status of T-cells in HCV-chronically infected patients as measured by Tim-3 and PD-1 expression was elevated when compared to that observed in HC. Successful antiviral therapy reduces the exhaustion, suggesting immune recovery.
Currently, the research is focused on the evaluation of exhaustion status of HCV-specific T cells and HCV genetic heterogeneity.

Participation in conferences: -

Publications, scientific presentations as a leading author:

Kamila Caraballo Cortés, Sylwia Osuch, Karol Perlejewski, Agnieszka Pawełczyk, Justyna Kaźmierczak, Maciej Janiak, Joanna Jabłońska, Khalil Nazzal, Anna Stelmaszczyk-Emmel, Hanna Berak, Iwona Bukowska-Ośko, Marcin Paciorek, Tomasz Laskus. Marek Radkowski
Expression of programmed cell death protein 1 (PD-1) and T cell immunoglobulin and mucin domain-containing molecule-3 (Tim-3) on peripheral blood CD4+CD8+ double positive T cells in patients with chronic hepatitis C virus (HCV) infection and in subjects who spontaneously cleared the virus. Journal of viral hepatitis. 10 April 2019 26(8):942-950. doi: 10.1111/jvh.13108

Maciej Janiak, Karol Perlejewski, Piotr Grabarczyk, Dorota Kubicka-Russel, Osvaldo Zagordi, Hanna Berak, Sylwia Osuch, Agnieszka Pawełczyk, Iwona Bukowska-Ośko, Rafał Płoski, Tomasz Laskus and Kamila Caraballo Cortes
Hepatitis C virus (HCV) genotype 1b displays higher genetic variability of hypervariable region 1 (HVR1) than genotype 3 Submitted to Scientific Reports

Successful chronic hepatitis C (CHC) treatment with direct-acting antiviral drugs (DAA) has impact on expression of T-cell exhaustion markers: PD-1 (*programmed cell death*-1), Tim-3 (T-cell immunoglobulin and mucin domain-containing protein 3) and IL-10 (interleukin 10). In preparation.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Konrad Pagacz	Year of studies: 1st

Title of the project: Detection of ovarian cancer using serum miRNA expression profiles

Name of the supervisor: Wojciech Fendler, MD, PhD

Affiliation: Medical University of Lodz, Department of Biostatistics and Translational Medicine

AIMS of the project:

Ovarian cancer is the seventh-most common cancer in women and the eighth most-common cause of death from cancer worldwide and the fourth most-common cause of death from cancer in Poland. Five-year survival rate is mediocre at 45% in USA and slightly lower at 42% in Poland. The low survival rate origins from diagnostic troubles of detecting the cancer. Patients usually present with unspecific symptoms delaying the correct medical decisions and are diagnosed with the disease in the late stages III and IV, which are burdened by very low survival rates (30-50%) compared to the earlier stages (75% survival rate). The importance of early detection is emphasized in patients who are particularly at risk of developing the cancer. This high-risk group consists mostly of people with mutations known to have a high penetration rate of the cancer phenotype, amongst them the most numerous are people with mutations in BRCA1 and BRCA2 genes. A cost-effective, fast and non-invasive test for detection of ovarian cancer would hugely benefit both low and high-risk groups of patients. Previously published serum miRNA signature of ovarian cancer showed some promise in delivering such a test, as such the aims of my project were as follow:

1. Refine an existing serum miRNA signature of ovarian cancer in people with non-mutated.
2. Create a serum miRNA signature of ovarian cancer in people with a germinal BRCA1/BRCA2 mutation.

Methods:

I gathered datasets of serum miRNA expression data from next generation sequencing of ovarian cancer patients and healthy controls. The acquired raw .fastq files were first quality checked, then kit-specific adapters were trimmed, low quality reads filtered out, all others collapsed. Later, I used a sequential mapping scheme, starting with human miRNAs from miRbase. Remaining unmapped reads were mapped to piR_human and snoRNABase, lastly to the human genome (hg38) and GtRNAdb (tRNAs). miRNA mapping allowed for 1bp modification. Lastly the tpm normalization was performed adjusted for miRNA lengths. I used an already published serum miRNA signature of 14 miRNAs as well as perform my own miRNA selection using three approaches:

1. False Discovery Rate-based: using FDR values calculated from t-tests between cancer and control group.
2. Fold change-based: using FC values calculated between cancer and control group
3. Correlation Feature Selection-based: using a correlation-based metric to distringuish miRNAs, which convey the most information.

Then I used logistic regression and neural network models to create a suitable classifier. They were compared using accuracy as the primary and AUC, sensitivity and specificity as secondary metrics.

Results:

I gathered 6 datasets of serum miRNA expression with 767 patients in total. Two datasets were already published as a result of NECC and ERASMOS studies. One was acquired from a biobank led by Jan Lubinski. One was acquired from Judith Garber and two cohorts (Indian and American) were

recruited and analyzed during the study. Out of all patients, 184 had BRCA1 or BRCA2 mutation. 305 of them had ovarian cancer and were sampled sooner than 2 years prior to the diagnosis.

NECC and ERASMOS patients were used to develop the already published signature of 14 miRNAs (miR-203a, miR-320c, miR-320d, miR-335-5p, miR-450b-5p, miR-1246, miR-1307-5p, miR-23b-3p, miR-29a-3p, miR-32-5p, miR-92a-3p, miR-150-5p, miR-200a-3p, miR-200c-3p), therefore I only validated them on the other datasets. Due to the strong batch effect in Indian and Lubinski datasets making a direct implementation of the model impossible, I used only the American cohort for the validation – excluding all BRCA positive samples. The original signature scored 62.55% accuracy on the American cohort. After refining it, the accuracy increased to 81.54%. The FDR-based model scored 81.82% accuracy (20 miRNAs), CFS-based 85.67% (14 miRNAs) and FC-based 91.18% (22 miRNAs). Using only the 14 already published signature miRNAs it was possible to tweak the model to achieve 55% sensitivity and 99% specificity at the threshold of probability of cancer of 0.6380. This model made only a single false positive prediction. The newly refined classifier based on the 14 already published miRNAs scored very low on the previously excluded BRCA samples (AUC 0.3390). I overcame this by developing a classifier specific for BRCA positive patients. The BRCA specific classifier scored 88,21% accuracy with 82% sensitivity and 89% specificity on BRCA samples.

Participation in conferences:

18.11.2019-21.11.2019 – Gliwice Scientific Meeting

16.05.2019 – 18.05.2019 – XX Conference of Polish Diabetes Association

Publications, scientific presentations as a leading author:

1. A. Michalak*, K. Pagacz*, W. Młynarski, A. Szadkowska, W. FendlerDiscrepancies between methods of continuous glucose monitoring in key metrics of glucose control in children with type 1 diabetes.Pediatric DiabetesApril201910.1111/pedi.12854IF = 3.347MNiSW = 25
2. P. Kucharski*, K. Pagacz*, A. Szadkowska, W. Młynarski, A. Romawski, W. Fendler. Resistance to Data Loss of Glycemic Variability Measurements in Long-Term Continuous Glucose Monitoring. Diabetes Technology & Therapeutics. November 201810.1089/dia.2018.0247. IF = 2,921. MNiSW = 25.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Joanna Knap	Year of studies: 1st

Title of the project: Definition of targetable signaling pathways in chronic lymphocytic leukemia patients with rationale to novel therapy with *EZH2* inhibition

Name of the supervisor: Prof. dr hab. n. med. Krzysztof Giannopoulos

Affiliation: Medical University of Lublin

AIMS of the project:

The main aim of this study is to define, which targetable signaling pathways might be effectively used for future therapy in chronic lymphocytic leukemia (CLL) depending on molecular mutations and gene expressions. The clinical course is highly heterogenic, that reflexes CLL's marked molecular diversity. Since CLL remains incurable it is desirable that development of new personalized therapies should be based on the existence of individual mutations or differential gene expression. The rapid progress in the next generation sequencing (NGS) technology has significantly contributed to define molecular aberrations associated with the disease progression and response to treatment in CLL patients. NGS is essential to determine a mutations profile for each CLL patient and to propose genes which could be utilized as targets in personalized therapies. Despite the recent progress in developing therapeutic options in CLL and improving patient survival, benefits are still insufficient - CLL remains largely incurable and its course in particular patient is difficult to predict. Moreover currently available treatment in CLL are not enough specific. The individual mutations profile and determination genes expression are essential to improve effectiveness of treatment. A one promising target is *Enhancer of zeste homolog 2* (EZH2), which has a critical role in multiple biological processes via epigenetic regulation of gene transcription. In addition, high levels of *EZH2* expression were significantly correlated with CLL poor prognosis. Taking into consideration all reports concerning EZH2 signaling pathway, we suspect that pharmacological inhibition of EZH2 may represent a potential novel therapeutic approach for a subgroup of CLL patients. Moreover this enticing idea is readily achievable, since EZH2 pharmacological first-in-class inhibitor is currently validated in clinical trials for other types of B-cell malignancies.

The main aim will be achieved through the implementation of these specific aims:

1. specific aim: to determine the mutation and clinical profile for approximately 50 CLL patients;

2. specific aim: to determine the expression of *EZH2* gene;

3. specific aim: to specify the sensitivity of CLL cells to EZH2 inhibitor;

4. specific aim: analysis the biological consequences of EZH2 pathway inhibition.

Methods:

Ethical approval was granted by local review committees of Medical University od Lublin. The study includes 50 newly diagnosed and previously untreated patients with CLL under informed consent. Clinical and biological data about the patient cohort was collected. Peripheral blood mononuclear cells were isolated from peripheral blood samples using Biocoll density gradient centrifugation and cryopreserved at minus 150°C to the time of analysis. For DNA preparation, QIAamp DNA Blood Mini Kit was used according to the manufacturer's instructions. DNA was quantified by BioSpec-nano as well as Qubit 2.0. Targeted enrichment strategy using custom designed capture probes followed by NGS on Illumina platform for 15 DNA samples from CLL patients was employed. The custom panel enabled us to analyze 193 selected oncogenes and tumor suppressors. First, Genom

Libraries were prepared following manufacturer's optimized PCR-based protocol. Resulting libraries were normalized, pooled and then loaded into a flow cell for sequencing. All steps were performed according to the manufacturer's protocol. Sequencing Data Results will be analyzed in collaboration and under supervision of Dr Tomasz Stokłosa (Immunology Department, Medical University of Warsaw).

Results:

1. Collection of blood samples from 50 patients with CLL, PBMCs and DNA isolation.

2. Collection of clinical data of patients for statistical and bioinformatical analyses.

3. Optimization targeted NGS-based diagnostic protocol and implementation of above protocol into practice.

4. Collection of Sequencing data (*EZH2, TP53, MYD88, NOTCH1, XPO1* and next 188 genes implicated in human cancer were included) from 15 CLL patients and preparation this date to detailed analysis.

5. Future plans: We are planning to analyze NGS data and continue NGS with targeted enrichment approach in next 35 samples from CLL patients. Functional study of consequences overexpression and silencing *EZH2* will be performed as part of my NCN PRELUDIUM 16 grant obtained in this year.

Participation in conferences:

1. *24th EHA Congress*, 13-16 June 2019, Amsterdam, The Netherlands
2. *Jak leczę w 2019r*., 28-30 March, Wroclaw, Poland
3. *9. Current & Future Perspectives of MM & Other Hematological Malignancies Treatment*, 08 -09 March 201, Warsaw, Poland
4. *VIII Hematology Experts Forum*, 22-23 February 2019, Warsaw, Poland
5. *Amyloidoza – II Interdyscyplinarna Konferencja*, 19-20 November 2019, Warsaw, Poland.

Publications, scientific presentations as a leading author:

Publications

1. The prognostic value of mean platelet volume in cancer patients. Masternak M, Knap J, Giannopoulos K. Acta Hematologica Polonica – in preparation.
2. Deregulation of the immune system in patients with chronic lymphocytic leukemia. Skórka K, Kot M, Knap J, Giannopoulos K. Post. Hig. Med. Dośw 2019 t. 73 s. 117-132.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Michał Soin	Year of studies: 1st

Title of the project: Investigation of the origin and evolution of mutation signatures in hematopoietic neoplasms.

Name of the supervisor: prof. dr hab. med. Wiesław Wiktor Jędrzejczak/dr.n med. Marta Libura

Affiliation: Katedra i Klinika Chorób Wewnętrznych, Hematologii I Onkologii, Medical University of Warsaw

AIMS of the project:

Developments in sequencing techniques in recent years allowed to discover and describe mutational patterns in neoplasms. Those patterns termed mutational signatures, arise from frequencies of specific nucleotide substitutions in relations to their immediate surrounding. Furthermore study of those emerging patterns allowed to ascribe some of them to distinct mechanisms of mutagenesis (i.e. signatures with high C>G and C>T associated with faulty APOBEC or C>A with aflatoxin). This study aims to explore mutational patterns of hematopoetic neoplasms mainly acute leukemias. Using different next generation sequencing approaches from targeted panels, through whole exome to whole genome sequencing relationships between mutations in acute leukemias will be studied. Firstly, targeted NGS panels will be performed on broad spectrum of patients to determine mutation incidence in known leukemia associated genes, as well as frequency of cooccurrence between them. Secondly exerts from patient cohorts for whom material from diagnosis post treatment an relapse points will be available, as well as somatic control will be studied using WES/WGS to determine primary mutational signatures, and their shift during treatment and relapse. Collected data will be confronted with clinical outcome.

Methods:

Isolation of mononuclear cells (MNC) from peripheral blood and bone marrow followed by
isolation of RNA using Chomczyński and Sachi protocol,
isolation of RNA using MagnaPure (Roche) system,
isolation of DNA using MagnaPure (Roche) system,
isolation of DNA using spin-columns (Qiagene).

Initial genetic characterization of acute leukemias and MPNs using following procedures:
RT-PCR and nested RT-PCR for fusion genes (BCR-ABL1, RUNX1-RUNX1T1, CBFB-MYH11, PML-RARA, KMT2A -AFDN, KMT2A -AFF1, STIL-TAL1, NUP214-ABL1, PICALM-MLLT10, ETV6-PDGFRB, FIP1L1-PDGFRA)
genomic PCR followed by Sanger sequencing (CEBPA, IDH1, IDH2, KIT, JAK2),
genomic PCR followed by GeneScan detection (FLT3-ITD, NPM1)
ARMS-PCR, Double ARMS-PCR followed by GeneScan detection (JAK2 V617F, MPL W515X)
Real Time-PCR for RNA quality control (ABL1).

Results:

During the 1st year of the PhD project, patients diagnostic material was collected within 2 biobanks: (1) local biobank with material from patients with myeloid malignancies and acute leukemias diagnosed and treated in the Hematology and Oncology department UCK WUM in Warsaw, as well as (2) genetic and cellular material from Acute myeloid leukemia (AML) patients diagnosed in 20 Hematological Units from all over Poland treated within Polish Adult Leukemia Group (PALG) prospective clinical trial PALG-AML1/2016.

The material collected within local biobank consisted of bone marrow aspirates and if not available, also peripheral blood, and was treated differentially within the pre-analytical phase, depending on the patients' diagnosis: in case of MDS dry cell pellets after erythrocytes lysis were archived, while in case of fully transformed acute leukemias, MNCs were isolated using density gradient method and then nucleic acids (both DNA and RNA) were extracted and stored in -20 and -80 C. Excess of MNCs was frozen in the form of dry cell pellets or cell suspensions embedded in DMSO. For all patients with myeloproliferative disorders erythrocytes lysis was performed followed by DNA and RNA isolation.

Within local biobank following diagnostic samples were archived from patients with myeloid malignances: 75 MDS, 25 AML, 2 APL, 21 CML, 51 MPN, 43 cases with suspected MPN; 6 from lymphoblastic acute leukemias.
For all diagnostic samples initial genetic work up was performed according to European LeukemiaNet 2017 (ELN 2017) recommendations and WHO 2016 criteria, including detection of standard panel of fusion genes and mutations by PCR-based approaches. Throughout this 1st year, following genetic profile was established for diagnostic samples archived within local biobank:
Out of 25 AML patients: FLT3-ITD was detected in 4 pts (16%), FLT3 TKD – 1 pts (4%), NPM1 mutation – 7 pts (28%), CEBPA mutation– 1 pts (4%), CBFB-MYH11– 2 pts (8%);
2 APL with PML-RARA fusion genes;
18 CML patients with BCR-ABL1 fusion, other MPNS with ETV6-PDGFRB fusion in 1 case, JAK2 - V617F mutation- 48, CARL mutation- 2.
6 ALLs, with 1 case accompanied by NUP2014-ABL1 fusion and 1 case with BCR-ABL1 fusion gene.
Mutational profile of remaining samples (4 ALLs, 10 AMLs, as well as 43 cases with suspected MPN) could not be determined using standard diagnostic work up.

PALG biobank consisted of AML samples from patients diagnosed in 20 polish hematologic centers and treated within PALG clinical trial within the years 2017-2019. Within this national initiative samples were collected during different time points: at the moment of diagnosis and follow up. Biobank consisted of DNA, RNA and dry cell pellet samples. Parallel to diagnostic leukemic sample, non-leukemic tissue was also collected with perspective of NGS whole genome comparative studies. Currently PALG biobank consists of 150 diagnostic AML samples, which are in most cases accompanied by follow up samples as well.
In future continuation of studies are programed according to aims presented above.

Participation in conferences:

1. IX Zjazd Polskiego Towarzystwa Genetyki Człowieka, Bydgoszcz 2018
2. Wyzwania Hematologii MDS/MPN, Kraków 2018
3. II Interdyscyplinarna Konferencja Amyloidoza Warszawa 2018
4. X Spotkanie Laboratoriów Hematologii Molekularnej, Kraków 2018
5. XI Spotkanie Laboratoriów Hematologii Molekularnej, Kraków 2019

Publications, scientific presentations as a leading author: N/A

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Anna Deręgowska	Year of studies:4th

Title of the project: The role of telomeric complex in genomic instability leading to resistance to targeted therapy and disease progression in chronic myeloid leukemia

Name of the supervisors: dr hab. Maciej Wnuk, prof. UR; dr n.med. Tomasz Stokłosa

Affiliation: Department of Genetics, Faculty of Biotechnology, University of Rzeszow

AIMS of the project:

Introduction of tyrosine kinase inhibitors (TKIs) to the therapy of chronic myeloid leukemia (CML) changed outcome for the majority of patients, but still significant number of patients develop resistance and in consequence may progress to advanced phase with limited therapeutic options. As in most cancers, reduction in telomere length is one of the features of CML cells and may result in increased genomic instability associated with natural progression of the disease from chronic phase (CML-CP) to the blastic phase (CML-BP). However, the precise role of telomere-associated proteins, including shelterin complex in BCR/ABL1-mediated genomic instability in CML progression and resistance to TKI have not been fully elucidated. The aim of the study is to determine the role of the members of the shelterin complex in aberrant telomere maintenance mechanisms in the pathogenesis of CML and identify new mechanisms of disease progression and drug resistance in leukemia cells.

Methods:

We employed CML CD34+ primary cells isolated from peripheral blood leukocytes or bone marrow of CML patients (following written consent from each patient and after applicable ethical committee approval) at different stages of disease: CML-CP, CML-BP and CML-TKIres (samples from patients with clinical resistance to TKI), as well as imatinib resistant-human CML cell lines (K-562 and MEG-A2). To generate imatinib-resistant cells, K-562 and MEG-A2 cells were incubated in stepwise increased imatinib concentration (50-1000 nM) and imatinib-supplemented culture medium was refreshed every 2-3 days. The length of telomeres was determined by PNA-FISH technique and Southern Blot. Enzymatic activity of telomerase was measured immunoenzymatically, while expression of subunits of telomerase, shelterin complexes and telomeric repeat containing RNA (*TERRA*) was examined by RT-qPCR and/or by Western Blot. Role of alternative lengthening of telomeres (ALT) in BCR/ABL1 positive cell lines was measured using immunofluorescence. Metabolic phenotype was analyzed using a Seahorse Analyzer.

Results:

The telomere shortening was observed during CML progression as determined by Southern blotting and PNA FISH technique, also in available paired samples from the same patients in different stages of the disease. In addition, the negative correlation between mean telomere length and expression of *BCR-ABL1* in CML samples was observed. Dynamic changes in telomere length were neither associated with expression of *TERT/TERC* nor with enzymatic activity of telomerase (TA) in the course of the disease. We found that the expression of *RAP1*, *DKC1* and *Tankyrase1* was increased in CML-BP as compared to CML-CP. However, in samples from CD34+ CML-TKIres patients in comparison to CML-CP patients, an increase in telomere length was observed. The link between telomere-associated proteins and telomere maintenance mechanism has been verified in the model of imatinib resistant-cell lines. We showed that telomeres in the resistant cells were shorter which was associated with *TERRA* overexpression. We also found that the changes in telomere length in TKIres cells were accompanied by the metabolic phenotype changes. In conclusion, we postulate that the

dynamics the telomere length s in CML cells is related to the level of expression of *BCR-ABL1* and cell metabolism, which stimulates increased expression of *TERRA* and selected shelterin genes, such as *RAP1*, *Tankyrase1* and *DKC1*. This may play important role in CML progression, clonal selection and resistance to targeted therapy with TKIs.

Participation in conferences: -

Publications, scientific presentations as a leading author:

c-Myc activation promotes cofilin-mediated F-actin cytoskeleton remodeling and telomere homeostasis as a response to oxidant-based DNA damage in medulloblastoma cells; Lewinska A., Klukowska-Rötzler J., Deregowska A., Adamczyk-Grochala J., Wnuk M.; Redox Biol. 2019 Jun;24:101163

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Kacper Guglas	Year of studies: 1st

Title of the project: Biological role of YRNAs in head and neck squamous cell carcinomas

Name of the supervisor: dr hab. n. med. Katarzyna M Lamperska

Affiliation: Wielkopolskie Centrum Onkologii

AIMS of the project:

YRNAs are components of Ro60 ribonucleoprotein particle which is a target of antibodies in patients with rheumatic autoimmune diseases and consist of 80-110 nt. YRNAs are involved in DNA replication and Ro60 activation. They were found in serum and plasma of healthy and nonhealthy patients. Deregulation of YRNAs was confirmed in many diseases, also in cancers. However, in the case of head and neck squamous cell carcinoma (HNSCC) there are no studies considering their influence on the development of the HNSCC. It is believed that YRNAs promote cell proliferation and metastasis, as it was found in other cancer types. The main aim is to determine the biological role of YRNAs in the carcinogenesis of head and neck squamous cell carcinomas. Next, the expression of YRNAs in patients as well as in cell lines will be examined. Finally, their usefulness as potential HNSCC biomarkers will be evaluated.

Methods:

Four head and neck squamous cell carcinoma cell lines were cultured according to the instructions from the DSMZ and ATCC: SCC-040 (oral cancer model), SCC-025 (tongue cancer model), FaDu (hypopharyngeal cancer model) and Cal27 (tongue cancer model). As a reference DOK cell line (model of dysplastic mucosa) was cultured according to instructions described by Sigma-Aldrich. Patients samples were obtained from Greater Poland Cancer Centre from patients surgically treated in 2010 and 2011. The study material included 20 matched cancer tissues and normal epithelium tissue taken at minimum of 2 cm distance from the tumours margins and were included in discovery cohort. For validation cohort 70 FFPETs of HNSCC patients were collected. Total RNA from the cell lines was isolated using a High Pure miRNA isolation kit, according to the isolation protocol for total RNA from cell line samples. For discovery cohort, HNSCC tissues were frozen in -80°C immediately after surgery and total RNA was isolated using TRI reagent. Next, samples were concentrated and purified using the High Pure miRNA Isolation Kit according to the protocol for isolation of total RNA. For validation cohort, FFPETs with HNSCC were isolated using the High Pure FFPET Isolation Kit according to the protocol for isolation of total RNA. RNA was quantified using NanoDrop 2000 spectrophotometer. Complementary DNA was synthesized using iScript cDNA Synthesis Kit. Quantitative PCR was performed using 2x concentrated SYBR Green Master Mix with specific primers to detect YRNA1, YRNA3, YRNA4 and YRNA5. The real-time PCR reactions were performed on a LightCycler 96 and melting curve was performed to discriminate non-specific products of PCR reaction. All real-time PCR data was analyzed by calculating the $2\text{-}\Delta C_T$, normalizing against the mean of HPRT1 and B2M expression, as the refference genes. All statistical analyses were performed using Graphpad Prism 5: Shapiro-Wilk normality test, T-test, or Mann–Whitney U test, One-way ANOVA; for Disease-Free Survival and Overall Survival analyses Log-rank (Mantel-Cox) Test, Spearman Rank Correlation TesT and Gehan-Breslow-Wilcoxon Test were used; in all analyses p-val<0.05 was considered as significant. The Cancer Genome Atlas (TCGA) expression data for YRNA1 was downloaded from cBioPortal (Head and Neck Squamous Cell Carcinoma, TCGA, Provisional, 530 samples). Clinical data of patients was obtained from the UALCAN database and StarBase v3.0. All data is available online, and access is unrestricted. Patients consent or other permissions are not required. The use of the data does not violate the rights of any person or any institution.

Results:

It was found that YRNA1 and YRNA5 are significantly downregulated in head and neck squamous cell carcinoma cell lines compared to reference DOK cell line (p=0.0008 and p=0.0470 respectively). Furthermore YRNA1 was found to be significantly downregulated in paired patients' tumour samples and matched-adjacent healthy tissues (1247±440.9 vs 322.8±130.5; p=0.0109), YRNA3, YRNA4 and YRNA5 do not show significant differences. Next, the expression of YRNAs was examined considering tumour localization, however the correlation of YRNAs' expression and tumour localization was not established. It was also noted that YRNA1 (p=0.0211), YRNA3 (p=0.0339), YRNA4 (p=0.0357) and YRNA5 (p=0.0071) was significantly upregulated in T4 stage of head and neck squamous cell carcinoma. Next, Receiver Operating Charactericstic Curve (ROC Curve) analysis was performed. It was noted that YRNA1 showes highest sensitivity percentage (AUC=0.7975±0.07486; p=0.001295) of all examined YRNAs, suggesting its role as a potential disease biomarker. Next, the analysis based on YRNA1 expression data obtained from The Cancer Genome Atlas (TCGA) were performed. First, the clinical-pathological properties were examined. Only in the case of HPV p16 status (p=0.0002) significant difference is observed. Next, the samples were divided into three groups: oral cavity (n=76), pharynx (n=19) and larynx (n=21). Expression analysis of RNY1 showed no significant differences. Furthermore, Disease-Free Survival (DFS) and Overall Survival (OS) of patients with examined YRNA1 expression was evaluated. The low expression group for DFS analysis was defined as expression levels below -2.526 (n=21) and the medium+high expression group included all samples above -2.526 (n=62). It was noted that patients with RNY1 low expression profile have longer median DFS periods compared to medium+high levels expression group 23.59 vs 61.07 months respectively (p=0.0130; HR=2.924; 95% CI: 1.254-6.818). Furthermore, the low expression group for OS analysis was defined as expression levels below -2.598 (n=29) and the medium+high expression group included all samples above -2.598 (n=87). The OS analysis showed that patients with RNY1 low expression survive shorter median periods compared to medium+high expression group 15.08 vs 48.16 months respectively (p=0.0083; HR=2.195; 95% CI: 1.225-3.934). Furthermore, predicted target *in silico* analysis was performed. The RNY1 expression values derived from TCGA data was divided into low and high expression groups. It was noted that RNY1 expression is associated with many other genes, considered as RNY1 predicted targets. These genes are associated with many crucial processes that occur in cancer cells, such as: tumour metastasis, cancers stem cells development, apoptosis, epithelial to mesenchymal transition and cell cycle. Evaluation of those targets is in progres, as well as the possible sponge effect between YRNA1 and miRNA. So far, few possible miRNAs were found to be correlated with YRNA1 as sponges: let-7a-5p (p=0.0003; R = -0.3449), miR-3934-5p (p=0.0299; R = -0.2131) and miR-24-3p (p=0.0484; R = -0.1931). These *in silico* studies will be evaluated in the nearest future.

Participation in conferences:

11th International Conference of Contemporary Oncology

Publications, scientific presentations as a leading author:

Guglas K, et al. lncRNA Expression after Irradiation and Chemoexposure of HNSCC Cell Lines. Noncoding RNA. 2018 Nov 14;4(4).
Kolenda T, Kopczyńska M, Guglas K, et al. EGOT lncRNA in head and neck squamous cell carcinoma. Pol J Pathol. 2018;69(4):356-365.
Kolenda T, Guglas K, et al. Low let-7d and high miR-205 expression levels positively influence HNSCC patient outcome. J Biomed Sci. 2019 Feb 13;26(1):17.
Kolenda T, Guglas K, et al. Oncogenic role of ZFAS1 lncRNA in Head and Neck Squamous Cell Carcinomas. Cells. 2019 Apr 21;8(4).

Kolenda T, Rutkowski P, Michalak M, Kozak K, Guglas K, et al. Plasma lncRNA expression profile as a prognostic tool in *BRAF*-mutant metastatic melanoma patients treated with BRAF inhibitor. Oncotarget. 2019 Jun 11;10(39):3879-3893.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Jakub Stawicki	Year of studies: 2nd

Title of the project: Analysis of protein distribution in primary brain tumors evaluated in histopathological preparations.

Name of the supervisor: Prof. dr hab. n. med. Maciej Wiznerowicz

Affiliation: Poznan University of Medical Science, Medical Biotechnology

AIMS of the project:

Until now, the processes of formation of primary brain tumors are unknown. Long-term studies of these cancers help us to understand the processes of mutation and the transformation of cells into a more malignant form. Microscopic assessment and the type of proteins created during the mutation will allow us to bring closer the processes that affect the transformation of this group of cancers from the low-differential (LGG) to the highly diverse form (HGG) and in the next stage to assess the formation of subsequent mutations and recurrences of primary tumors.

The main goal of the PhD thesis is to compare stained proteins in histopathological preparations. The primary and secondary malignancies of the brain as well as the recurrence of these tumors will be used for the analyses.

Methods:

1. GBG proteogenomic analysis of patients qualified for the CPTAC project.
    a. Tumors collected for histopathological examination during surgery are included in the CPTAC project. A proteogenomic evaluation of these tumors is made.
    b. Sequencing of proteins, DNA and RNA using high throughput technologies;
    c. The proteins that occur most often will be selected.
    d. Cancer preparations will be stained and evaluation of these preparations will be made.
    e. Biological material obtained from other clinical centers included in the CPTAC project will be used for further research, cooperation with the center: Central Clinical Hospital Banacha, Provincial Specialist Hospital No. 5 them. St. Barbara Sosnowiec.
    f. Data analysis and interpretation within the CPTAC GBM Disease Working Group; monthly meetings
2. Archive material
    a. In the group of approximately 150 patients operated between 2006 and 2018 with initial diagnosis of LGG and re-operated due to recurrence, transformation of the cancer to GBM.
    b. The location of blocks of histopathological preparations in the archive
    c. Dyeing and molecular evaluation of histopathological preparations under the microscopic changes taking place during the transformation of the tumor from LGG to GBM.

Results:

Proteogenomic analyzes of GBM patients qualified for the CPTAC project.

1. Collection of biological material (brain tumor) from 47 patients.
2. Blood collection from this group of patients.
3. Surveys were carried out and consents were obtained from qualified patients.

4. 7 patients were qualified for the CPTAC project.
5. Introduction of detailed data on qualified patients to the CPTAC system.
6. Conducting an annual observation of patients participating in the program follow up.
7. Searching for histopathological results obtained from patients operated on in the years 2006-2018.
8. Tumor recurrence collection from patients qualified in the project and young patient (age up to 30)

Archive Material

1. Creating a group of patients
2. Identification of histopathological preparations in the Department of Pathomorphology.
3. Preparation of a list of patients with primary brain tumors based on histopathological results

Participation in conferences:

1. Scientific session of the Polish Society of Spine Surgery
2. Autumn School SMM
3. IIMO Workshop: Analyses of TCGA Genomic and Epigenomic Data.

Publications, scientific presentations as a leading author: -

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Bartosz Czapski	Year of studies: 4th

Title of the project: Role of mutations in IDH1/2 in epigenetic deregulation of TGF-B signaling pathway in human glioblastoma

Name of the supervisor: prof. Bożena Kamińska

Affiliation: Nencki Institute of Experimental Biology

AIMS of the project:

Given that the IDH1/2 mutation alone is able to significantly alter the phenotype of methylation in gliomas (tumors display a hypermethylator phenotype, G-CIMP), we hypothesized that it may be a mechanism affecting gene expression and functions of the TGF-B signaling pathway in these tumors. We sought to explore if IDH1/2 mutations affect the methylation status of the TGF-B signaling pathway genes and how it is correlated with gene expression. Confirmation of such correlation would allow a more accurate understanding of the molecular mechanisms leading to the development of glioma. In addition, it would allow to establish a risk group that is particularly vulnerable to glioma development among low grade tumors.

Methods:

RNA and DNA was isolated from frozen glioma samples. The fragments carrying mutated *IDH1* or *IDH2* gene was amplified by PCR and subjwcted to Sanger sequencing. RNA sequencing was performed on selected glioma samples. Correlations of selected gene expression and *IDH* mutation status, histopathology and Kapplan-Meier survival curves were analyzed. Genes of TGF-B signaling pathway were selected from known databases based their expression dependence on *IDH* mutations and their biological significance (participation in regulation of cell growth and differentiation, EMT and invasion). The expression of selected genes was determined by quantitative PCR in total RNA from previously harvested glioma tissue samples. Routinely 1 µg was used to synthesize cDNA. Real-time PCR amplifications were performed in technical duplicate on cDNA equivalent to 25 ng RNA in 10-µl reaction volume. The expression of each product was be normalized to GAPDH, which will be used as an internal reference gene. Methylation profile at promoters of selected genes was determined by single-strand DNA methylation analysis. Correlation of methylation status with gene expression profiles, tumor grade and the presence of *IDH1/IDH2* mutation was made.

Results:

The computational analysis of the expression of 54 TGFβ signaling related genes and immune/inflammatory genes in RNA-seq data from 28 mut*IDH1* and 56 wild type gliomas was performed to search for correlation of *IDH1/2* mutational status. The analysis of expression of *TGFB1, TGFBR1, BMP7, AVCR1, SMAD3, SMAD6, SMAD7, SMAD9, SNAI2, ZEB1* and *S100A4* genes showed correlation between the presence of *IDH1/2* mutations and grade of the tumor. Further analyses on collected samples were done by qPCR to validate results. Moreover, in-house collected data on DNA methylation in gliomas of low and high grades were used to examine whether the methylations occur in CpG sites of those genes and what is their biological meaning (correlation with gene expression in RNAseq data). Our data support our notion that the *IDH1* mutation has a direct impact on the TGF-B signaling via gene promoter silencing through their excessive methylation. It affect the expression immune/inflammatory genes and shape immune microenvironment of GBM. *AVCR1* and *BMP7* genes tend to be the most affected by IDH-related hypermethylation.

Participation in conferences:

European Training Course in Neurosurgery – Tumour, 5-8 may 2019, Mosow, Russia
World Federation of Neurosurgery 2019 International Meeting, 21-24 march 2019, Belgrade, Serbia.

Publications, scientific presentations as a leading author:

Consequences of IDH1/2 Mutations in Gliomas and an Assessment of Inhibitors Targeting Mutated IDH Proteins. Kaminska B, Czapski B, Guzik R, Król SK, Gielniewski B.Molecules. 2019 Mar 9;24(5).

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Agnieszka Anna Koppolu	Year of studies: 4th

Title of the project: Molecular mosaicism in endometrial tissue

Name of the supervisor: Prof. dr hab. n. med. Rafał Płoski

Affiliation: Department of Medical Genetics, Medical University of Warsaw

AIMS of the project:

The aim of the study is to investigate ectopic and eutopic glandular epithelium (GE) of endometrial tissue with the use of laser-capture microdissection (LCM) in order to identify somatic mutations through NGS panel sequencing. Detected somatic variants may indicate the direction towards better understanding the genetic factors driving/involved in the onset of endometriosis.

Methods:

Eutopic endometrial tissue, ectopic lesions and blood samples were collected from 13 women (mean age 33.3 years, SD = 5.3 year, range 25-42 years) with deep infiltrating endometriosis of the recto-vaginal space confirmed by laparoscopic and histopathological evaluation, diagnosed and hospitalized at the Gynecology Departments of St. Sophia Hospital, Warsaw, Poland. All cases in this study were classified during surgery as stage III/IV of endometriosis according to the revised criteria of the American Society Reproductive Medicine (rASRM)[2] and B1/B2 in the revised Enzian classification. The study was performed on specimens taken solely from deep lesions of uretrosacral ligaments (Deep Infiltrating Endometriosis). The surgery was performed during continuous progestin therapy or on day 5–10 of the menstrual cycle. None of the patients suffered from any other inherited or chronic disorder, hematologic malignancy or cancer. All but two patients were never pregnant before the surgery. All tissue samples were rinsed in saline, immediately immersed in O.C.T. Medium (Tissue-Tek), and snap frozen. Peripheral blood was collected to EDTA vials (BD Vacutainer) and immediately frozen. Tissue and blood samples were stored at -80°C until further processing.

Paired tissue specimens of each patient (eutopic endometrial biopsies and ectopic lesions) were cut with a cryostat (CM 1860, Leica, USA) on 8µm sections, which were mounted on glass slides (SuperFrost®, Menzel, Germany) and underwent standard H&E staining, followed by an initial assessment of the presence of endometrial glands in both samples. LCM (Zeiss PALM MicroBeam, Germany) was performed in order to obtain single endometrial glands from each sample. In order to obtain a sufficient amount of the DNA for NGS sequencing three technical repeats of whole-genome amplification (WGA) were performed for each sample. NGS panel libraries were prepared on endometrial DNA as well as peripheral blood DNA using custom panel probes (Roche, 1296 genes related to cancer) and pair-end sequenced on Illumina HiSeq 1500 2x100bp to the mean coverage 150x. NGS raw data was processed according to the pipeline established in the Department of Medical Genetics, WUM. The processing involved removal of poor quality reads, alignment to the reference genome (Hg19) and variant calling. The results were grouped and consolidated for each patient.

Somatic variants were detected based on comparison of the sequencing results of the glands against the blood. All detected variants were manually verified in Integrative Genomics Viewer (IGV). Non-silent variants were analyzed against consolidated lists of endometrial and ovarian cancer driver genes (IntOGen). For both tissues, the chance of mutation in driver and non-driver genes were calculated. In order to verify whether the chance of a driver gene mutation is significantly higher in ectopic than eutopic tissue, Chi-square test was performed.

Selected variants were verified in all available samples and technical repeats by Amplicon Deep Sequencing (ADS).

Results:

NGS panel sequencing revealed somatic variants in both ectopic and eutopic GE in all except one patient. Altogether, 82 non-silent variants were detected in all the samples, including 34 variants in ectopic GE, 42 in eutopic GE and 4 in both GE. All detected somatic variants were of low mutated allele frequency (<10%). One ectopic GE sample carried a *TP53* variant and another sample harbored one *KRAS* variant (previously mentioned by other authors). ADS verification confirmed the presence of *TP53* variant in both available WGA technical repeats and the *KRAS* variant in one of two available technical repeats. No recurrent variants were observed.

Out of 1296 genes and gene hot spots represented in our panel, 85 were classified as driver genes and the remaining 1211 genes were classified as non-driver genes. Overall, in the ectopic tissue, out of 24 detected variants, 5 were located in driver genes while the remaining 19 were in non-driver genes. Therefore, the chance of driver gene and non-driver gene mutation in the ectopic tissue was 0,059 (5/85) and 0,016 (19/1211) respectively. In the eutopic tissue out of 31 detected mutations 2 were located in driver genes and 29 in non-driver genes. Therefore, the chance of mutation in both types of genes was equal (2/85≅29/85≅0,024). The predominance of mutations in driver genes in ectopic GE is statistically significant (Chi-square test; p=0,015).

Participation in conferences: ----------

Publications, scientific presentations as a leading author:

- *FARSA mutations mimic phenylalanyl-tRNA synthetase deficiency caused by FARSB defects.* Krenke K et. al. Clin Genet. 2019 Jul 29. doi: 10.1111/cge.13614. [Epub ahead of print]
- *FGF12p.Gly112Ser variant as a cause of phenytoin/phenobarbital responsive epilepsy.* Paprocka J et. al. Clin Genet. 2019 Sep;96(3):274-275. doi: 10.1111/cge.13592. Epub 2019 Jul 10.
- *A Novel Monoallelic Nonsense Mutation in the NFKB2 Gene Does Not Cause a Clinical Manifestation.* Kotlinowski J et al. Front Genet. 2019 Feb 26;10:140. doi: 10.3389/fgene.2019.00140. eCollection 2019.
- *Clinico-pathological correlation in case of BRAT1 mutation.* Szymańska K et al. Folia Neuropathol. 2018;56(4):362-371. doi: 10.5114/fn.2018.80870.
- *A case of severe trichothiodystrophy 3 in a neonate due to mutation in the GTF2H5 gene: Clinical report.* Michalska E et al. Eur J Med Genet. 2018 Oct 22:103557. doi: 10.1016/j.ejmg.2018.10.009. [Epub ahead of print]
- *Mapping of breakpoints in balanced chromosomal translocations by shallow whole-genome sequencing points to EFNA5, BAHD1 and PPP2R5E as novel candidates for genes causing human Mendelian disorders.* Murcia Pienkowski V et al. J Med Genet. 2019 Feb;56(2):104-112. doi: 10.1136/jmedgenet-2018-105527. Epub 2018 Oct 23.
- *Novel calcineurin A (PPP3CA) variant associated with epilepsy, constitutive enzyme activation and downregulation of protein expression.* Rydzanicz M et al. Eur J Hum Genet. 2019 Jan;27(1):61-69. doi: 10.1038/s41431-018-0254-8. Epub 2018 Sep 25.
- *Evidence for HNRNPH1 being another gene for Bain type syndromic mental retardation.* Pilch J et al. Clin Genet. 2018 Oct;94(3-4):381-385. doi: 10.1111/cge.13410. Epub 2018 Aug 2.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Marcin Leja	Year of studies: 2nd

Title of the project: Integrating cochlea-specific multi-gene panel and whole genome sequencing to uncover molecular basis of autosomal dominant hearing loss

Name of the supervisor: Dr hab. n. med. Monika Ołdak

Affiliation: Postgraduate School of Molecular Medicine, Medical University of Warsaw

AIMS of the project:

Hearing Loss (HL) is the most common sensory disorder affecting more than 5% of the world's population, with approximately half of the cases attributable to a genetic cause. Autosomal Dominant Hearing Loss (ADHL) is the second most common form of inherited HL with an onset usually after the second decade of life. It affects mainly high frequencies and progresses over time. To date, more than 170 loci related to human HL have been mapped and 118 genes identified, including 45 genes involved in ADHL development. Due to high genetic heterogeneity, providing a molecular diagnosis for HL patients is challenging. Current knowledge on the genetic aspects of ADHL in Polish patients is limited, which significantly affects the diagnosis, genetic counselling and prevents prediction of disease progression. With the aim to develop advanced diagnostic tools to investigate the genetic basis of hearing impairment, we designed a targeted next-generation sequencing (NGS) panel including 237 genes involved in non-syndromic and syndromic HL.

Methods:

In 2019, NGS was performed for 15 families with a vertical interitance pattern of HL Enrolment of patients and ascertainment of their family history, collection of peripheral blood and testing of two major HL genes (GJB2, GJB6) genes was performed at the Department of Genetics,Institute of Physiology and Pathology of Hearing. . All recruited probands were negative for pathogenic variants at the DFNB1 locus (GJB2 and GJB6 genes). DNA was isolated from peripheral blood with a standard procedure or oral cavity swabs using Maxwell FSC DNA IQ Casework Kit (Promega, USA). For all families the cochlea-specific multi-gene panel was performed, libraries were prepared using KAPA HyperPlus Kit (KAPA Biosystems Inc, USA) and were enriched using a custom SeqCap EZ Choice kit (Roche NimbleGen Inc, USA), following the manufacturer's instructions. The obtained libraries were sequenced on a MiSeq platform ((Illumina Inc, USA). Sequence reads were aligned to the GRCh38 human reference genome assembly. Poor quality variants and variants with an allele frequency above 0,01 in general population, according to the open access databases (gnomAD, ESP6500, UK10K) were filtered out. Next, variants with a potential functional effect (missense, splice site, indels, stop gained) were chosen. Variants potentially related to ADHL were selected based on the prediction scores from computational algorithms (CADD, LRT, FATHHM, MutationTaster, PolyPhen-2, SIFT). Sanger sequencing was performed to confirm the presence and segregation of the identified probably pathogenic variants in all studied families (n=95).

Results:

The research carried out in 2019 has provided new insights into of ADHL, showing an advantage of using cochlea-specific multi-gene panel with 237 HL genes for studying the molecular basis of the genetically heterogeneous disorder. Genetic cause of HL was found in 46,7% (7/15) of the examined families and in 85,7% (6/7) of the families the genetic variant was novel. In 53,3% (8/15) of the studied families, the variant accountable for HL was not identified.

This is due to the lack of segregation of selected variants (*MYH14, EYA4, DFNA5, TBC1D24, PTPRQ)* or currently carried out analysis of results (*TJP2, PSIP1, DFNA5, TBC1D24).*

In our study, we identified and confirmed one known missense (c.5668C>T, p.Arg1890Cys) variant in *TECTA* gene associated with HL corresponding to the family phenotype. Furthermore, we characterized one novel probably pathogenic missense variant in *TBC1D24* gene c.905T>G, p.Leu302Arg, two novel pathogenic missense variant in *KCNQ4* gene: c.746T>C, p.Leu249Pro and c.650T>G, p.Met217Arg, three novel probably pathogenic variants in *MYO6* gene: a missense variant c.2596G>A, p.Glu866Lys; splice site variant c.1984-1G>A and a stop gained variant c.1489C>T, p.Gln497*. Introduction of NGS has improved the genetic diagnosis of HL but the diagnostic efficiency of our testing is currently below 50% and a significant part of the genetic causes of HL still remain unknown. Interestingly, for one family two probably pathogenic variants were selected in *PTPRQ*, *TBC1D24* genes.

These variants segregated with ADHL in five family members. Only further studies questioned the possible pathogenicity of the variants. This example indicates that in ADHL, the largest number of family members should be taken for testing. It is also important to extend the NGS panel analysis of functional studies created to investigate the specific pathogenic effect of the identified variants.

Participation in conferences:

1. Oziębło D., Leja M., Sarosiak A., Skarżyński H., Ołdak M.: Novel variants in known genes – results of genetic testing in families with autosomal dominant hearing loss. European Human Genetics Conference 15-18.06.2019 Gothenburg, Sweden
2. Oziębło D., Leja M., Sarosiak A., Skarżyński H., Ołdak M.: Genetic basis of autosomal dominant hearing loss in pediatric patients. 32nd Politzer Society Meeting and 2nd World Congress of Otology, 28.05-01.06.2019 Kajetany, Warsaw, Poland
3. Oziębło D., Leja M., Sarosiak A., Skarżyński H., Ołdak M. Wariant patogenny w genie DIAPH1 – wspólna przyczyna niedosłuchu i makrotrombocytopenii. XLI Krajowa Konferencja Naukowo-Szkoleniowa „Problemy otorynolaryngologii dziecięcej w codziennej praktyce" 18–20.11.18, Kajetany, Polska

Publications, scientific presentations as a leading author:

Szychowski KA., Kaminskyy DV., Leja M., Kryshchyshyn AP, Lesyk RB., Tobiasz J., Wnuk M., Pomianek T., Gmiński J. "Anticancer properties of 5Z-(4-fluorobenzylidene)-2-(4-hydroxyphenylamino)-thiazol-4-one" Scientific Reports, 2019, 9:10609

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Ewelina Użarowska	Year of studies: 2nd

Title of the project: Development of new diagnostic methods of atypical hemolytic uremic syndrome.

Name of the supervisor: dr hab.n.med. Anna Wójcicka

Affiliation: Genomic Medicine, Department of General, Transplant and Liver Surgery, Medical University of Warsaw; "WARSAW GENOMICS Spółka z ograniczoną odpowiedzialnością" sp. k.

AIMS of the project:

The aim of the project is to make a diagnosis of atypical hemolytic uremic syndrome (aHUS) more accurate. This disease is extremely rare and its diagnosis is problematic. The greatest difficulty lies in differentiation of aHUS from others kidney diseases, such as hemolytic uremic syndrome or C3 glomerulopathy. The aim of my project is to create a genetic test which will clearly states whether patient is affected by aHUS or not. In treatment of aHUS it is essential to know the risk of developing of end-stage renal disease which finally ends up with patients kidney transplantation. In such situation it is worth knowing the risk of relapse disease in transplanted organ. The risk depends on the genetic background of patients, i.e. existence of haplotype in *CFH* or *CD46*. Moreover, an important stage in aHUS diagnosis is determination of anti-CFH antibody's level – it's necessary before making a decision of Eculizumab treatment. During this project I want to analyze genetics variants that are present in aHUS patients and whether thy correspond to stage of the disease. There is also no obvious inheritance of aHUS, the analysis of proband's family will be considered. The literature indicates that genetic variants connected with aHUS occur also out of complement pathway genes (which are the most frequently analyzed), therefore, we added a few genes to genetic panel. The next step is to perform the whole exome sequencing in aHUS and C3 glomerulopathy patients in order to obtain relevant data.

Methods:

DNA is isolated from peripheral blood (alternatively from saliva, if patient had blood transfusion recently). Quantity and quality of DNA is verified using fluorometer and spectrophotometer. Then the libraries of DNA are prepared using standard protocol validated in Warsaw Genomics. Sequencing is performed on NextSeq500 or HiSeq4000, obtained data are analyzed by bioinfoirmatics. Genetic variants which are found in patients are compared with literature data. For each patient also an elisa test is conduct in order to assay of anti-CFH antibody level. It is perform with the use of serum with commercially available elisa kit. The results of elisa test are compared to control group, which was also examined in Warsaw Genomics (it is a group of healthy volunteers).

Results:

After sequencing every patient obtains a result of diagnostic panel (ADAMTS13; C3; CD46; CFB; CFH; CFHR1; CFHR2; CFHR3; CFHR4; CFHR5; CFI; DGKE; THBD), which contains a list of genetic variants which are found. Each variant is compared with literature in order to found information about relationship with disease or way of treatment. Unfortunately, these variants are rare and in literature there aren't many details about significance of variants. Almost all of them in ClinVar are classified as a benign what is the reason of difficult interpretation. During the two years of my project I collected samples from around 80 patients, for which sequencing was performed were tested (there were people diagnosed aHUS and also who were suspected of having aHUS). Also, in each of those patients elisa test was conducted. In addition, elisa test was performed about 50 times, also in

patients being on different stages of Eculizumab treatment. After literature search, I have decided to add to diagnostic panel genes, which are relevant to the development of the disease (PLG, C5, MMACHC, CD59). Currently, we are waiting for the sequencing result of new panel.

Participation in conferences: -

Publications, scientific presentations as a leading author:

„Genetyczne podłoże atypowego zespołu hemolityczno-mocznicowego i jego wpływ na przebieg choroby i efekty leczenia" Genetic background of atypical hemolytic-uremic syndrome and its influence on the course of disease and therapeutic effects. Mgr Ewelina Użarowska[1,2,3], Michał Kościółek[1], dr hab. n. med. Anna Wójcicka[1,2]
[1] Warsaw Genomics, 01-682 Warszawa,[2] Zakład Medycyny Genomowej, Warszawski Uniwersytet Medyczny, 02-097 Warszawa, [3] Studium Medycyny Molekularnej, Warszawski Uniwersytet Medyczny, 02-091 Warszawa; Wiadomości Lekarskie; in a review.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Rishikesh Kumar Gupta	Year of studies: 1st

Title of the project: Role of Stim2a protein in the neuroprotection in *Danio rerio*

Name of the supervisor: Prof. Jacek Kuźnicki

Affiliation: International Institute of Molecular and Cell Biology in Warsaw

AIMS of the project:

My project is planned to identify mechanisms that lead to neuroprotection without Stim2 protein and potential drug targets of the pathways using s*tim*2a$^{-/-}$ and *(stim2a; stim2b)*$^{-/-}$ mutant zebrafish line.

Methods:

The knockout line *stim2a*$^{-/-}$ was generated using CRISPR/Cas9 technology. *Stim2a* mutants were recognized and confirmed by using the restriction-digestion method. *Stim2a*$^{-/-}$ were crossed with *Tg(HuC: GCaMP5G)* to create the *stim2a*$^{-/-}$*: GCaMP5G* transgenic line to use *in vivo* calcium imaging experiments. *Stim2a*$^{-/-}$ *were crossed with stim2b*$^{-/-}$ to create the *(stim2a; stim2b)*$^{-/-}$ mutant. Adult zebrafish and larvae were kept in E3 medium at 28.5°C in 14/10h light/dark cycle. Calcium imaging in the Light Sheet Fluorescence Microscopy (LSFM) using line expressing GCAMP5 and wild type Stim2 was performed to establish the methodology.

Results:

Genotyping to recognize and confirm the homozygous *stim2a*$^{-/-}$ was done by using the restriction-digestion method. Further next, homozygous *stim2a*$^{-/-}$ fish was analyzed by RT-PCR, which confirmed the significant downregulation of stim2a. At present *stim2a*$^{-/-}$ were crossed with *Tg(HuC: GCaMP5G)* to create the *stim2a*$^{-/-}$*: GCaMP5G* transgenic line. *Stim2a*$^{-/-}$*: GCaMP5G* transgenic line is growing, and their offspring will be used for *in vivo* calcium imaging experiments using LSFM. Data analysis was performed using MATLAB for the wild type Stim2 fish expressing GCaMP5G. *Stim2a*$^{-/-}$ were crossed with *stim2b*$^{-/-}$ to create the *(stim2a; stim2b)*$^{-/-}$ mutant to create the *(stim2a; stim2b)*$^{-/-}$*:GCaMP5G* transgenic line.

Participation in conferences:

1. Gupta RK, Wasilewska I, Palchevska OP, Kuznicki J, Decoding the spontaneous *in vivo* Ca^{2+} oscillations in zebrafish brain neurons. 14th International Congress of the Polish Neuroscience Society (ICPNS), Katowice, Poland, Aug-2019; *Poster planned*.
2. Wasilewska I, Palchevska OP, Gupta RK, Kuznicki J, Stim2 role in response to oxidative stress. 14th International Congress of the Polish Neuroscience Society (ICPNS), Katowice, Poland, Aug-2019; *Poster planned*.
3. Gupta RK, Wasilewska I, Palchevska OP, Kuznicki J, Encoding and Decoding of Ca^{2+} oscillations in zebrafish brain neurons by in vivo Ca^{2+} imaging. FEBS Congress, Krakow, Poland, July-2019, Poster.
4. Wasilewska I, Gupta RK, Palchevska OP, Kuznicki J, Identification of zebrafish calcium toolkit genes and their expression in the brain. What do we know about Orai:STIM signaling?- Medical University of Graz, Austria, Feb-2019; Poster.

5. Palchevska OP, Wasilewska I, Gupta RK, Palchevskyy SS, Kuznicki J, Zebrafish as a model for calcium signaling studies *in vitro*. What do we know about Orai:STIM signaling?- Medical University of Graz, Austria, Feb-2019; Poster.

Publications, scientific presentations as a leading author:

1. Wasilewska I, Gupta RK, Palchevska O, Kuźnicki J. Identification of Zebrafish Calcium Toolkit Genes and their Expression in the Brain. *Genes (Basel)*. 2019; 18;10(3). PMID: 30889933.
2. Maciąg F, Majewski Ł, Boguszewski PM, Gupta RK, Wasilewska I, Wojtaś B, Kuznicki J. Behavioral and electrophysiological changes in female mice overexpressing ORAI1 in neurons. Biochim Biophys Acta Mol Cell Res. 2019; 4889(19)30005-9. PMID: 30659848.
3. Gupta RK, Wasilewska I, Palchevska OP, Kuznicki J, Encoding and Decoding of Ca^{2+} oscillations in zebrafish brain neurons by in vivo Ca^{2+} imaging. What do we know about Orai:STIM signaling?- Medical University of Graz, Austria, Feb-2019 (Oral presentation).

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Maria Wypchło	Year of studies:1st

Title of the project: Decoding genetic predisposition to rare neurodevelopmental disease: Integration of genome, trascriptome and methylome studies with genome engineering of human induced pluripotent stem cell (hiPSC) based neuronal modeling

Name of the supervisor: Paweł Lisowski, PhD, DSc, Eng.

Affiliation: Department of Medical Genetics, Warsaw Medical University

AIMS of the project:

The aim of the PhD thesis is to discover genetic determinants of hitherto undiagnosed neurodevelopmental disorders (NDs) inherited in a recessive way (two affected siblings, healthy parents) with the focus on non-coding genomic regions. Functional genomics screening on neuronal cells differentiated from patient derived induced pluripotent stem cells (hiPSCs) aimed to guide the interpretation of the whole genome sequencing (WGS) results allowing for discovery of the novel causative genetic variants.

In particular, in depth NGS analysis of transcriptome and methylome along the neuronal lineage of patient derived neuronal cells in comparison to unrelated controls as well as isogenic cell lines will identify aberrant gene expression and alternative splicing events and/or aberrant methylation of associated regulatory regions which will be consistent with genomic information. The putative causative variants identified using WGS will be validated by multiplex genome editing using innovative combination of designed high fidelity Cas9 nucleases (correction of more than one risk variant(s) in the patient derived hiPSCs and/or introduction of variant(s) in control hiPSCs).

Methods:

WES, WGS, RNASeq

Two siblings, girl and boy, with neurodevelopmental health issues have been found so far. Blood samples for the DNA analysis have been collected, peripheral blood mononuclear cells (PBMCs) were reprogrammed into iPS cells and then differentiated into neurons including healthy controls.

The DNA samples have been extracted using QIAamp DNA Mini Kit, QIAGEN. Using the SureSelect QXT Library Prep Kit, samples have been prepared to the Whole Exome Sequencing. In the next step the same DNA samples have been used to the Whole Genome Sequencing. Libraries have been prepared using the Nextera DNA Flex Library Prep Kit.

RNA extracted from human induced pluripotent steam cells (hiPSCs) and human neuronal progenitor cells lines (hNPCs) have been used for the transcriptome analysis. hiPSCs reprogrammed from PBMCs using Sendai virus under feeder-free conditions have been converted into human neural progenitor cells (hNPCs) using small molecules.

The RNA has been extracted using QIAshredder columns and RNeasy Mini Kit, QIAGEN. Using TruSeq Stranded Total RNA Library Prep Gold all samples have been prepared for the total RNASeq. Row datasets were subjected for QC using FastQC, mapped to human genome GRCh38 with Hisat2 and analyzed by Cuffquant and Cuffmerge.

Results:

Since deep analysis of Whole Exome Sequencing gave negative results the comparison of these results with both patients' severe neurodevelopmental disorders we assume the genetic background may be connected with specific gene expression, aberrant alternative splicing events and/or aberrant methylation of associated regulatory regions

Current WGS and RNASeq data are under analysis and interpretation to narrow down the WGS regions of interest. Causing mutations in non-coding genomic regions using WGS are expected. To support more accurately the WGS findings the RNA sequencing was performed in the two time-points of neuronal differentiation.

Participation in conferences:

Young Scientists Conference on Molecular and Cell Biology 2019, Warsaw

Innowacyjne rozwiązania do hodowli komórek ssaczych, Warsaw

Publications, scientific presentations as a leading author:

Scientific abstract:

Neuroscience 2019, the 49th annual gathering of the Society for Neuroscience, will be held Oct. 19-23 in Chicago.
M. WYPCHLO, T. HAHN, M. RYDZANICZ, A. PRIGIONE, R. KUHN, A. RYBAK-WOLF, S. DIECKE, R. PLOSKI, P. LISOWSKI
Integration of clinical WGS supported by patient specific hiPS cell-based disease modeling and functional genomics translational approach for decoding of unknown neurodevelopmental disorders (UNDs)

Publication:

Grzechocińska B, Warzecha D, Wypchło M, Ploski R, Wielgoś M. Premature ovarian insufficiency as a variable feature of blepharophimosis, ptosis, and epicanthus inversus syndrome associated with c.223C > T p.(Leu75Phe) FOXL2 mutation: a case report. BMC Med Genet. 2019 Jul 31;20(1):132. doi: 10.1186/s12881-019-0865-0.

1 publication is after firs review.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Agata Dziedzic	Year of studies: 1st

Title of the project: DNA methylation analysis of glioma samples: Comparison of DNA methylation patterns in gliomas of various grades and different IDH gene status.

Name of the supervisor: dr Michał J Dąbrowski

Affiliation: Instytut Podstaw Informatyki PAN

AIMS of the project:

DNA methylation profiles have important implications for understanding how epigenetic changes might play a role in a specific disease, including tumours. Methylation status of regulatory regions, such as promoters and enhancers, may activate or repress the regions activity causing changes in gene expression levels. Recently, much evidence has been gathered which suggests that DNA methylation may play a crucial role in the glioma pathogenesis. We had following aims:

1) To analyse raw bisulphite-sequancing data (from fastq files to methylation valuses for each sequenced cytosine) together with quality assesment of the data.

2) To identify the group of genes whose expression is affected by DNA methylation, as well as the pattern of DNA methylation itself, all in relation to various tumour grades based on 26 patients representing various glioma grades: I (n = 8), II - III (n = 7) and IV (n = 11).

3) To analyse methylation patterns of various transcription factors (TF) motifs - to examined the hypothesis that methylation of TFs would affect gene expression.

4) To identify differentially methylated sites in respect to different tumor grades or IDH status

The results will elucidate the today unknown role of transcriptional and epigenetic dysfunctions in brain tumors and likely identify novel predictions that may allow further research on better diagnostics. We may reveal novel targets for the therapy of lethal tumors.

Methods:

For methylome analysis we used SeqCap Epi CpGiant Methylation panel and performed bisulphite conversion followed by Illumina sequencing. Sequences were obtained for a set of three different WHO glioma grades: *pilocytic astrocytoma* (PA; grade I), *diffuse astrocytoma* (DA; grades II and III) and *glioblastoma* (GB; grade IV) from the Polish patients samples.
We developed a tool that we developed is based on Roche pipeline - it combines together a set of open source software packages dedicated to bisulphite data analysis. We compiled them together and used for analysys of raw sequencing data. All following analyses are based on open soure bioinformatic tools and custom made R scripts and R packages.

Results:

We obtained at average 23 mln methylation sites per each sample from which ~ 3.3 mln sites were in CpG context and ~ 19.7 mln were in non-CpG context. For further study we only selected sites that were in CpG context and with coverage above 10 reads.
The tool we developed outputs distribution of DNA methylation across all samples with: (i) annotation regarding hypo and hypermethylation, (ii) division into CpG islands, shores and open seas, (iii) specification of genomic region (i.e. promoter, enhancer, open chromatin, transcription factor binding site). Typically, in healthy mammalian tissue 70-80% of CpG sites are methylated and the remining sites are unmethylated (Lister et al. 2009). In our data most of the samples had around 50% of CpG

sites unmethylated. This indicates global hypomethylation of analysed samples. We were also able to differentially methylated CpG sites/ regions in relation to a predefined feature from the data e.g. tumour grade. We performed differentional methylation calling and found 209,260 differentially methylated sites for GB vs. PA and 215,923 differentially methylated sites for IDH mutant vs. IDH wild-type. Then we annotated those sites in respect to the genome parts (promoter, gene body, CpG islands). There are more hypermethylated sites in GB samples comparing to PA – most of this hypermethylated sites are within CpG islands of genes promoters regions. . There were more hypermethylated sites in IDH mt samples comparing to IDH wild type.

Participation in conferences:

1) 8th Clinical Epigenetics International - Düsseldorf, Germany

2) Young Scientists Conference on Molecular and Cell Biology

3) Brain Tumors Meeting 2018 - From Biology to Therapy

Publications, scientific presentations as a leading author: -

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Justyna Jędrychowska	Year of studies: 3rd

Title of the project: The role of voltage-gated potassium channels in the development of ear in zebrafish

Name of the supervisor: dr Vladimir Korzh, prof dr hab. Jacek Kuźnicki

Affiliation: Laboratory of Neurodegeneration, International Institute of Molecular and Cell Biology in Warsaw

AIMS of the project:

The aim of this project is to investigate the role of Kcnb1 in the development and function of zebrafish ear.

Methods:

Zebrafish (*Danio rerio*) mutant of Kcnb1 (*kcnb1*$^{-/-}$ line) and control (wild-type) animals were used in the experiments.

To characterize number and morphology of the hairs cells in the inner ear of *kcnb1* mutants, immunohistochemistry (IHC) using antibody against acetylated tubulin and staining with phalloidin were used. Lightsheet fluorescence microscopy was used to image embryos.

Behavioral analysis was conducted to assess hearing and vestibular function of mutants. To obtain a sufficient number of embryos with mutant phenotype, microinjections of kcnb1 morpholino were used.

RT-qPCR was used to validate the expression of the genes important during ear development.

Three mutations in human *KCNB1*: I199F, V378A, G379R were selected to study the effect of these mutations on developing zebrafish ear. In humans, these mutations have been linked to early infantile epileptic encephalopathy (EIEE). Vectors for *in vitro* mRNA expression of mutant versions of KCNB1 were generated by standard cloning protocols. Then mRNAs were synthesized and microinjected into the yolk of developing wild-type 1-cell stage embryos. Light microscopy was used to assess changes of the phenotypes in the developing embryos.

Results:

Kcnb1 mutants developed significantly smaller ears and otoliths comparing to wild-type controls. The immunohistochemistry revealed changes in the morphology of the hair cells in the ear of *kcnb1*$^{-/-}$ with changes in orientation of their kinocilia.

Using qRT-PCR upregulation and downregulation of some ear marker gene (for example *otogl*, *otofa*, *otop1*, *hapln1a*) was confirmed.

Behavioral tests showed abnormal swimming pattern and hearing dysfunctions of mutants.

Overexpression of human wild-type and mutated KCNB1 by mRNAs microinjections led to abnormalities in the ear, such as reduction of its size, the occurrence of additional (third) otolith or absence of one otolith.

In summary, these results support a hypothesis that Kcnb1 plays a role during the development of zebrafish ear and its function in hearing and spatial orientation.

Participation in conferences:

Jędrychowska J., Gasanov E., Kuźnicki J., Korzh V. Kcnb1 plays a role in the development and function of ear in zebrafish. 14th International Congress of the Polish Neuroscience Society. Katowice, Poland, 2019

Jędrychowska J., Gasanov E., Kuźnicki J., Korzh V. Kcnb1 plays a role in the development of ear in vertebrates. The 14th International Zebrafish Conference. Suzhou, China, 2019

Jędrychowska J., Kuznicki J., Korzh V.: The role of voltage-gated potassium channels in thedevelopment of ear in zebrafish. COST Action BM1408, Seillac, France, 2018

Publications, scientific presentations as a leading author:

Jędrychowska J., Gasanov E., Kuznicki J., Korzh V. The role of Kcnb1 in the development of ear in zebrafish. Young Scientists Conference on Molecular and Cell Biology Warsaw, Poland, 2019, oral presentation.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Ranju Kumari	Year of studies: 2nd

Title of the project: The role of TMCC2 in the development and maintenance of the inner ear mechanosensory hair cells.

Name of the supervisor: dr Piotr Kaźmierczak

Affiliation: Centre of New Technologies, University of Warsaw

AIMS of the project:

Inner ear sensory hair cells are responsible for auditory signal transduction as they respond to sound-induced vibrations and convert these mechanical signals into electrical impulses in a process known as mechanoelectrical transduction. Hair cells display remarkable apico-basal polarization and develop in a highly ordered architecture of the organ of Corti, surrounded by supporting cells and in contact with innervating neurons. The molecular mechanisms that control the development and maintenance of hair cells are only partially understood but it is clear that these cells express a large number of proteins that serve hair cell specific functions. Regulation of their expression as well as precise control of their intracellular localization and turnover are necessary for hair cell function and disturbances in these processes can lead to hearing deficits. Identification of novel protein markers expressed in hair cells has been one of the successful approaches to deciphering the mechanisms of hearing and the causes of deafness. We identified one such novel hair cell specific marker; a protein called transmembrane and coiled-coil 2 (TMCC2). It is particularly interesting because it resides in intracellular membranes such as ER and Golgi and preliminary experiments suggest that *Tmcc2* mRNA is expressed exclusively in hair cells and the protein is localized to the cell body. Additionally, recent findings implicate its close paralog in intracellular protein sorting, raising the possibility that the loss of this protein might influence the development of hair cells.

1. Establish the role of TMCC2 in inner ear development and hearing
2. Determine the expression pattern of *Tmcc* family genes in the inner ear
3. Define the molecular function of TMCC2

Methods:

In order to achieve the objectives of my studies, I used different approaches ranging from mouse genetics and basic molecular biology to techniques like immunohistochemistry, and co-immunoprecipitation. We used CRISPR/Cas9 to ablate the gene and confirmed creation of the knockout mouse line by genotyping, western blots and immunofluorescent staining in whole mount cochlea.

1. Immunofluorescent staining in *Tmcc2* knockout mice for evaluating cochlear morphology
2. TMCC2 subcellular localization using specific ER and Golgi markers available commercially
3. Studying TMCC2 interactions using co-immunoprecipitation from wild-type mice and *Tmcc2* knockouts as a negative control
4. Expressing potential interacting proteins in HEK 293 cells to confirm and supplement the results obtained from mouse tissue.

Results:

We used CRISPR/Cas 9 mediated deletion of *Tmcc2* gene to characterize the role of TMCC2 in development and hearing, and we isolated genomic DNA from tail to check the genotype of mice and we found mendelian type of inheritance for mice. The deletion of TMCC2 protein was observed by

absence of band in *Tmcc2* knockout protein extracts in western blotting. We looked for the subcellular localization of TMCC2 by immunostainings and the staining of TMCC2 is absent in the knockouts, also we have detected the presence of TMCC2 in the Golgi apparatus in developing wild-type hair cells at the age of P5. We compared the staining pattern of a known hair cell marker Myo7a at P3, P5, P7 and P18 in hair cells and found it was normally distributed among different developmental stages between wild-types and knockout. With the use of immunostainings, we investigated hair bundle structure visualized by actin and tubulin, and the localization of the component of interstereociliary links CDH23 at P5, P7 and P18 in wild-types and knockouts and no differences were observed among them. We have screened nearly 40 novel mouse commercial antibodies to identify hair cell markers to build a toolset for probing and studying the cytoarchitecture and colocalization of novel knockouts and identified several unpublished useful tools. Further, we characterised three anti-TMCC2 antibodies by expressing proteins in cell lines and confirmed with western blotting and immunostainings. Previously we have reported a preliminary observation of long stretches of extra outer hair cells in some Tmcc2 knockout mice. Since then we have gathered additional data indicating that this feature is not specific to the knockouts and is instead a peculiar characteristic of the genetic background on which our knockouts were raised. Currently, we are conducting co-immunoprecipitation experiments in transfected cells.

Participation in conferences:

56th Inner Ear Biology Workshop "Hearing Research: from History into the future" on 7th-10th September, 2019 at University of Padua, Padua, Italy.

Publications, scientific presentations as a leading author:

Poster presentation at 56th Inner Ear Biology Workshop, Hearing Research: from History into the future on "Identification of TMCC2 as novel hair cell marker and characterization of antibodies for phenotypic study of hair cell deficits." At Padova, September 7-10, 2019.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Filip Maciąg	Year of studies: 3rd

Title of the project: Transgenic mice with elevated basal level of calcium ions in neurons as a model of age-induced neurodegeneration of sporadic Alzheimer's disease

Name of the supervisor: prof. dr hab. Jacek Kuźnicki

Affiliation: International Institute of Molecular and Cell Biology in Warsaw

AIMS of the project :

Sporadic Alzheimer's disease (sAD) is the most widespread form of dementia. The key risk factor for sAD is age. It has been shown that during ageing, sustained changes in neuronal Ca^{2+} homeostasis occur, giving rise to the 'calcium hypothesis of aging'. The main objective of the project is to understand how the process called Store Operated Calcium Entry (SOCE) may contribute to neurodegeneration. In our research we use transgenic mice that overexpress STIM1, STIM2 or ORAI1 proteins, key players in SOCE. SOCE is a process by which the depletion of Ca^{2+} in the endoplasmic reticulum (ER) causes STIM proteins, ER Ca^{2+} sensors, to move to plasma membrane and activate ORAI channels, causing an influx of Ca^{2+} from the extracellular space and refilling of the ER. Our objective is to analyse the phenotype of mice overexpressing SOCE proteins. Having characterised the STIM1 and ORAI1 lines (Majewski et al., 2017; Maciąg, Majewski et al., 2018), we are now focused on double transgenic STIM2/ORAI1 animals. Our group has shown that neuronal STIM2 and ORAI1 overexpression increased basal cytosolic Ca^{2+} level (Gruszczynska-Biegala et al., 2011). Therefore, mice that overexpress the two proteins may be an interesting potential model of neurodegenerative diseases such as sAD.

Methods:

To validate the observation of increased basal cytosolic Ca^{2+} level in cortical cultures that were transfected with both STIM2 and ORAI1 proteins, we aimed to perform Ca^{2+} imaging in acutely isolated hippocampal slices from double transgenic STIM2/ORAI1 mice. Ratiometric Ca^{2+} indicator (Fura-2 AM) was used as the probe. In contrast to routinely performed imaging of 2D neuronal cultures, Fura-2 Ca^{2+} imaging in *ex vivo* tissue is a challenging and rarely used approach. Therefore, a protocol that would enable successful loading of the dye into the cells had to be established. To obtain acute tissue slices, mice were sacrificed by cervical dislocation and hippocampi were quickly retrieved. 350 μm thick slices were prepared according to (Ting et al, 2018, *J. Vis. Exp.*). It was experimentally determined that a 20 min local application of 250 μl of 50 μM Fura-2 AM solution in ACSF at 33 °C resulted in successful loading of neuronal somas. Addition of 2.5% Pluronic F-127 in DMSO in the loading solution was mandatory to achieve successful loading of the dye. Slices that were obtained from animals up to approximately 5 weeks after birth could be effectively loaded with the established protocol. Quantification of the signal that was obtained after excitation at 360 nm, the wavelength at which Fura-2 signal is not dependent on Ca^{2+} concentration, was used as a control for the reproducibility of the loading procedure. The intensity of the control signal was comparable during each experiment. Initial trials aimed to calibrate the obtained signal and convert it to absolute Ca^{2+} concentration values failed due to compromised viability of the slice and high variability of the responses that were obtained during the calibration protocol. Therefore, the responses were represented as standard ratio of the signal (340 nm to 380 nm). To measure Ca^{2+} signals in the loaded neurons, an automated perfusion system was used to perform a modified Ca^{2+}-addback assay. First, basal Ca^{2+} level in the cytoplasm was measured in standard ACSF that contained 2.5mM Ca^{2+}, followed by application of 100 μM glutamate to load the cells with Ca^{2+} and provide reference maximal signal. After relaxation of the signal to the baseline, extracellular Ca^{2+} was removed by the addition of 0.5 mM EGTA. Next, Ca^{2+} release from the ER was triggered by blocking the SERCA pump by cyclopiazonic acid (CPA), which was followed by Ca^{2+} readdition to assess the activity of store-operated Ca^{2+} channels. Fluorescent signals were imaged with an epi-fluorescent microscope coupled with EM-CCD camera, under 40x objective. The data were analysed using MetaFluor and Microsoft Excel software.

Results:

The established imaging protocol was used to compare Ca^{2+} signals in wild-type and double transgenic STIM2/ORAI1 mice. The analysis revealed no changes in the basal cytoplasmic Ca^{2+} level. Therefore, the initial hypothesis of the project was not confirmed. Similarly, the maximal signal upon the addition of glutamate was similar in both groups. Also the peak corresponding to Ca^{2+} efflux from the ER and the influx of these ions after readdition of Ca^{2+} following store depletion were not changed in STIM2/ORAI1 mice compared with wild-type controls. However, following the application of glutamate, in STIM2/ORAI1 mice the signal did not relax to the level that was observed before the agonist addition, but remained slightly elevated. This difference in the average Ca^{2+} level persisted throughout the entire protocol. The obtained result may indicate impaired extrusion of Ca^{2+} from the cytoplasm, and/or that the processes responsible for importing Ca^{2+} ions into the intracellular stores are affected. It will be intriguing to determine whether it is the STIM2 or ORAI1 protein alone that is responsible for the observed difference, or the two partners need to act in concert to cause this effect. To this end, analogous experiments are currently being performed on single transgenic STIM2 and ORAI1 mice. Next, to assess whether the observed difference translates to any changes in basic electrophysiological properties, local field potential and patch-clamp recordings from neurons overexpressing both proteins will be performed. The experiments will include measurements of basal synaptic transmission and short- and long-term plasticity.

Participation in conferences:

1. *What do we really know on Orai/Stim complex* – meeting of COST action BM140; poster presentation entitled *Investigating synaptic properties of neurons overexpressing key store-operated calcium entry proteins*, 12-14th February 2019, Graz, Austria.

2. *1st Young Scientists Conference on Molecular and Cell Biology*; poster presentation entitled *Use of imaging and electrophysiological methods to elucidate the role of store-operated calcium entry proteins in neural function*, 11th April, 2019, Warsaw, Poland.

Publications, scientific presentations as a leading author:

Maciąg F., Majewski Ł., Boguszewski P.M., Gupta R.K., Wasilewska, I., Wojtaś B., Kuznicki J.; *Behavioral and electrophysiological changes in female mice overexpressing ORAI1 in neurons*; Biochimica et Biophysica Acta – Molecular Cell Research, Vol 1866 (7), p 1137-1150, 2019; *equal rights

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Iga Wasilewska	Year of studies: 3rd

Title of the project: Involvement of STIM2 protein in neurodegeneration in *Danio rerio*.

Name of the supervisor: Prof. dr hab. Jacek Kuźnicki

Affiliation: International Institute of Molecular and Cell Biology in Warsaw

AIMS of the project:

STIM proteins are important players in cellular calcium homeostasis as they are required to store-operated calcium entry (SOCE) process. STIM2 is highly expressed in the rodent brain and its properties are far less understood than that of STIM1. Dysregulated Ca^{2+} homeostasis is a feature of many neurodegenerative disorders. STIM2 seems to be especially important in this context of neurodegeneration process but its role in this process is still unclear.

Recently, new evidences indicating that STIM proteins may react not only to changes in Ca^{2+} level inside the endoplasmic reticulum (ER) have appeared. STIM1 reacts to changes in the temperature and is involved in the reaction to oxidative stress. However, not much is known about STIM2 in this context.

Aim of the project is to reveal the unknown functions of Stim2. Impact of Stim2 on neuronal Ca^{2+} homeostasis in the conditions of oxidative stress is investigated. We want to know if Stim2 may react to reactive oxygen species, modulated intracellular Ca^{2+} level and affect mitochondria function. I believe that investigating Stim2 functions in neurons bring us closer to understanding the role of this protein in pathological processes.

Methods:

I use zebrafish as a model organism. In order to investigate Stim2 functions we use a mutant line with suppressed expression of Stim2b.

I compared the expression of genes encoding Ca^{2+} signaling toolkit (CaTK) members between wild-type (WT) and *stim2b*$^{-/-}$ fish using RT-PCR arrays. Also, changes in expression of genes encoding mitochondrial proteins and those involved in the response to the oxidative stress were checked.

Level of ROS was compared between WT and mutant fish using CellROX Green dye. Cell death was assessed by acridine orange staining.

stim2b$^{-/-}$ line was crossed with two transgenic zebrafish lines expressing CEPIA Ca^{2+} probe targeted to the mitochondria. Changes in Ca^{2+} levels in response to oxidative stress are being analyzed using these lines. *In vivo* Ca^{2+} imaging was performed using Light Sheet Fluorescence microscopy. Anesthetized larvae were mounted in 2% agarose and treated with 2mM hydrogen peroxide for 40 min and then signals from optic tectum were recorded.

Results:

We have already observed that mRNA of genes encoding Stim and Orai proteins is present in the zebrafish brain (Wasilewska et al., 2019). Also, we observed the expression of vast majority of CaTK members.

RT-PCR arrays showed differences in expression of several mitochondrial genes in the brain of *stim2b*$^{-/-}$ fish, however repetition of these experiments with a standard RT-PCR didn't confirm these

results. Also, expression of oxidative stress-related genes was not affected in the mutant. Accordingly, neither level of ROS in the brain or the extent of cell death appeared to be noticeably changed in mutant fish.

In vivo Ca^{2+} imaging was performed to establish the methodology.

Participation in conferences:

- COST BM-1406 meeting: "Ion Channels and Immune Response toward a global understanding of immune cell physiology and for new therapeutic approaches", Graz, 12-14.02.2019
- "The 15th International Meeting of the European Calcium Society (ECS)", Hamburg, 09-13.09.2018

Publications, scientific presentations as a leading author:

- Wasilewska, I.; Gupta, R.K.; Palchevska, O.; Kuznicki, J. Identification of Zebrafish Calcium Toolkit Genes and their Expression in the Brain. *Genes* 2019, *10*, doi:10.3390/genes10030230.
- Short talk: "Identification of zebrafish calcium toolkit genes and their expression in the brain" I. Wasilewska, R.K. Gupta, O. Palchevska, J. Kuznicki, during COST BM-1406 meeting, Graz, 2019

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Anna Sarosiak	Year of studies: 3rd

Title of the project: Development of a zebrafish model for interpreting human genetic variation in hearing loss

Name of the supervisor: dr hab. n. med. Monika Ołdak

Affiliation: 1. Department of Histology and Embryology, Center of Biostructure Research, Medical University of Warsaw, Warsaw, Poland; 2. Department of Genetics, Institute of Physiology and Pathology of Hearing, Warsaw, Poland

AIMS of the project:

Autosomal dominant hearing loss (ADHL) is the second most common form of hereditary hearing loss (HL) characterized by a large genetic heterogeneity. Detection rate of a causative genetic variant in ADHL reaches approximately 50% leaving the remaining half unexplored. Due to evolutionary conserved features and a high accessibility for genetic manipulations zebrafish model can be used to dissect the underlying mechanism of action of the newly discovered genes and alleles involved in HL development.

Novel probably pathogenic variants of the *WFS1* and *TBC1D24* genes were detected in ADHL patients of the Institute of Physiology and Pathology of Hearing. The major aim of this stage of the project was to develop genetic knock-down zebrafish models by injection of morpholino oligonucleotides (MOs) to study the involvement of *wfs1* and *tbc1d24* genes in hearing in zebrafish. The complementary aim was to prepare a referent zebrafish HL model using an ototoxic drug – gentamicin (GNT) to cause GNT-induced hair cell death in the zebrafish lateral line as a representative paragon for the latter studies on HL in zebrafish. The study aimed to asses hearing defects by (i) evaluating the morphology of zebrafish auditory system by dimensional measurements of the inner ear structures, (ii) applying imaging techniques to asses structure and function of neuromasts - small sensory patches containing hair cells, using a different vital dyes, (iii) use of behavioral studies for testing hearing responses - different types of movements and reactions dependent on function of the hearing apparatus.

Methods:

To induce damage of neuromast sensory hair cells, 5 dpf (days post-fertilization) zebrafish larvae were incubated in embryo medium containing GNT for 6 h at a range of concentrations from 50 to 400 μM. After the treatment larvae were subjected for neuromast live cells staining with DASPEI and Yo-Pro1 dyes (Thermo Fisher Scientific, Massachusetts, USA). Neuromast imaging was performed using fluorescence stereomicroscopy and confocal microscopy. Locomotor activity after GNT administration was measured with the ZebraBox System (ViewPoint, Civrieux, France) using tracking mode before and after acoustic stimulus. The *wfs1*- and *tbc1d24*-blocking MOs were injected into the yolk of the one-cell stage zebrafish embryos at different dosages to obtain phenotype spectrum and observed in 5 dpf. Phenotypic evaluation included (i) neuromast live cells staining with Yo-Pro1 and FM1-43FX dyes (Thermo Fisher Scientific, Massachusetts, USA), (ii) inner ear, saccular and utricular otoliths size measurements and (iii) locomotor activity and movement characterization with ZebraBox System (ViewPoint, Civrieux, France) using the tracking mode before and after acoustic stimulus.

Results:

In 5 dpf zebrafish larvae treated with GNT, vital dyes' fluorescent signals were gradually lost in neuromasts in a dose-dependent manner indicating a GNT-induced cell death. All GNT-treated groups showed a statistically significant reduction in locomotor activity also indicating a lower sensitivity to acoustic stimuli. The obtained phenotype was assessed to be related to GNT ototoxicity and a consequent hearing loss in zebrafish larvae. The developed model can be used in a further analyses of HL mechanisms in zebrafish.

In *tbc1d24* morphants a gradual and MO dose-dependent loss of fluorescent light signal was observed both in Yo-Pro and FM1-43FX neuromast stainings which indicated the negative effect of *tbc1d24* knockdown on neuromast size and viability and the probable disruption in hair cells mechanotransduction system. In this group, a statistically significant MO dose-dependent reduction in locomotor activity and response to auditory stimulus was observed.

In *wfs1* morphants the Yo-Pro1 staining showed a gradual loss of fluorescent light signal in larvae injected with higher doses of MO. The effect was less pronounced when compared to *tbc1d24* morphants. In this group the FM1-43FX fluorescent signal did not vary noticeably between the larvae injected with different dosages of *wfs1*-MO and was comparable to control. A statistically significant reduction in locomotor activity and response to auditory stimulus was observed in this group and specific *wfs1*-MO doses showed a different effect on larvae locomotor activity. For *wfs1* morphants a statistically significant reduction of saccular otolith size was observed, suggesting an impaired otolith formation in this group.

The developed GNT-induced HL model and knock-down models showed a range of features related to disrupted sound processing mechanisms and structures in zebrafish. The workflow based on behavioral characterization and imaging applied on *wfs1*- and *tbc1d24*-knockdowns will enable to establish a model useful for further studies on the pathogenicity of human *WFS1* and *TBC2D24* genetic variants.

Participation in conferences:

Oziębło D., Sarosiak A., Leja M., Skarżyński H., Ołdak M.: Evidence for causative role of *PTPRQ* in autosomal dominant hearing loss development. 2nd World Congress of Otology, 28.05.-1.06.19, Warsaw, Poland

Sarosiak A., Minota I., Kozioł K., Ołdak M.: Comprehensive chromosome screening of human first polar bodies and oocytes using four different whole genome amplification methods and single-cell next-generation sequencing. European Society of Human Genetics Conference, 15-18.06.19, Göteborg, Sweden.

Publications, scientific presentations as a leading author:

Sarosiak A., Minota I., Kozioł K., Ołdak M.: Recent advances and prospects of preimplantation genetic testing (Part I. and Part II.). Journal of Health Study and Medicine, accepted for publication

Oziębło D., Sarosiak A., Budde B., Tacikowska G., DiDonato N., Bolz H.J., Nürnberg P., Skarżyński H., Ołdak M.: First confirmatory study on *PTPRQ* as an autosomal dominant non-syndromic hearing loss gene. Journal of Translational Medicine, under review

Sarosiak A., Leja M., Oziębło D., Domagała S., Madejska A., Skarżyński H., Ołdak M.: Screening for *WFS1* gene variants in families with low frequency hearing loss and development of zebrafish knock-down model of the orthologous gene *wfs1*. 2nd World Congress of Otology, 28.05.-1.06.19, Warsaw, Poland (oral presentation)

Sarosiak A., Minota I., Kozioł K., Ołdak M.: The use of single-cell next generation sequencing for comprehensive chromosome screening in human first polar bodies and oocytes. Young Scientists Conference on Molecular and Cell Biology, 11.04.19, Warsaw, Poland (oral presentation).

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Karol Nowosad	Year of studies: 2nd

Title of the project: Characterization of cis-regulatory landscape of SMOC2 and DACT2, genes co-expressed during development of the synovial joint

Name of the supervisor: Prof. Przemysław Tylżanowski

Affiliation: Department of Biochemistry and Molecular Biology; Medical University of Lublin

AIMS of the project:

Our research focuses on identification of *SMOC2-DACT2 cis*-regulatory elements and its 3D chromatin organization. Both genes are modulators of BMP and Wnt signaling, the key pathways involved in joint development. Moreover, the *SMOC2* presents altered expression in Osteoarthritis (OA), the most common joint disease characterized by loss of articular cartilage and concomitant subchondral bone sclerosis, synovitis and osteophyte formation. The misexpression of SMOC2 in OA imply that regulatory elements may be important etiological factors in this disease. Therefore, the significance of interrogation of *SMOC2-DAC2 cis*-regulatory landscape goes well beyond the field of limb development and enters the basic concepts postnatal joint disease.
The conservation of the locus across species, permitted us to use the developing chick model system to study interzone biology.

Methods:

Interzone collection: One of the key challenges when studying the chromatin structure in chicken joint interzones is the tissue collection. The microdissection of joint interzones and adjacent phalanges from chicken embryos (Hamburger developmental stage 32 (HH32), an equivalent of E14.5 in mice) was performed under a microscope. To reduce the noise caused by contamination from the cells adjacent to interzone we carried out the quality controls to assess the molecular purity of collected material. The purity of isolation was tested by checking the expression of markers known to be associated exclusively with either of these structures. Specifically, as molecular markers, we carried out RT-qPCR for GDF5 and DACT2 (joint interzone) and COL2A1 and MANT-1 (phalange cartilage).

Targeted Chromatin Capture (T2C) study: The previously optimized protocol for T2C, a high resolution variant of Chromosome Conformation Capture (3C) technique was applied to interrogate the chromatin structure within ~3.4 Mb region encompassing *SMOC2* and *DACT2*.

Chromatin Immunoprecipitation (ChIP): The optimized protocol for the *in vivo* samples (with antibodies against H3K27ac histone modification) was used to investigate the putative enhancer regions.

Results:

The molecular purity of isolated interzones/phalanges was confirmed by RT-qPCR. The joint interzones presented high expression of GDF5/DACT2 and low expression of COL2A1/MANT-1, whereas phalanges presented high expression of COL2A1/MANT-1 and low expression of GDF5/DACT2.

Then, we interrogated the chromatin architecture of ~3.4 Mb region encompassing *SMOC2* and *DACT2*. The samples passed all quality controls before library preparation, including *Apo*I digestion control, ligation control, *Dpn*II digestion control and concentration measurement. Then, for

enrichment of fraction containing the region of interest, we used custom oligos designed to hybridize with fragments located within *SMOC2- DACT2* ~3.4 Mb region. Finally, the libraries were sequenced on an Illumina HiSeq2500 sequencer. Paired-end reads were generated of 100 base-pairs in length. Now, the samples are bioinformatically processed by our collaborators form the Department of Cell Biology, ErasmusMC, Rotterdam, Netherlands.

In parallel, we applied the ChIPseq technique to investigate the enhancer-specific epigenetic marks, such as H3K27ac. We optimized the protocol for interzone/phalanges tissues and performed the quality control using qPCR (% of input ~0,5-2%). Then, the libraries were prepared according to manufacture protocol (QIAseq Ultralow Input Library Kit) and sequenced on an Illumina sequencer. For data analysis we created the custom bioinformatic script which include: (a) raw data quality control using fastQC; (b) sequence alignment to the reference [galGal6] using Bowtie2; (c) comparison of the number of mapped reads between two samples (input and IP sample) using bamCompare; (d) peak calling using MACS2 for identification of genome regions enriched with aligned reads. Based on the obtained data we identified two enhancer candidate regions with high enrichment of H3K27ac and conservation between chicken, mouse and human. Importantly, both regions are located within one topologically associating domain (TAD), up to 1Mb from SMOC2 promoter region. Currently, we are waiting for the ChIPseq data with antibodies against H3K4me1 and P300 to verify the abovementioned putative enhancer regions.

Conclusions:

- We successfully collected molecularly pure material for chromatin analysis experiments
- We applied T2C to interrogate the chromatin architecture of ~3.4 Mb *SMOC2* and *DACT2* region.
- We used ChIPseq technique to identify potential cis-regulatory elements specifically regulating either SMOC2 or DACT2 in developing joint interzone.

Participation in conferences:

- Young Scientists Conference on Molecular and Cell Biology; poster presentation: "Transcriptome Analysis of the Developing Chick Joint Interzones"; April 11, 2019 , IIMCB, Warsaw; Best Poster Award

Publications, scientific presentations as a leading author: none

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Ewelina M. Olech	Year of studies: 2nd

Title of the project: Identification of novel genetics variants as the cause of the premature closure of the cranial sutures in children using novel sequencing techniques and array comparative genomic hybridization

Name of the supervisor: Associate Prof. Aleksander Jamsheer, MD, PhD

Affiliation: Poznan University of Medical Sciences

AIMS of the project:

The aim of the project was to identify pathogenic variants in a group of patients affected by craniofacial disorders, i.e. craniosynostosis (CS) and facial dysostoses (FD).

Methods:

We used two distinct next-generation sequencing genes panels to screen a cohort of patients affected with various craniofacial disorders. The confirmation and segregation study of each probably pathogenic variants was performed by means of Sanger sequencing analysis.

Results:

1. We proved successful adaptation of SureSelect hybridisation-based target enrichment protocol for the sequencing on the Ion Torrent S5 platform, which is designed to work preferably with amplicon-based panels. In our study, we applied a custom NGS panel to screen a cohort of unrelated patients affected by craniosynostosis.

2. We affirmed the clinical utility of NGS targeted gene panel approach as a valuable first-tier testing in the molecular analysis of patients presenting with facial dysostoses. We have delineated phenotypes and genotypes of eleven individuals presenting with distinct forms of facial dysostoses, in whom we described six novel and two previously described pathogenic variants.

Participation in conferences:

European Human Genetics Conference 2018. Gothenburg, Sweden 15-18 June 2019

Publications, scientific presentations as a leading author:

Publications:

1.Ewelina M. Olech, Karolina Matuszewska, Michał Piechota, Anna Latos-Bieleńska, Aleksander Jamsheer. Phenotypic description of two adult brothers presenting with mild form of Smith-Lemli-Opitz syndrome. Clin Dysmorphol. 2019 Mar 28:154–156. doi: 10.1097/MCD.0000000000000276.
2.Ewa Hordyjewska-Kowalczyk[#], Anna Sowińska-Seidler[#], Ewelina M. Olech, Magdalena Socha, Renata Glazar, Anna Kruczek, Anna Latos-Bieleńska, Przemko Tylzanowskiand Aleksander Jamsheer. Functional analysis of novel RUNX2 mutations identified in patients with Cleidocranial dysplasia. Clin Genet. July 26, 2019 vol. doi: 10.1111/cge.13610 [#]These two first authors contributed equally to this work.

Posters:

1. Anna Sowińska-Seidler, Magdalena Socha, Ewelina M. Olech, Aleksander Jamsheer. Tytuł: The effect of genomic structural variants on long-range chromatin interactions in patients affected by congenital limb malformations. EMBO Workshop, Kylini, Grecee 20-24 May 2019

2.Ewelina M. Olech, Anna Sowińska-Seidler, Delfina Popiel, Grzegorz Koczyk, Magdalena Socha, Joanna Walczak-Sztulpa, Anna Materna-Kiryluk, Anna Latos-Bieleńska, Renata Posmyk, Robert Śmigiel, Adam Dawidziuk, Aleksander Jamsheer. Tytuł: Diagnostic value of tarheted next-generation sequencing in craniosynostosis. European Human Genetics Conference 2018. Gothenburg, Sweden 15-18 June 2019

3. Ewelina M. Olech, Delfina Popiel, Grzegorz Koczyk, Anna Materna-Kiryluk, Magdalena Badura-Stronka, Marzena Wiśniewska, Anna Latos-Bieleńska, Aleksander Jamsheer. Targeted next-generation sequencing in the diagnosis of facial dysostoses. European Human Genetics Conference 2018. Gothenburg, Sweden 15-18 June 2019.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Mohit Kumar Sharma	Year of studies: 2nd

Title of the project: Design of proteins with unique geometry

Name of the supervisor: dr Jonathan G Heddle, prof. UJ

Affiliation: Malopolska Centre of Biotechnology, Jagiellonian University, Krakow

AIMS of the project :

Proteins are an excellent example of the relationship between form and function: The linear amino acid sequence typically folds into a precise three-dimensional structure with specific features which must be present for the protein to function. This is perhaps best demonstrated in enzymes where a protein structure may be necessary within tolerances at the Angstrom level to lower energy barriers between reactants. To be able to design de novo protein structures would clearly be very useful in both medicine and industry. Some progress has been made, precisely predicting the structure of proteins of any significant size is currently not practicable. It is possible to use existing protein building blocks of known structure and arrange them according to standard simple symmetries to make useful shapes, specifically hollow protein cages. However, it may also be possible to arrange proteins in non-standard geometries and to do so offers an interesting *in silico* challenge as well as the possibility of discovering new structures with associated novel capabilities.Protein cages such as virus capsids are clearly of medical importance because of their involvement in disease pathology. Protein cages have been extensively explored as functional entities in bio nanotechnology as drug delivery or gene delivery vehicles. The scope of functionalities and applications of protein cages can be significantly broadened if they are combined with synthetic polymers on their surface or within their interior.

TRAP-cage is a breakthrough but is only one type of cage of a certain size and characteristics. As a lab we are interested in carrying out basic research to understand how these cages form and to design and build new cages with different characteristics so that eventually in the years to come we will have a toolbox of protein cages which we can use in different medical and materials science applications. A part of this, my thesis has three major goals. The main aims are

1. One is to design software that is able to design novel protein cages.

2. The second part is to actually design and produce a new novel protein cage.

3. To design new, more complex protein cages.

Methods:

(1). RCSB-PDB: I used the RCSB-PDB protein databank (PDB) which provides public access to experimentally determined 3D-structures of biological macro-molecules (proteins, peptides and nucleic acids). It was used as a source of protein structures to be queried.

(2) Python & Biopython: Python programming language was used to write the script for finding the diameter of three dimensioanl protein structures for particular symmetry (cyclic symmetry). It is a dynamic, readable language that is a popular platform for all types of bioinformatics work, from simple one-off scripts to large, complex software projects. Python programming language (2x and 3x version), Biopython Modules, NumPy and Scipy packages.

(3) *In-silico* Design of 10mer TRAP: By using the Protein design and modelling software PyMol, Chimera we designed the the model of 10mer TRAP ring.

(4).Production and Purification of TRAP5 protein: I am doing experimetal work to validate our *insilico* generated model of TRAP ring (10mer). I am using the Bacterial culture: pET21b plasmid, BL21(DE3) bacterial strain. Protein purification: Cell lysis by sonication, Ni-NTA affinity chromatogrphy, Size Exclusion Chromatography (SEC)

Results:

(1). We have generated the tool based on different modules of python which tells us the diameter of proteins 3D structures with graphical representation. calcDiameter: a method to find the maximum diameter of a ring-shaped protein complex of symmetry Cn. The tool is developed using Python 3.7 version. (*The tool has been developed in collaboration with Dr. Jan Zaucha from Technical University of Munich, Germany*). Apart from this I have also generated the python scripts to probe the PDB database for finding the atom distance of CA atoms, to extract the cordinates, to fetch the structures by using pdb ids.

(2). We designed a gene in which 5 copies of the TRAP protein are fused together. When this protein expresses it will produce a single polypeptide chain that will consist of 5 copies of TRAP monomer covalently linked together. The first design I am trying is based on TRAP ring protein. The rotational symmetry is important for deciding the type of shape that is made – i.e. how many "sides" the protein has. Our TRAP ring has 11 sides.But we have imagined what would happen if we have a 10 sided TRAP, that means a TRAP ring where one monomer is removed. We have modelled the 10 sided TRAP ring design *in-silico.*Our TRAP 10mer acts like a pentamer and what shape is formed from pentamers – the classic 20-sided dodecahedron.

(3). The results after protein expression and purification are encouraging I found that the protein can be expressed and purified and that size exclusion chromatography (SEC) confirms the size of 42 kDa (approx). Now I am trying to make it possible TRAP-5 the next stage will be to react with a source of gold to cause the cage to form. This can then be characterised for example to see heat stability, disassembly in reducing agents etc. I would also like to see if it can be used encapsulate cargoes . As the structure will be completely new it will be interesting to try Cryo-EM and we have cryo-EM experts in the lab from whom I can learn this technique as well as access to the only high-end cryo -EM in Eastern Europe which will be at the Solaris synchrotron next door to MCB which we will be able to use.

Participation in conferences:

(1). Participated in MCB- Summer School held at Zakopane w.e.f 23- 24th May 2019.

(2). Participated in Bionano 2018 conference w.e.f 17-18th September 2018 at Institute of Zoology, Jagiellonian University, Krakow, Poland.

Publications, scientific presentations as a leading author:

(1). Presented poster entitled "Design of Proteins with unique geometry" in MCB- Summer School at Zakopane w.e.f. 23-24th May 2019.

(2). Presented poster entitled "PDBSPyer: Tool to probe the PDB (RCSB) Database" in Bionano 2018 conference w.e.f 17-18th September 2018 at Institute of Zoology, Jagiellonian University, Krakow, Poland.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Mantu Kumar	Year of studies: 2nd

Title of the project: Designing and production of highly programmable DNA-Protein hybrid Nano-structure for therapeutic cage delivery

Name of the supervisor: Prof. Jonathan Heddle

Affiliation: Malopolska Centre of Biotechnology, Jagiellonian University, Krakow

AIMS of the project:

There are following aims in my project: (1) To develop nanostructures which can carry multiple therapeutic agents to the cells. For this, we are designing a modular protein cage structure utilizing the unique property of an archaeal ferritin *Thermotoga maritima* (TmFtn), here cage assembly (dimer to 24-mer) triggered by metal/salt. (2) Tuning the cage assembly and stability by structural modification and genetic mutation. (3) Designing the DNA nanostructures for specificity and modularity. (4) Encapsulation and delivery of therapeutic molecules to the target cells.

Methods:

We are utilizing various biochemical and biophysical techniques in this study.

(1) Cloning, purification and mutant generation: cloning and expression of TmFtn gene done in pET vectors and BL21 (DE3) cells respectively. Mutants were generated using PCR based method-site directed mutagenesis (SDM).

(2) Characterization of TmFtn mutant: After cage assembly Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS) were used to measure morphology and hydrodynamic diameter respectively; all mutants imaged and accessed for the cage formation in different metals.

(3) Encapsulation was confirmed by the measuring encapsulated GFP fluorescence in size exclusion chromatography (co-elution of cage and GFP) and SDS PAGE. Further in TmFtn, Lysozyme was encapsulated and superlattice was formed using gold nanoparticles.

(4) Crystallization and structure determination of TmFtn mutants to decipher the role of the interdimeric interface in cage assembly and stability. Structure analysis was performed in chimera and coot.

(5) Click chemistry to combine protein cage and DNA structure; the labeled aptamer is conjugated with ferritin cage, confirmed by fluorescent measurement and Native PAGE.

Results:

We have successfully cloned and purified the *Thermotoga martima* ferritin, which is used for the encapsulation of enzymes and therapeutic molecules. The assembly of TmFtn performed by the addition of metal/salt, we found that TmFtn assembly efficiently triggered by divalent cations, we also observed, excess of salts can mediate cage formation. We have characterized cage formation by measuring cage hydrodynamic diameter in DLS and visualized under TEM imaging. Reversible assembly and disassembly of TmFtn confirmed. Excessive dilution and EDTA chelation break cage into intermediate/dimeric state. In the elution profile of SEC two individual peaks corresponding to the cage and dimer observed, which is visualized under TEM for their cage and intermediate state.

Further, we have encapsulated an enzyme (Lysozyme) in TmFtn and formed an enzymatically active cage. Encapsulation protects enzyme from extreme temperature and protease action. Enzyme filled

cage is used to form superlattice using gold nanoparticles, this result we have published recently in Nano Letters.

Based on crystal structure information we have designed a series of mutants, basically by changing charged residue at the interdimeric interface. We could able to tune the cage assembly and stability. E65K/R mutation reversed the TmFtn into canonical ferritin, which is self-assembled and extremely thermal stable up to 95^{o}C and can tolerate pH from 4-10. While E65Q/D shows weaker cage. From Native PAGE, SEC and TEM, we have confirmed TmFtn mutants structure shows differential assembly and stability.

To decipher the role of interface residues in cage formation we have crystallized TmFtn mutants and solving structures. A single mutant at interface seems crucial and able to reverse the cage assembly completely. We have utilized the TmFtn cage structure to conjugated the DNA nanostructures, and characterized, which will be further developed as a programmable cargo carrier. TmFtn proven to protect encapsulated cargo and can tolerate multiple modifications. The easy reversible assembly of TmFtn cage can be advantageous over existing method to package any therapeutic cargo inside and deliver to the target cells.

Participation in conferences:

1. M. Kumar, S. ChakrabortiI, J. Heddle. *An investigation to understand structural flexibility and salt tolerance mechanism of Thermotoga maritima ferritin*. Poster presented at 44th FEBS congress, from molecules to living system, Krakow, Poland, 2019

Publications, scientific presentations as a leading author:

1. Soumyananda Chakraborti, Antti Korpi, Mantu Kumar, Piotr Stępień, Mauri A. Kostiainen, Jonathan G. Heddle. *A three-dimensional protein cage array capable of active enzyme capture and artificial chaperone activity,* Nano letters, 2019. https://doi.org/10.1021/acs.nanolett.9b01148

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Karolina Majsterkiewicz	Year of studies: 3rd

Title of the project: Understanding the role of gold in production of protein-protein links and ordered protein assemblies

Name of the supervisor: dr Jonathan Heddle, prof. UJ

Affiliation: Malopolska Center of Biotechnology, Jagiellonian University, Krakow

AIMS of the project:

Work in the Heddle Laboratory has suggested that certain gold nanoparticles and gold(I) compounds can interact with a cysteine-containing mutant of TRAP (trp-RNA attenuation protein) to promote protein-protein bonds, which can result in production of highly stable artificial protein cage structures. The particular and so-far unique role of the GNPs and gold(I) compounds in TRAP cage formation is yet to be investigated.

Aim of the project is to establish the exact mechanism and kinetics of cage formation (small and large cage), in particular the role of gold and to apply this finding to the other proteins/peptides in order to build novel, designed, stable structures.

Methods:

Protein concentration measurement: absorption at 280 nm. Cage assembly: reaction was prepared by mixing suitable ratios of solutions of TRAP and gold compound (and/or other metal compounds) in suitable buffer, followed by incubation for at least 1h at room temperature; variant – in presence or absence of oxygen. Cage purification: SEC (size exclusion chromatography, Superose 6 /Superdex). Cage quality check and visualisation: native electrophoresis (Bis-Tris system, 3-20% gradient gels) / DLS / TEM (copper grids, negative staining with tungsten)). Protein chemical modification: reaction was prepared by mixing suitable ratios of solutions of TRAP and modifier (IAA – iodoacetamide, benzyl bromide, NEM – N-methylmaleimide, DTNB – Ellman's Reagent) in suitable buffer, followed by incubation for 1h at room temperature, protected from light. Protein and cage characterization: mass (SEC with RALS detector), stability tests (against: chaotropic agents – urea/guanidine hydrochloride; reducing agents: DTT/TCEP/glutathione; detergents: SDS; all of the agents were incubated with TRAP cage overnight; temperature; mechanical stress (AFM – atomic force microscope)), chemical modification (MS – mass spectrometry), structure (AFM).

Results:

- Large cage formation reaction in absence of oxygen showed no difference to reaction in presence of O_2. It indicates that O_2 does not play a major role in cage formation mechanism (it was speculated that oxygen may oxidise one of the reaction products, thus shifting reaction equilibration towards higher yield of cage formation),
- Cage formation can be promoted by other metals than Au(I), namely Ag(I), Cd(II), Hg(II) suggesting that metal-driven cage formation requires water-stable, d^{10} metal ions with preferred two-ligand linear geometry,
- TRAP purified with DTT (in the final, pure sample of TRAP there is no DTT) is more stable over time (less precipitation) and retains ability to form cages for longer periods of time than TRAP purified without DTT. Moreover, TRAP purified without DTT can form small cages more easily. That would suggest that Cys modification influences large to small cage ratio.

- Controlled chemical modification of Cys in TRAP (by thiol-blocking agents as IAA, benzyl bromide, NEM, DTNB) resulted in complete blocking of cage formation or inducing small cage formation (in condition previously used for large cage formation). That would suggest that Cys modification influences large to small cage ratio.
- Mass and AFM measurements (angle between rings) suggests 12 to 14 building blocks in small cage. This information can be used to prepare model of small cage.
- Stability tests indicate lower stability of small cage compared to large cage.

Future plans:

- Further analysis of chemical modification of Cys influence on small cage formation.
- Establishing model of small cage structure and confirmation by cryo-EM.
- Large cage formation step by step using iSCAMS (interferometric scattering mass spectrometry).
- Control over metal-driven cage formation by manipulation of coordination environment by chemical modification of Cys or protein engineering.

Participation in conferences:

1) GRC Physical Virology: Mechanistic Virology and Virus-Mimetics, 20 – 25.01.2019, Ventura, USA
2) Biomolecules and Nanostructures 7, 15 – 19.05.2019, Pomlewo near Gdańsk, Poland
3) E-MRS 2019 Fall Meeting, 15 – 19.09.2019, Warszawa, Poland.

Publications, scientific presentations as a leading author:

- Malay A.D., Miyazaki N., Biela A., Chakraborti S., Majsterkiewicz K., Stupka I., Kaplan C.S., Kowalczyk A., Piette B.M.A.G., Hochberg G.K.A., Wu D., Wrobel T.P., Fineberg A., Kushwah M.S., Kelemen M., Vavpetič P., Pelicon P., Kukura P., Benesch J.L.P., Iwasaki K., Heddle J.G., "An ultra-stable gold-coordinated protein cage displaying reversible assembly", *Nature*, vol. 569, no. 7756, pp. 438–442, May 2019
- Majsterkiewicz K., Biela A., Kowalczyk A., Piette B.M.A.G., Stupka I., Heddle J.G., "Gold(I) concentration and other factors as means to control protein cage assembly", poster at 1)
- Majsterkiewicz K., Biela A., Kowalczyk A., Piette B.M.A.G., Stupka I., Heddle JG., "Metal ion induced protein cage assembly – factors controlling the process", poster at 2)
- Majsterkiewicz K., Chakraborti S., Kowalczyk A., Piette B.M.A.G., Maity S., Roos W.H., Heddle J.G., „Large and small artificial protein cage – using gold to control size", poster and oral prersentation at 3).

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Katarzyna Bazydło-Guzenda	Year of studies: 2nd

Title of the project: Development of an innovative GPR40 agonist as a potential therapeutic in therapy of the type II diabetes.

Name of the supervisor: prof. Bożena Kamińska, dr Jerzy Pieczykolan

Affiliation: Celon Pharma S.A.

AIMS of the project:

A type II diabetes is a civilization disease that now has reached an epidemic status. GPR40 (or FFAR1-Free Fatty Acid Receptor 1) belongs to the G-protein coupled receptors (GPCRs) family and is strongly expressed in β-pancreas cells and in the brain. This receptor is activated by medium and long fatty acids and the receptor activation increases the calcium ions concentration in the cytosol by IP3-mediated Ca2+ ions release from the endoplasmic reticulum. The increase in the concentration of calcium ions in cytoplasm of the β-pancreas cells is a signal for insulin secretion. This mechanism makes a GPR40 agonist a potential therapeutic for use in diabetes therapy.

The aim of this project is to select a small molecule for further development of an innovative and unique anti-diabetic drug, which will induce insulin secretion without the risk of hypoglycaemia and side effects characteristic for competitive drugs.

Methods:

GLP-1 secretion: the compounds' activity in GLP-1 secretion was determined using STC-1 cells (ATCC) in KRBH buffer. GLP-1 levels after stimulation of the compounds at 10 and 100 μM in assay buffer was measured using Mouse GLP-1 ELISA test (EMD Millipore Corporation, USA).

Cytotoxicity/proliferation abilities: the compounds cytotoxicity/proliferation was determined in HepG2, SHSY5Y, MIN6, A375, T-47D cell lines after 48 hours of compounds stimulation using real-time glo test (Promega).

Microsomal stability studies: were performed on hepatic microsomes of dog, monkey and mini pigs (Invitrogen) in phosphate buffer in the presence of phase I and II metabolism cofactors (NADPH, UDPGA).

Glucose tolerance test (GTT): *in vivo* effect was examined in 12-14 weeks old male GK rats (GK/MolTac; Taconic) or 8-9 weeks old male db/db mice (BKS.Cg-Dock7m+/+ Leprdb/J,Charles Rivers) using an intraperitoneal glucose tolerance test (IPGTT). Animals were fasted for 6 hours (with free access to water) and at the time point t = -60 min received a single dose of the compound administered per os (p.o.) and then at t=0 glucose bolus (2 mg/kg) administered interperitoneal (i.p.) The glucose level in time points was measured by a standard Accu-Chek®Performa glucometer.

Pharmacokinetic study: the study was performed on male Wistar HAN (Crl: WI (HAN)) outbred rats from TAZD-CBU breeding, age 8-10 weeks or C57BL6/cmdb mice from CMDB breeding, age 8-10 weeks. Animals were fasted for 12 hours from t = -12h (with free access to water). At the time point t = 0 animals received a single dose of the compounds administered p.o. or intravenously (i.v.). Compounds concentration in rats serum and mouse serum, liver, brain, pancreas after compounds administration was measured in a different time points (up to 24h) using LC-MS/MS method.

Results:

Four GPR40 agonists selected from the screening phase have been tested in *in vivo* pharmacokinetic (PK) study in rats and the PK parameters like AUC, Cmax, Tmax, T1/2 or F were determined. For 2 most promising compounds with good pharmacokinetic parameters, glucose tolerance test (GTT) in diabetic models (GK rat and db/db mice) as well as PK study in mice were determined. One compound

– CPL207-280 with activities comparable or better than the reference GPR40 agonist – TAK-875 (Takeda Pharmaceulicals) was identified.
The selected compound CPL207-280 underwent preliminary ADME characterization (using microsomal stability test in monkey, dog and mini pig microsomes) and the ability to GLP-1 secretion in mice enteroendocrin cells (STC-1) was tested. Next the cytotoxicity/proliferation abilities of the compound in a few selected cell lines (HepG2, SHSY5Y, MIN6, A375, T-47D) was determined. As a result, one active *(in vitro* and *in vivo*) and safe compound with good PK parameters, leading structures – compound CPL207-280 - was identified.

Participation in conferences:

1.Young Scientists Conference on Molecular and Cell Biology, Warsaw, April 11, 2019

Publications, scientific presentations as a leading author:

1. Mach M., Dzida R., Smuga D., Stelmach F., Matłoka M, Bazydło K, Dubiel K, Wieczorek M., Pieczykolan J. "3-phenyl-4-hexynoic acid derivatives as GPR40 agonists". Patent application PCT/EP2019/050194.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Dominika Oziębło	Year of studies: 3rd

Title of the project: Genetic testing for hearing loss: implications for a more personalized partial deafness treatment

Name of the supervisor: Dr hab. n. med. Monika Ołdak

Affiliation: Medical University of Warsaw

AIMS of the project

Hearing loss (HL) is one of the most common sensory disorders that affects almost 466 million people worldwide. Each year, approximately 1-6 out of every 1000 children are born with severe to profound HL. For this group of patients cochlear implantation (CI) is the treatment of choice and early auditory intervention is crucial for their optimal cognitive development. Nevertheless, despite the advantage in CI technology there are still differences in children auditory development and there are only few studies attempting to combine the etiology of HL with CI outcomes.

In the majority of deaf children HL is genetically determined and usually two pathogenic variants are detected in the *GJB2/GJB6* genes (DFNB1 locus). In the last two years we have thoroughly analyzed auditory development of patients with pathogenic variants in DFNB1 locus and now we are focused on the non-DFNB1 group of implanted patients.

The aim of the study was to dissect the genetic background of HL in CI patients without DFNB1 locus pathogenic variants and to analyze their auditory development.

Methods:

The study group (n=50) was recruited from patients with isolated profound prelingual deafness who received cochlear implants before the age of 24 months. All patients were negative for DFNB1 locus pathogenic variants and had excluded both environmental HL risk factors as well as factors affecting auditory development. Detailed medical history including temporal bone imaging data was analyzed.

Genomic DNA was isolated from blood samples of deaf probands and available family members. In 34 patients whole exome sequencing (WES) was performed. Libraries were constructed using Twist Human Core Exome Kit (Twist Bioscience, USA) according to the manufacturer's protocol and sequenced on NovaSeq 6000 platform (Illumina Inc, USA) with read length 2x 100 bp.

After bioinformatics analysis, all pathogenic and probably pathogenic variants involved in HL development were selected based on their population frequencies (<1%), functional consequences (e.g.: missense, frameshift, splice-site) and pathogenic predictions (CADD, LRT, FATHHM, MutationTaster, PolyPhen-2, SIFT). Validation of selected variants and family segregation analysis were performed using standard Sanger sequencing and 3500xL Genetic Analyzer (Applied Biosystems, USA).

Evaluation of the auditory development was performed with the LittlEARS questionnaire (LEAQ) and relative auditory development delay (RADD) at 6th month after CI activation was calculated. Age of hearing aids (HAs) fitting, duration of hearing experience with HAs, effect of audibility provided by HAs and age at CI were analyzed.

Results:

In each of the tested subjects exome sequencing revealed at least 5 pathogenic variants located in HL related genes. Based on clinical features (age of HL onset, HL severity) as well as known inheritance pattern of HL characteristic for particular genes, causative variants were selected in 21 patients. The majority of detected variants are localized in *MYO15A* gene (n=7), followed by *MITF* (n=3), *TMC1* (n=3), *CDH23* (n=2), *OTOF* (n=2), *PCDH15* (n=1), *SLC26A* (n=1), *TMPRSS3* (n=1) and *TRIOBP* (n=1) genes. Despite the recruitment of patients with isolated form of HL, we have identified three patients with dominantly inherited Waardenburg syndrome due to heterozygous *MITF* variants and one patient with Usher type I syndrome and homozygous *PCDH15* pathogenic variant. In one patient with inner ear malformation (IP2, incomplete partition type 2) one known pathogenic variant in *SLC26A4* gene was identified. In the second allele Sanger sequencing detected the presence of previously described haplotype predisposing to the development of this malformation. Among the identified 38 disease causing alleles, 55% (21/38) contained novel genetic alterations. In the remaining 13 families with, so far, undetected genetic cause of HL, novel candidate variants and genes are selected and tested in probands and their families.

Mean RADD was 0.27 and varied between -0.40 and 0.82. There is no statistically significant difference in RADD between patients implanted early (before 12 months of age) and patients implanted late (after 12 months of age) (0.21 vs 0.36; p=0.12).

In order to determine the exact link between genetic background of HL and the CI outcome further genetic and clinical analysis are required. There is a possibility of involvement of novel genes or presence of pathogenic variants in regulatory regions of known HL genes in patients with still unidentified genetic cause of the disease.

Participation in conferences:

Author and co-author of 27 presentations and posters at domestic and international conferences.

Publications, scientific presentations as a leading author:

1. Ołdak M., Lechowicz U., Pollak A., Oziębło D., Skarżyński H. Overinterpretation of high throughput sequencing data in medical genetics: first evidence against *TMPRSS3/GJB2* digenic inheritance of hearing loss. J Transl Med, 2019, 17(1), 269.
2. Ołdak M., Domagała S., Oziębło D., Skarżyński H.: Genetics in otosclerosis. Now Audiofonol, 2018; 7(4): 11–18

1. Oziębło D., Leja M., Sarosiak A., Tacikowska G., Kochanek K., Skarżyński H., Ołdak M..: First independent confirmation of TBC1D24 as an autosomal dominant hearing loss gene and audiological characteristics of affected individuals. 32nd Politzer Society Meeting and 2nd World Congress of Otology, 28.05-01.06.2019 Kajetany, Warsaw, Poland

2. Oziębło D., Domagała S., Leja M., Skarżyński H., Ołdak M.: Genetic association study in Polish otosclerosis patients ‘ analysis of RELN and TGFB1 variants. 32nd Politzer Society Meeting and 2nd World Congress of Otology, 28.05-01.06.2019 Kajetany, Warsaw, Poland

3. Oziębło D., Obrycka A., Lorens A., Skarżyński H., Ołdak M.: Cochlear implantation outcome in patients with DFNB1 locus pathogenic variants - implications for precision medicine. European Human Genetics Conference 15-18.06.2019 Gothenburg, Sweden

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Weronika Bystrzycka	Year of studies: 3rd

Title of the project: The impact of di- and tri-valent metal ions on the creation and release of Neutrophil Extracellular Traps (NETs)

Name of the supervisor: dr hab. n. med. Olga Ciepiela

Affiliation: Department of Laboratory Diagnostics and Clinical Immunology of Developmental Age, Medical University of Warsaw

AIMS of the project:

Neutrophils are the most abundant type of immune cells in human and are described as the first line of defense against pathogens. To this end neutrophils engage several strategies including degranulation, phagocytosis, generation of reactive oxygen species (ROS) and netosis. Netosis is a form of neutrophil activation leading to release of decondensed chromatin into extracellular space. These fragile web-like fibers are composed of chromatin decorated with histones and antimicrobial proteins and are termed as neutrophil extracellular traps – NETs. According to recent research, it has been found that NETs, apart from DNA and antimicrobial proteins, include also trace elements as well as contain calprotectin and lactoferrin.

The overall aim of my studies is to advance the knowledge on the molecular basis of the interaction between vital microelements such as zinc and iron and netosis.

Methods:

Studies were carried out on neutrophils isolated by density gradient centrifugation and subsequent polyvinyl alcohol sedimentation for removal of erythrocytes from the blood of healthy donors. Cells were incubated with zinc and holo-transferrin (hT). Immunodetection of citrullinated histone H3 and the conversion of soluble LC3I to lipid bound LC3II were performed by Western blot analysis. Bactericidal activity of NETs after stimulation by *E.coli* was analyzed by counting the survived bacteria in released NETs.Phagocytosis and degranulation were analyzed by flow cytometry.

Murine neutrophils were isolated from the bone marrow by negative magnetic selection by depletion of non-target cells.Subsequently, neutrophils were incubated with the studied microelements and CI, LPS 0.128 or PAF were added to stimulate NETs release. Release of NETs was analyzed under the inverted fluorescent microscope and amount of extracellular DNA was quantified by fluorometry.Intracellular production of ROS was analyzed by fluorometrical analysis usingDHR123. Phagocytosis and degranulation were analyzed by flow cytometry.

Results:

Immunodetection of citrullinated histone H3 revealed that zinc inhibits citrullination-dependent NETs release. Moreover, zinc altered degranulation in neutrophils and affected the bactericidal activity of NETs towards *E.coli*.

The delivery of zinc, not hT, to murine neutrophils inhibited netosis after stimulation with CI. However, hT and zinc did not affect NETs release after stimulation with natural stimuli. Visualization of NETs by fluorescence microscopy confirmed the results of the quantification method.

Participation in conferences:

1. A.Manda-Handzlik, W.Bystrzycka, A.Cieloch, A.Skrobot, U.Demkow,M.Wachowska.Reactive nitrogen species are synthesized during neutrophil extracellular traps (NETs) formation and enhance NETs release. 5thInternational Conference of translational medicine on pathogenesis and therapy of immunomediated diseases. Milan, 16-18.05.2019
2. A.Manda-Handzlik, A.Skrobot, A.Cieloch, W. Bystrzycka, Z.Homoncik, M.Wachowska. Neutrophil extracellular traps formation is dependent on PI3K activity and autophagy – independent. 5thInternational Conference of translational medicine on pathogenesis and therapy of immunomediated diseases. Milan, 16-18.05.2019
3. Manda-Handzlik A, Wachowska M, Bystrzycka W,Ciepiela O, DemkowU.Crosstalk of autophagy and reactive oxygen species synthesis in reactive nitrogen species-induced neutrophil extracellular traps formation.
4. Ciepiela O, Bystrzycka W,Holka J, Tarnowska P, Manda-Handzlik A, Wachowska M. Stimulation of MAC-inhibitory protein (CD59) but no complement decay-accelerating factor (CD55) induces release of neutrophil extracellular trap (NETs). 5th Euroepan Congress of Immunology ECI 2019, Amsterdam, Netherlands 2-5.09.2018

Publications, scientific presentations as a leading author:

Increased temperature facilitates adeno-associated virus vector transduction of colorectal cancer cell lines in a manner dependent on heat shock protein signature". A.Bieńkowska, W.Bystrzycka, O.Ciepiela, J.Ochocki, M.Małecki. Biomed Res Int. 2019 – articleaccepted, in press.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Monika Turska	Year of studies: 3rd

Title of the project: Role of glutamate mechanisms in metabolism and proliferation of normal and pathological skin cells exposed to UVB radiation – *in vitro* study.

Name of the supervisor: dr hab. n. farm. Tomasz Plech, dr n. med. Katarzyna Walczak

Affiliation: Department of Pharmacology, Medical University of Lublin,

Postgraduate School of Molecular Medicine, Medical University of Warsaw

AIMS of the project:

The aim of the study was to determine the effect of memantine and kynurenic acid on expression and phosphorylation of selected DNA damage response proteins in melanoma cells exposed to UVB radiation.

Methods:

In order to determine the effect of memantine and kynurenic acid on expression and phosphorylation of selected DNA damage response proteins in melanoma cells A375 cells were treated with memantine (100 pM - 100 μM) or kynurenic acid (100 nM – 5 mM), exposed to UVB radiation at a dose of 500 mJ/cm^2 or 25 mJ/cm^2 and incubated for 2 or 24 hours, respectively. The similar experiment was performed with A375 cells treated with memantine (100 pM - 100 μM) or kynurenic acid (100 nM – 5 mM) for 2 and 24 hours without exposition to UVB. The effect of memantine and kynurenic acid on phosphorylation and expression of selected UV induced DNA damage response proteins: phosphorylation of cdc25c, Chk1 and ATR and expression of RPA32/RPA2, ATRIP and microcephalin-1/BRIT1 was determined with the use of Western Blot. Expression of β-actin was used as a loading control.

Results:

UVB radiation at a dose of 25 mJ/cm^2, which caused inhibition in proliferation of melanoma A375 cells after 24h, marginally affected phosphorylation and expression of selected UV induced DNA damage response proteins. Therefore, higher dose of UVB radiation – 500 mJ/cm^2 was used. It was chosen in the initial experiments after exposure to different doses of UVB – 50, 100, 500, 1000 mJ/cm^2. Cells were incubated for 2h due to cell death after longer time of incubation and high dose of UVB radiation.

UVB radiation at a dose of 500 mJ/cm^2 highly intensified the phosphorylation of Chk1 and slightly increased phosphorylation of ATR, ATRIP and expression of RPA32/RPA2. Kynurenic acid and memantine did not influence phosphorylation and expression of selected proteins under standard conditions.

In cells exposed to UVB radiation at a dose of 500 mJ/cm^2 kynurenic acid highly reduced phosphorylation of Chk1 and expression of RPA32/RPA2 induced by UVB. In addition it slightly lowered phosphorylation of ATR and ATRIP.

In cells exposed to UVB radiation at a dose of 500 mJ/cm^2 memantine reduced phosphorylation of ATRIP induced by UVB. The effect on other selected UV induced DNA damage response proteins was not evident.

Summing up, both kynurenic acid and memantine affect selected UV induced DNA damage response proteins. However, kynurenic acid has stronger influence on A375 cells exposed to UVB radiation at a dose of 500 mJ/cm^2 than memantine.

Participation in conferences:

- 24th Congress of Dermatology, Milan, Italy, 10-15 June 2019 - Effect of UVB on melanoma cells viability inhibited by kynurenic acid - in vitro study.
- IV Nadwiślańskie Spotkania z Dermatologią, Sandomierz, Polska, 16-18 maja 2019 – "Bliznowce - leczenie skojarzone", "Róg skórny".

Publications, scientific presentations as a leading author:

- Presence of kynurenic acid in alcoholic beverages – is this good news, or bad news? M. Turska, R. Rutyna, M. Paluszkiewicz, P. Terlecka, A. Dobrowolski, J. Pelak, MP. Turski, B. Muszyńska, W. Dąbrowski, T. Kocki, T. Plech. Medical Hypotheses 2019 Jan; 122:200-205.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Anna Salerno-Kochan	Year of studies: 3rd

Title of the project: Structural characterization of Mei-P26 protein - a central regulator of RNA biosynthesis during stem cell fate decision

Name of the supervisor: dr Sebastian Glatt

Affiliation: Malopolska Centre of Biotechnology, Jagiellonian University, Krakow

AIMS of the project:

Post-transcriptional gene regulation via RNA-binding proteins (RBPs) plays essential roles in the regulation of stem cell fate decision during early development of the D. melanogaster organism. In detail, nanosRNA Repressive Complex promotes stem cells differentiation by forming ribonucleoprotein particles (RNPs) with cis-elements present on 3' untranslated regions (3' UTR) of nanosRNA and repressing protein expression of the most important maintenance factors: Nanos (Nos) and Pumilio (Pum). In addition, stem cell fate decision might be regulated by bone morphogenetic protein (BMP) pathway or small interfering RNA pathway. Those two pathways are responsible for promoting germline stem cells (GSCs) maintenance via repression of the translation of differentiation factors.

Current PhD project focuses on structural and functional characterization of Mei-P26 protein, which has multiple functions during stem cells self-renew and differentiation processes. Mei-P26 is one of key factors during regulation of germline differentiation – (I) it is an integrated component of the nanosRNA Repressive Complex and block the Nos-Pum pathway and (II) it binds to Argonaut (Ago1) to repress the miRNA pathway. Moreover, mutations in mei-P26 lead to accumulation of undifferentiated cysts, which results in tumor formation in the ovary and testis. Interestingly, loss of mei-P26 impairs BMP signaling, suggesting that Mei-P26 is crucial both for stem cell self-renewal and differentiation. Mei-P26 belongs to TRIM (tripartite motif containing) – NHL (NCL-1, HT2A, LIN-41) protein family. Like other members of this group, Mei-P26 has a single RING domain with potential E3 ligase activity, B-Box coild-coil domain which is probable place for protein-protein interaction and NHL domain capable of binding RNA sequences and proteins.

The main aim of the PhD thesis is to obtain structural and functional insights into Mei-P26 protein and its interactions with its molecular partners (proteins and RNA) in order to understand the mechanism by which Mei-P26 participates in the regulation of the cell fate decision.

Methods:

Full-length Mei-P26, N- and C-terminal domains were obtained using Bac-to-Bac Baculoviruses Expression System, followed by affinity and size-excision chromatography purification. To solve the C-terminal domain structure *Biomolecular X-ray Crystallography* approach was used. NHL crystals were grown at room temperature using sitting drop diffusion method for two weeks in a presence of various commercially designed crystallization screens conditions and subsequently were fished, cryoprotected and snap frozen in liquid nitrogen. Data sets were collected at Synchrotron Radiation Source BESSY II Helmholtz-Zentrum Berlin. Mei-P26 NHL structure was computationally solved using standard programs for processing (*XDS*), phasing (*Phaser*), model building (*COOT*) and refinement (*Phenix*).

To access Mei-P26 mRNA binding specificity, *Microscale Thermophoresis (MST)* and *Electromobility Shift Assay (EMSA)* were performed with Cy-5 labeled oligonucleotides. As protein quality and proper folding controls SDS-page electrophoresis and *Thermalshift assay (TFA)* were conducted.

Results:

Our first goal was to get insight into an atomic resolution structure of the 33 kDa Mei-P26 NHL domain, located on C-terminus of the protein and crucial for protein-mRNA interaction. The domain had crystallized in several tested conditions and we have solved its structure at 1.6Å using molecular replacement approach with Brat NHL as an initial phasing model. Mei-P26 NHL folds into six-blended β-propeller with the blends distributed in radial fashion around central axis forming "donut-like" shape. Each of multiple repeated units is composed of four antiparallel β-sheets connected by flexible loops with different length and degree of order.

Additionally, we have conducted comparative analysis of *Drosophila melanogaster* Mei-P26 with *Drosophila melanogaster* Brat and *Danio rerio* Lin41 NHL domains that have the highest similarity score based on DALI structure alignment search. Importantly, all three proteins are the members of TRIM-NHL family and have the ability to interact with mRNA to fine-tune distinct cellular processes. Despite conformational resemblances between tested domains, we have spotted major differences in sequence alignment manifested by low conservation of amino acids important for protein-RNA interactions and highly diversity of top surface charges distribution between those domains, which together lead to different RNA target specificity.

Next, to access the Mei-P26 NHL ability to nucleic acids recognition, we have performed binding assay using short fragment of DNA, double stranded RNA (dsRNA) and single stranded RNA (ssRNA). Our data has clearly proven exclusive binding of ssRNA by Mei-P26. To gain complete knowledge about Mei-P26 target recognition specificity, we have collaborated with Bujnicki Laboratory to reanalyze the data form RNA compete experiments for Mei-P26 and Brat NHLs performed by Hughes Group. We have predicted 7-mer oligonucleotides sequences with the highest probability to be bound by Mei-P26 and subsequently experimentally validated the findings. In detail, we have tested six sequences with the highest Z-scores. Mei-P26 NHL binds four out of six sequences. The highest binding affinity was observed for UUUUACA sequence, which stays in agreement with the predictions. The comparable values we have obtained for UUUUUUU, UUUUUAC and AUUUACA. The estimation of binding affinities was not possible for the sequences UUUACAA and UUUACAC. Furthermore, we have extended the oligonucleotides library for several modified versions of previously tested sequences in order to check various working hypothesis. In summary, by performing binding affinity experiments we confirmed that Mei-P26 NHL has different target specificity than other known NHL domains and recognizes ssRNA with polyU stretches.

Moreover, by looking on local surface charges distribution and by Mei-P26 NHL-U-rich motif model analysis, we have hypothesized that the top surface works as mRNA binding platform. Therefore, we designed the set of Mei-P26 NHL mutated variants and performed the binding affinity experiments with UUUUACA and UUUUUUUUU sequences. We have spotted that single mutations of residues: K1172, R1018 or R1075 abolish the binding with UUUUACA sequence (but interestingly not with UUUUUUUUU), making them crucial spots for specific target recognition by Mei-P26 NHL.

Now, our short-term goal is to conclude Mei-P26 NHL studies in a form of a publication by combining structural, biochemical and biophysical data with a modeling (Bujnicki Group, Warsaw) and *in vivo* studies regarding Mei-P26 physiological target recognition (Medenbach Group, Regensburg). Moreover, the ultimate aim is to obtain the structural information about full-length Mei-P26 or Mei-P26 in the complex with target mRNA using Cryo-EM technique in order to better understand the function of the protein in the context of stem cell decision. To fully accomplish current project, the internship in Max Planck Institute of Biochemistry in Germany (Conti Laboratory) supported by ETIUDA 7 program (National Science Center) is planned for the next academic year.

Participation in conferences:

6/2019 RNA meeting, Krakow, Poland
11/2018 Microscale Thermophoresis (MST) Workshop, Nanotemper, Krakow, Poland

Publications, scientific presentations as a leading author:

6/2019 RNA meeting, Krakow, Poland – poster presentation
ETIUDA 7 Grant fellowship (for 2019/2020)

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Izabela Stupka	Year of studies: 3rd

Title of the project: Engineering of protein cages and their potential applications

Name of the supervisor: dr Jonathan Heddle, prof. UJ

Affiliation: Malopolska Centre of Biotechnology, Jagiellonian University, Krakow

AIMS of the project:

The aim of the project it to characterize TRAP protein cages obtained with the use of bismaleimidohexane (BMH) and dithiobismaleimidoethane (DTME) and to develop new ones with novel functionalities by using different crosslinking reagents. The main interest was to obtain the novel protein cage assembled by a photolabile crosslinker which would give us the opportunity to control its assembly by light irradiation.

Methods:

BMH/DTME crosslinked TRAP cage assembly and biophysical testing

$TRAP^{CS}$ were produced as described previously (Malay et al. Nature, 2019) in the presence of 2 mM DTT. 20 mM cross-linker stocks were prepared by dissolving BMH or DTME in DMSO. Cross-linked TRAP-cages were produced by 1 h incubation of fresh $TRAP^{CS}$ in PBS with 5-fold molar excess of either DTME or BMH supplied with 5 mM EDTA at room temperature with stirring. Concentrations of reactants were typically as follows: 0.5 mM TRAPCS and 2.5 mM DTME or BMH. Assembled cages were then purified by size exclusion chromatography using a Superose 6 Increase 10/300 GL column (GE Healthcare). Fractions containing cross-linked TRAP cages were pooled and concentrated using an Amicon Ultra 100 kDa MWCO centrifugal filter unit. Protein concentration was determined by Bicinchoninic acid assay (BCA) method using a commercial kit. The purity of obtained cross-linked TRAP cages were checked by native PAGE, and fidelity by negative staining transmission electron microscopy (TEM).

In order to establish the size of assembled particles, dynamic light scattering (DLS) was used. Their molecular mass was assessed by using right/low angle light scattering (RALS/LALS) method.

Photolabile TRAP cage assembly

Gold-induced TRAP cages were prepared as described previously (Malay et al. Nature, 2019). 1,3-bisbromomethyl-4-nitrobenzene was purchased from a commercial vender and dissolved in *N, N*-dimethyl formamide (DMF). 2 molar excess (to TRAP monomer) of 1,3 - bisbromomethyl – 4, -nitro-benzene was mixed with freshly purified gold-induced TRAP cage in 50 mM sodium phosphate buffer, pH 7.4 containing 5 mM EDTA while stirring at room temperature for 1 hour. 10 mM TCEP was then added to the reaction to capture Au(I). The sample was then purified by size exclusion chromatography using a Superose 6 Increase 10/300 GL column (GE Healthcare).

Photoinduced disassembly of 1,3 - bisbromomethyl – 4- nitro-benzene TRAP cage was tested by exposing the samples for varied time with 365-nm wavelength light in the presence of 1 mM DTT to quench the free radical species. The cage morphology and the crosslinker-cleavage process was monitored using dynamic light scattering (DLS) on a Zetasizer (Malvern), SDS and native PAGE.

Results:

Native PAGE gel electrophoresis results suggested production of novel TRAP cages obtained by using specific crosslinking agents which was then proved by TEM imaging. DLS method indicated the size of BMH/DTME TRAP cages to be approx. 25 nm and their molecular mass around 2.2 MDa.

In case of photolabile crosslinker it was not possible to use the same method of cages assembly as for the BMH/DTME crosslinkers probably due to the difficulties in controlling the right orientation of the rings. The novel templating method, using previously assembled gold-induced TRAP cages, enabled us to obtain the first photolabile TRAP cages which were visualized by TEM. DLS method indicated the size of 1,3 - bisbromomethyl – 4, - nitro-benzene cages to be approx. 24 nm making them smaller than the previously described ones due to the presence of the shorter crosslinker. SDS PAGE showed a clear appearance of TRAP dimers after crosslinking reaction proving the presence of covalent bonds in the photolabile TRAP cages which disappear after UV irradiation.

In vitro testing of photolabile disassembly indicated the need of a quencher in the cages solution during the irradiation process to prevent the reversibility of the cleavage. The optimization of this process indicated the minimal quencher (DTT) concentration to be approx. 1 mM. Native PAGE method showed that the photolabile TRAP cages need to be irradiated for around 2 minutes to be disassembled in tested concentrations and DLS method proved that the disappearance of the band corresponding to the cage occurs due to a disassembly process rather than random aggregation.

Participation in conferences:

1. GRC Physical Virology: Mechanistic Virology and Virus-Mimetics, 20 – 25.01.2019, Ventura, USA
2. Biomolecules and Nanostructures 7, 15 – 19.05.2019, Pomlewo near Gdańsk, Poland

Publications, scientific presentations as a leading author:

1. Malay A.D., Miyazaki N., Biela A., Chakraborti S., Majsterkiewicz K., Stupka I., Kaplan C.S., Kowalczyk A., Piette B.M.A.G., Hochberg G.K.A., Wu D., Wrobel T.P., Fineberg A., Kushwah M.S., Kelemen M., Vavpetič P., Pelicon P., Kukura P., Benesch J.L.P., Iwasaki K., Heddle J.G., "An ultra-stable gold-coordinated protein cage displaying reversible assembly", Nature, vol. 569, no. 7756, pp. 438–442, May 2019
2 Stupka I., Biela A., Miszczak K., Heddle J.G., "Highly-stable, chemically-induced protein cages, poster and oral presentation at GRC Physical Virology: Mechanistic Virology and Virus-Mimetics", 20 – 25.01.2019, Ventura, USA
3 Stupka I., Biela A., Miszczak K., Heddle J.G., "Chemical crosslinking as a novel method for obtaining artificial protein cages"; awarded best poster presentation at Biomolecules and Nanostructures 7, 15 – 19.05.2019, Pomlewo near Gdańsk

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Łukasz Mazurek	Year of studies: 3rd

Title of the project: The molecular mechanisms of pentapeptide repeat proteins action on DNA gyrase elucidated by structural biology methods

Name of the supervisor: Prof. Jonathan Heddle, Dr Dmitry Ghilarov

Affiliation: Małopolska Center of Biotechnology, Jagiellonian University, Krakow

AIMS of the project

Topoisomerase acting pentapeptide repeat proteins (TA-PrPs) are a group of proteins sharing a common fold which are able to act on bacterial topoisomerase II – gyrase. DNA gyrase remodels the bacterial chromosome by introducing negative supercoils, playing an essential role in compacting the genome and solving topological challenges associated with transcription and replication. TA-PrPs are known to protect gyrase against fluoroquinolones (Qnr) and bacterial toxins targeting gyrase such as albicidin (AlbG). TA-PrPs are postulated to be DNA mimicking proteins owing to their approximate organization and surface charge similar to dsDNA. The exact mechanism of TA-PrPs action is still unknown. Presently there are three hypothesized models of how TA-PrPs act: 1. By directly competing for the DNA in the active site of DNA gyrase (i.e. mimicking a G-segment); 2. By selectively binding to the formed gyrase-DNA-drug complex which leads to the dissociation of drug; or 3. By mimicking the T(transported)-segment and being recycled through the enzyme.QnrB1 as a model TA-PrP has been purified and studied in order to analyse its interactions with DNA gyrase and establish new up to date model of TA-PrPs action.

Methods:

Evolved Methanocaldococcus jannaschii aminoacyl-tRNA synthetase(s) (aaRS)/suppressor tRNA pairs were used to introduce unnatural amino acids (UAA): 4-Benzoyl-L-phenylalanine or 4-Azido-L-phenylalanine. The system incorporates UAA in response of amber (TAG) stop codon. Abovementioned amino acids are photoreactive and can be crosslinked upon UV light radiation. This allows mapping of interaction the UAA-containing. Several amber stop codon mutants of QnrB1 protein were created based on residue position and conservation. In total 29 mutants were created using overlap extension PCR. In Vivo crosslinking was performed first in order to find crosslinking residues. *For* in vivo *photo crosslinking experiments Dharmacon ™ GyrA and GyrB SPA tag strains* of DY330 *E. coli* were used. SPA contains triple FLAG epitope allowing to observe protein using western blot. QnrB1 protein posses his tag on N-terminal allowing to discriminate between QnrB1 and gyrA/B subunits. 10 ml culture of cells were grown in 37°C in a presence of 1 mM UAA until it reached OD600 = 0.6. Expression of QnrB1 protein was induced by addition of 10 mM arabinose and the culture was allowed to grow for a further 3 hours. Then the cells were placed upon λ=365 nm for 30 minutes in PBS buffer. The radiated and irradiated cells were compared using western blot. Positive *in vivo* photo crosslinking mutants were further purified using Ni-NTA chromatography and further studied *in vitro*. Purified gyrase complex or subunits were mixed with UAA containing QnrB1 proteins in different conditions to check influence of different drugs and nucleotides on crosslinking efficiency. The crosslinks were analysed using Biorad TGX™ gels Finally, mass spectrometry analysis was performed to locate the interaction position and provide quasi structural data of QnrB1 and DNA gyrase mechanism of interaction.

Results:

Out of 29 screened residues 4 gave positive result in crosslinking *In Vivo*. Resiudes Q51, R77, Y123 and R167 were crosslinking specifically to GyrB subunit. We have not observed any residues crosslinking to GyrA subunit. Four crosslinking residues are located on the same plane of the Qnrb1 protein surface. After purification of QnrB1_BpA mutants we identified residues Y123 an Q51 as strongest crosslinking partners. Detailed *In Vitro* analysis has showed that the QnrB1 crosslinks to B43 gyrase domain. B43 consists of ATPase and transducer domain. We were able to show that in the presence of non-hydrolysable analogue of ATP – ADPNP crosslinking efficiency is reduced drastically. This observation suggest that N-terminal gate of DNA gyrase needs to be open to let QnrB1 interact. Preliminary results from mass spectrometry analysis shows that QnrB1 protein is crosslinking to very end of ATPase domain. We have not observed crosslinking to ATPase domain alone which suggest whole B43 domain (ATPase + transducer) is required for appropriate interaction of DNA gyrase an QnrB1.

Collected data contradicts a simple G-segment-displacement model for interactions of PrPs with gyrase. Obtained results favours the T-segment mimicry model as a mode of TA-PrP action. Furthermore, it we have shown that homologous TA-PrP AlbG also crosslinks to DNA gyrase in a similar manner. The data suggests that proposed mode of action is similar among different TA-PrPs.

Participation in conferences:

- 2018 Bionano conference (Krakow, Poland)
  2019 FEBS conference (Krakow, Poland)
- 2019 EMBO Workshop on DNA topology and topoisomerases in genome dynamics (Les Diablerets, Switzerland)
- LS2 Satellite Meeting on DNA Topology and Topoisomerases in Genome Dynamics (Les Diablerets, Switzerland)
- Prot XRD worshops (Krakow, Poland)

Publications, scientific presentations as a leading author:

- 2019 FEBS conference (Krakow, Poland) – Poster presentation
- 2019 EMBO Workshop on DNA topology and topoisomerases in genome dynamics (Les Diablerets, Switzerland) – Poster presentation
- LS2 Satellite Meeting on DNA Topology and Topoisomerases in Genome Dynamics (Les Diablerets, Switzerland) – Oral presentation
- Prot XRD worshops (Krakow, Poland) – Poster presentation.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Karol Zakrzewski	Year of studies: 3rd

Title of the project: Structural insights into the role of Kti11 as Elongator regulatory factor in the context of tRNA modification process

Name of the supervisor: dr Sebastian Glatt

Affiliation: Małopolska Center of Biotechnology, Jagiellonian University, Krakow

AIMS of the project:

Process of decoding the information from sequences in messenger RNA (mRNA) and converting it to proteins is called translation. This one of the most crucial process for the cell's survival, requires precisely control to ensure the correct assembly of the proteins in specific time and cell department. One of the fundamental players responsible for tRNA modifications is a large, conserved and multi-protein complex called Elongator. Full complex is formed by two copies of each of its six highly conserved subunits (Elp1-6), which are necessary for complex assembly and enzymatic activity which in combination with additional modification pathways leads to formation of specific conversion of uridines in the wobble base position of tRNAs.

Current PhD project focuses on revealing and defining the role of regulatory factors (Kti11 and Kti13) of Elongator. Presently it is known that those regulatory elements are crucial for proper function of the tRNA modifying complex, nevertheless their individual functions and whole molecular mechanisms of regulation and interactions remain unclear.

The main aim of the PhD thesis is to gain structural and functional information about the exact interaction between Kti11 and Kti13 regulatory elements and catalytic domain of Elongator (Elp3) and define the other interaction network of this proteins to understand the mechanism of tRNA modification process.

Methods:

Proteins were obtained using E. coli and yeast *Saccharomyces cerevisiae* expression system, followed by affinity and size-excision chromatography purification. Application of Biomolecular X-ray Cystallography allowed to solved the structure of Kti11-Kti13 heterocomplex. Crystals were obtained by using sitting and hanging drop diffusion method in various commercially designed crystallization screens stored for three weeks in room temperature. Data sets were collected at synchrotron radiation source BESSY II Helmholtz-Zentrum Berlin.

The quality and purity of the proteins and domains were checked by SDS-PAGE. To analyze the interactions between individual proteins or sub-domains we were used GST-pull down assay in different orders.

Results:

Last year, we were able to gain *Chaetomium Thermophilum* Kti13 which allowed us to examine the importance and contribution of this protein for whole interaction network in the context of tRNA modification. In next step we purify the stable Kit11-Kti13 *Chaetomium Thermophilum* herocomplex using colysis approach. After the process of optimization protein purification conditions, we have achieved the purity of the complex allowing crystallization tests to be undertaken.

The complex had crystallized in several tested conditions and we have solved its structure at 2.8Å using molecular replacement approach with Saccharomyces cerevisiae Kti11-13 complex as an initial

phasing model. Despite the attempts made, no better resolution could be obtained. Current result is the first structure of the complex obtained from native proteins. Previous models from other organisms were obtained from fusion proteins or by truncation method. In the structure of complex, Kti11 indeed binds to Kti13 through the conserved hydrophobic region on top of the WD40 propeller and is twin to already known structures.

Additionally, it was possible to obtain and purify a stable complex of *Methanocaldococcus infernus* Elp3 with *Chaetomium Thermophilum* Kti11-Kti13. Currently, many crystallization attempts are underway in various conditions. Moreover, obtaining Kti13 allowed us examination the effect of this protein on discovered new link between Elongator tRNA modification with thiolation pathways. In detail, our previous results based on GST-pull down assays shows that Kti11 may have double role as a molecular switch between different tRNA modification pathways. Presently we investigate another important aspect of the project - the influence of Kti13 on the role of Kti11 in tRNA thiolation process.

Our data has proven that Kti13 did not interact with thiolation pathway and presumably is a switcher which determine role of Kti11 in tRNA modification process. Alone Kti11 could bind to thiolation pathway proteins and probably support this process, but when Kti11 forms heterodimer with Kti13 it leads to regulation of Elongator activity.

Now, our short-term goal is to receive structures of MinElp3-ChtKti11-ChtKti13 complex and structure of Kti11 with proteins involved in thiolation of tRNAs using Biomolecular X-ray Cystallography or cyro-electron microscopy. Moreover, we will investigate these interactions with fully assembled Elongator complex using Cryo-EM and subsequently, we will perform side directed mutagenesis, which allow us to define the role of exact amino acids in the whole process.

Participation in conferences:

21st Heart of Europe Bio-Crystallography Meeting 20th – 22nd September 2018 Quedlinburg, Germany
Young Investigator PhD Course EMBO 13th-20th January Heidelberg Germany

Publications, scientific presentations as a leading author:

Lin T. et. all "The Elongator subunit Elp3 is a non-canonical tRNA acetyltransferase." Nat Commun. 2019 Feb
Krutyhołowa R. Zakrzewski K. Glatt S. "Charging the code - tRNA modification complexes." Curr Opin Struct Biol. 2019 AprEpub 2019 May 16.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Mikołaj Sokołowski	Year of studies:3rd

Title of the project: Modification of the tRNA wobble base position by Ncs2/Ncs6

Name of the supervisor: dr Sebastian Glatt

Affiliation: Małopolskie Centre of Biotechnology, Jagiellonian University, Krakow

AIMS of the project:

Aims of the project encompass the study of Ncs2/6 protein complex and its role within the thiolation pathway – the addition of a unique sulfur atom modification to selected tRNAs at a specific position of the wobble base uridine within the anticodon. This gives rise to the s^2U_{34} tRNA modification. Lack thereofre has been associated with severe diseases in humans, and results in a plethora of cellular defects, in particular in the context of the stress response. Even though the primary protein components of the thiolation pathway have been identified, there is very little known about the regulation of the Ncs2/6 protein complex activity, specificity towards only a subset of the entire tRNA pool and the mechanism of the thiolation reaction itself. Therfore, we aim at biochemically characterizing Ncs2, Ncs6, Ncs2/6 proteins within the thiolation cascade, resolve the atomic structure of Ncs2 and Ncs6 using macromolecular crystallography as well as elucidate the thiolation reaction mechanism.

Methods:

Methods used include standard vector cloning and protein expression techniques in recombinant *E.coli* cells and subsequent purifications with a variety of liquid chromatography techniques. Purified protein samples are used in high throughput crystallization trials as well as studied using biochemical assays, such as: pulldown assay, thermofluor assay, microscale thermophoresis (MST), dynamic light scattering (DLS), mass spectrometry, electrophoretic mobility shift assay (EMSA) as well as analytical size-exclusion chromatograhy (SEC) for protein-protein interaction.

Results:

Individual, *E.coli* expressed, soluble subunits of Ncs2/6 complex were originally identified from *Chaetomium thermophilum*, allowing for extensive biochemical studies.
Ncs2, Ncs6, Ncs2/6 proteins were analyzed biochemically for their thermostability, stability over time over a temperature gradient as well as interactions with tRNAs, tRNA fragments and invidiual phosphonucleotides. While all proteins can be successfully expressed and purified in a soluble fraction, Ncs6 exhibits poor stability and tends to undergo proteolysis. To combat this, addition of phosphonucleotides, such as ATP/ADP/GTP can be added. Furthermore, complex formation of Ncs2/6 greatly increases the stability of the Ncs6 subunit. Therefore, complex formation was further studied using affinity pulldowns, ITC, and analytical SEC, revealing that Ncs2 and Ncs6 will readily form a heterodimer complex. Oligomeric state of Ncs2, Ncs6 and formed Ncs2/6 complexes was additional examined with RALS-LALS coupled detectors and DLS. Comparison of the elution retnetion to a range of mass standards, and further validation with light-scattering techniques revalead that Ncs2 elutes primarily as a monomer, Ncs2/6 complex as an equilibrium of dimers and tetramers, and Ncs6 as a broad range of high oligomers, with a small dimeric fraction. Interstingly, isolation of either Ncs6 peaks will reconstitute the original equilibrium when immediately reanalyzed. Moreover, unlike Ncs6, Ncs2/6 complex heterodimers are stable, and do not readily form larger oligomers.
Ncs2/6 place in the context of the entire thiolation cascade was examined by affinity pulldowns. Urm1 was found to specifically interact with Ncs6, but not Ncs2. Interestingly, Urm1 was shown to have

almost no affinity towards the Ncs2/6 heterodimer. Strikingly, Ncs2/6 isolated tetramer recapitulated the interaction with Urm1. This result points towards distinct conformations of Ncs2/6 complex. Moreover, Kti11, a regulatory factor of the Elongator complex was also found to specifically interact with Ncs6, but not Ncs2, indicating a previously unknown, direct protein link between the thiolation cascade, and Elongator-mediated tRNA modification. Surprisingly, a generated Ncs6 mutant with removed cysteines responsible for the coordination of an enzymatically critical iron-sulfur cluster (Ncs6 Fe/S C→S) shows loss of affinity towards Kti11.

Extensive efforts were dedicated to the generation of high quality protein crystals of either Ncs2, Ncs6, Ncs2/6. Initially, large amounts of highly pure individual protein components as well as Ncs2/6 complex were produced, and all available crystallization screens were tested, at various protein concentrations, without successful hits. Further mutants of Ncs2 and Ncs6 were developed, targeting disordered terminal loops or the putative dimerization interface. Regrettably, the majority of thus generated constructs were of poor stability and were prone to degradation. Subsequent crystallization strategy includes anaerobic resconstitution of the iron-sulfur cluster in Ncs6, using a customized anaerobic chamber and a high-throughput nanodispensing robot.

Participation in conferences: n/a

Publications, scientific presentations as a leading author: n/a

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Justyna Czarnik-Kwaśniak	Year of studies: 2nd

Title of the project: Antibodies against anti-citrullinated chemokines and their role in pathophysiology, clinical diagnosis and prognosis in rheumatoid arthritis

Name of the supervisor: dr Oleksandr Korchynskyy

Affiliation: Medical Faculty of University of Rzeszow

AIMS of the project:

Our main goal is to understand the actual functional importance of protein citrullination in the development and progression of RA and molecular mechanism that leads to aberrant citrullination of protein targets. On the way to succeed in our project we aimed to create an efficient procedure for preparation of stable citrullinated MCP-1/CCL2 chemokine, which can be used for different research and potentially diagnostic applications. At the current step, we have to confirm our hypothesis that citrullinated chemokines can be stabilized and protected from quick degradation by post-translational modifications, primarily glycosylation, occurring in mammalian cells.

Methods:

Cell culture: The studies were performed using immortalized human embryonic kidney 293T cells (HEK 293T) and codon-plus *Rosetta* strain of *E.coli*.

MCP-1/CCL2 DNA cloning**:** Full-length MCP-1/CCL2 complementary DNA (cDNA) was cloned from cDNA prepared from IL-1β-treated (10 ng/ml) primary synovial fibroblasts derived from RA patient. The cDNA fragment encoding mature protein was PCR-amplified using primers with an incorporated *Nco*I restriction site fused in a frame with upstream 6 codons encoding histidines in the forward primer along with an endogenous stop codon and the *Eco*RI restriction site in the reverse primer.

Transformation of *Rosetta E.coli cells* and purification of recombinant human MCP-1/CCL2
The codon-plus *Rosetta* strain of *E.coli* was used for transformation with bacterial expression vector pET19b to produce the full length mature 6His-tagged MCP-1/CCL2 chemokine.

Transfection of HEK 293T cells and purification of recombinant human MCP-1/CCL2: Verified full length mature 6His-tagged MCP-1/CCL2 cDNA insert was re-cloned into efficient mammalian expression vector pcDEF. All the mammalian expression vectors were transfected into HEK 293T cells using PEI reagent to collect supernatants that contain mature chemokine. 6His-tagged version of chemokine was transformed into codon-plus *Rosetta E.coli* cells and 2-liter cultures for chemokine were grown upon protein synthesis induction with IPTG for 6 hours. 6-His-tagged version of chemokine from bacterial or HEK293T cellular lysates were purified with ProBond nickel beads (Invitrogen) and eluted with 200 mM imidazole. A quality and a quantity of expressed recombinant proteins were assessed with appropriate ELISA duo-sets (R&D Systems) specific for these proteins and with coloidal Coomassie staining upon SDS-PAGE resolution.

In vitro citrullination of MCP-1/CCL2: After the concentration of purified MCP-1/CCL2 was measured using R&D Duo-Set ELISA kit, purified recombinant human chemokine was incubated with rabbit skeletal muscle PAD.

Detection of recombinant human MCP-1/CCL2 by ELISA and western blotting: Anti-modified citrulline ELISA-based method uses a commercial ELISA kit developed for the detection of total chemokine in a combination with Senshu's antibody that recognizes modified citrullines. Analysis of the experiment results was performed using western blotting.

Results:

In this part, the main goal of our work was to generate a procedure for preparation of stable citrullinated MCP-1/CCL2 chemokine suitable for research applications.
Using Western blot analysis we found, that both self-made bacterially produced MCP-1/CCL2 and commercially available recombinant MCP-1/CCL2 were quickly degraded during *in vitro* citrullination. Therefore, 6xHis-tagged version of MCP-1/CCL2 chemokine cDNA was further recloned into mammalian pcDEF expression vector in order to confer a high expression level of MCP-1/CCL2 chemokine. The mammalian expression vector was transfected into HEK 293T cells using PEI reagent. Cellular pellet with glycosylated full length mature 6His-tagged MCP-1/CCL2 was collected and chemokine was purified from lysate with ProBond nickel beads and eluted with imidazole. A quality and quantity of expressed recombinant protein from *E.coli* BL21 and HEK293T cells were determined using ELISA duo-sets specific for this protein and the absorbance was measured. It was shown that *in vitro* citrullinated mammalian cells-produced but not bacterially produced MCP-1/CCL2 chemokine can be efficiently used as the standards in ELISA. Glycosylation that is lucking in bacterially-produced proteins but occurs in mammalian cells stabilizes citrullinated MCP-1/CCL2 and protects the chemokine against rapid partial degradation. Besides, mammalian cells-produced properly glycosylated MCP-1/CCL2 can be further citrullinated *in vitro* and efficiently used as the standard in citrullination-specific ELISAs as well as in biological investigations or as a bait for generation of diagnostic kit for detection of anti-citrullinated chemokine antibodies.

Participation in conferences:

1. Co-author of poster presentation: "Glycosylation in mammals protects citrullinated chemokine MCP-1/CCL2 from partial degradation" Korchynskyy O., Czarnik-Kwaśniak J., et.al, EULAR, Madrid, June 2019

Publications, scientific presentations as a leading author:

1. „How genetic predispositions may have impact on injury and success in sport". Czarnik-Kwaśniak J., Kwaśniak K., Tabarkiewicz J. EJCEM (2018)
2. „The contribution of selected variants in ACE, MSTN and ADRB2 genes in the achievements of judo practitioners". Kwaśniak K., Myszka A., Czarnik-Kwaśniak J., Tabarkiewicz J. Archives of Budo (2018).

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Keerthiraju Ethiraju Ravichandran	Year of studies: 2nd

Title of the project: tRNA Modifications in Human Diseases- The structural and functional consequences of Urmylation on Ahp1 and tRNA thiolation pathway in response to Oxidative Stress

Name of the supervisor: Dr Sebastian Glatt

Affiliation: Max Planck Laboratory, Malopolska Centre of Biotechnology, Jagiellonian University, Krakow

AIMS of the project:

The prime aim of the project is to study the structural and functional consequences of urmylation on Ahp1 and the tRNA thiolation components during oxidative stress response. The decisive aim is to crystallize urmylated- Ahp1/Uba4/Elp/Ncs2/Ncs6 and/or urmylated-Ncs2-Ncs6, in case urmylation permits Ncs2/Ncs6 heterodimerization. I also hope to understand the nature of these ancient post-translational amendments, that stand at the interface amid prokaryotic sulfur relay systems and the eukaryotic ubiquitination pathway. Therefore, I aim to establish and optimize urmylation *in vitro*, recapitulate the regulatory networks under stress conditions and identify components that confer substrate specificity. The anticipated outcomes of this research work would afford profound molecular perceptions in to how urmylation auto-regulates Ahp1, Elongator, Uba4/Urm1-pathways and respective stress signaling pathways under oxidative stress conditions.

Methods:

I used standard molecular biology methods such as PCR, Cloning, mutagenesis, agarose gel electrophoreses, transformation and plasmid purification to generate expression plasmids that allow us to produce genes of interest in *E. coli*. Urm1-COSH was produced using established and described methodology by the collaboration partner (S. Leidel). Crystallization of the proteins was carried using an automated screening robot.

Results:

Preliminary interaction studies of Urm1COSH with Ncs2, Ncs6, and Ncs2/6 were performed successfully, but the predicted interaction with Elongator subunits could not be confirmed. Therefore, we decided to focus on studying the interaction and functions of Ahp1, which is the only protein shown to be urmylated also *in vivo*. Mutational analyses of Ahp1 in yeast (by our collaborator Prof. Dr. Raffael Schaffrath) have provided us with extremely useful information to obtain the complex between Urm1 and Ahp1 variants also *in vitro*. With a stable thiocarboxylated Urm1 and initial extensive studies of the post translationally modified protein, I established the first ever successful invitro Urmylation assay with the known invivo target from yeast, *Saccharomyces Cerevisiae* Ahp1. The *Invitro* urmylation was complemented with both the *Saccharomyces cerevisiae* and *Chaetomium thermophilum* Ahp1 and Thiocarboxylated Urm1 respectively. Strong results providing information on the requirement of thiocarboxylated Urm1 is needed for Urmylation and that the bond formed is a covalent one. This in fact sheds light on Urmylation's unique mechanism with only E1(activating step), exempting E2 (conjugation step) and E3 (termination step) from the evolutionary conserved Ubiquitin like proteins. Both *Saccharomyces cerevisiae* and *Chaetomium thermophilum* Urmylated Ahp1 complex were crystalized and now we have the *Chaetomium* Urmylated Ahp1 complex structure at 2.6 Å resolution need to be solved.

Lately, Urmylation of Uba4 has also been introduced, which have provided insight for the need of mechanism of Uba4's rhodanese domain in passing sulfur to the Urm1 and not the AT domain itself.

Initial evidence of *Invitro* Urmylation of Ncs6 and Elp1, the other targets shown to be urmylated *Invivo*.

Conclusions:

The post-translational modification of urmylation seems to be more intricate than previously anticipated. Once the complex structure of urmylated Ahp1 is solved, we will understand the nature of these ancient post-translational modification system. In addition, our work aims to decipher the connection between tRNA modifications and evolutionary conserved oxidative stress mechanisms, that are placed at the interface amid prokaryotic sulfur relay systems and the eukaryotic ubiquitination pathways.

Future plans:

My next ambitious plan is to get the *in vitro* complex of the Urmylated Uba4. By understanding the structural aspects of the complex formation of the urmylated-Ahp1 and urmylated-Uba4, we would also be able to comprehend urmylation in humans and its role in oxidative stress pathways and tRNA thiolation pathway.

Participation in conferences: N/A

Publications, scientific presentations as a leading author:

1. Harmen Hawer, Alexander Hammermeister, Keerthiraju Ethiraju Ravichandran, Sebastian Glatt, Raffael Schaffrath, and Roland Klassen. Roles of Elongator Dependent tRNA Modification Pathways in Neurodegeneration and Cancer. Genes, Review Dec 2018
2. Marta Pabis, Martin Termathe, Keerthiraju Ethiraju Ravichandran, Mikolaj Sokolowski, Roscislaw Krutyholowa, Sebastian Leidel, Sebastian Glatt. (Manuscript under preparation)
3. One research article is currently in preparation with our collaboration partner prof. Dr. Raffael Schaffrath (University of Kassel, Germany).

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Nour-el-hana Abbassi	Year of studies: 2nd

Title of the project: Biochemical and structural characterisation of Elp3

Name of the supervisor: Dr. Ting Yu Lin, Dr. Sebastian Glatt

Affiliation: Malopolska Center of Biotechnology, Jagiellonian University, Krakow

AIMS of the project:

Transfer RNA (tRNA) has a crucial role in protein expression as it is the only molecule that can properly match an mRNA codon to an amino acid. This process can be disturbed if the bound between the third nucleic acid on the codon and first on the anticodon (34) is too weak, we call it the wobble position. Only A and U can lead to such a loss in information, here we focus on the wobble U_{34} and the chemical modifications that life developed to overcome the mismatches. We will be focusing on the mcm^5 and ncm^5 modifications that are in part performed by the Elongator Complex. In Eukaryotes, this complex is composed of two copies of six subunits (Elp1-5) and is responsible for the methylation on the position ^{5} of the U_{34}. The catalytic subunit Elp3 is singlehandedly responsible for the tRNA binding and the hydrolysis of the cofactor Acetyl-coA (ACo). However, the proper mechanism of the modification is still elusive.

In this project we aim to establish the sequence of events for the modification using an archaea model of Elp3 (MinElp3) as well as establishing the amino acid residues responsible for the tRNA binding and the ACo Hydrolysis activity.

We also would like to build the human model and try to reconstitute in vitro the mutant related diseases and perform biochemical and structural assays to understand their role in the mechanism.

Methods:

The MinElp3 gene was amplified by PCR. The human Elp1,2 and 3 were ordered from Genescript. The human Elp4,5 and 6 were extracted from HEK Cells. Single point mutations or truncations were generated using Quikchange PCR protocol. Proteins of interest were expressed in *E.coli* for MinELp3 and using the Insect cell system for human Elp123. Purification was done using affinity chromatography, followed by a size exclusion column. The purified protein solution was mixed with equal volume crystallization solution in a hanging drop diffusion method in a 96-well plate. Crystals appeared within 3 days and were snap frozen in liquid nitrogen. The data was processed using XDS/XSCALE and the structures were resolved using Phaser. For Microscale Thermophoresis (MST) Cy5-labelled tRNA was mixed with different concentrations of protein (0.01 µM to 10 µM) and incubated at 25 °C for 30 min. The interactions of protein and tRNA were measured using the Nanotemper NT115. The Kds were calculated and analyzed using Nano temper Control for quantitative analysis. The Anaerobic chamber was filled with Nitrogen and contained between 0,5 and 1% of Oxygen. Every set of experiments was repeated at least three times.

Results:

This year my work has been divided in four projects: three of them performed on MinElp3 and the last one was to build the human model.

In the structure that we obtained from X-ray crystallography from MinElp3, we could not see the Iron/Sulfur cluster (Fe/S). We decided to perform reconstitution trials under anaerobic condition and

sent the samples for EPR measurement and analytical liquid chromatography. We could not see any reconstitution on our conditions.

In the structure we identified and then tested four point mutations in the ACo-binding pocket (K150A, K266A, Q461A and Y517A). The first two have been shown to still bind ACo (Kd WT: 135.4±55.3 μM, K150A 151.5±50.3 μM and K266A: >250 μM) even though they do not hydrolyze it, while the last two are involved in both the binding and the hydrolysis of ACo by Elp3. These point mutations however do not affect the binding affinity of tRNA to MinElp3.

Our next aim is to obtain a co-crystallization structure from MinElp3 with ACo and tRNA. In order to slow down the reaction enough for our purpose we chose the mutant K150A was chosen as it has a strong binding to ACo and tRNA and no hydrolysis activity. The trials are still ongoing.

Last but not least, we wanted to understand further how the eukaryotic complex worked so for the past year I have been assembling the six genes that code for the Human Elongator using the Gibson assembly method and I have been using the insect cells system to express the core complex (Elp123). We finally know that Elp123 can be expressed using this system. In the near future I will be introducing mutations in Elp3 and perform tRNA binding assays and ACO hydrolysis assays to assess the quality of those two activities in the context of Elp3 associated diseases.

Conclusion:

This year we published two crystallography structures that helped us understand better how to direct our mutations for our next biochemical assays. We also put a finger on which residues are exactly responsible for ACo binding and what direction to go for the next crystallography trials. We also built the human model that hopefully will give us some great opportunities to study the regulation of the complex.

Perspectives:

For the next year I would like to focus on how the Elp3 mutations affect the complex structure and tRNA binding and ACo hydrolysis. I would also like to obtain the structure of the full complex with its partners to confirm our binding data. And I will pursue the crystallization trials for MinElp3 with ACO and tRNA.

Participation in conferences:

- Nanotemper Symposium, November 2018, Krakow, Poland
- Poster Presentation at the Krakow Interdisciplinary Science Seminar, January 2019, Krakow, Poland.
- Scientific presentation at the High-throughput protein production and crystallization workshop, June 2019, Didcot, United Kingdom
- Scientific presentation at the MCB summer school, June 2019, Zakopane, Poland

Publications, scientific presentations as a leading author:

T-Y Lin, NH Abbassi, K Zakrzewski, A Chramiec-Głąbik, J Różycki and S Glatt. The Elongator subunit Elp3 is a non-canonical tRNA acetyltransferase. Nat. Comm. 2019

M.I. Dauden, M. Jaciuk, F. Weis, T-Y Lin, C. Kleindienst, NH Abbassi, H. Khatter, R. Krutyholowa, K.D. Breunig, J. Kosinski, C.W. Müller and S.Glatt. Molecular basis of tRNA recognition by the Elongator complex Sci. Adv. 2019

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Dhanasekaran Balakrishnan	Year of studies: 2nd

Title of the project: Fusion of nanodisc with membrane mediated by calcium ions

Name of the supervisor: Prof. Jonathan Heddle

Affiliation: Malopolska Centre of Biotechnology, Jagiellonian University, Krakow

AIMS of the project:

Membrane fusion is the heart of many biological process such as exocytosis, endocytosis and in the drug delivery. The main aim of my work is to fuse the nanodisc with the membrane mediated by the addition of the Ca^{2+} ions to the mixture. Understanding this fusion mechanism will provide us with insights to control the delivery of the membrane protein to the target membrane and it also helps us to understand the fate of membrane scaffold protein.

Methods:

Producing nanodisc:

I have prepared the nanodisc with flourescent lipids because we have decided to use fluorescence assay for checking this fusion and I used the ratio of 1:90 Membrane Scaffold Protein (MSP) lipids to assemble the nanodiscs. Lipids (DOPS in Sodium Cholate to Rhod-PE, NBD film) and MSP proteins were incubated along with biobeads for 4h at 4ºC. After incubation, biobeads were removed using the filters and the nanodisc was purified using the size exclusion chromatography. Fractions corresponding to the size of the nanodisc were collected and was verified using the transmission electron microscopy.

DOPS liposome production:

Production of liposome involves three steps I) preparing lipid films ii) mixing it with the desired buffer iii) Sonicate the mixture to prepare the liposomes. I have prepared the DOPS lipid film, mixed it with HEPES buffer and sonicated to prepare the liposomes and they were mono disperse obtained liposomes was checked in the dynamic light scattering.

Fluorescence Measurements:

Flourescence measurements has been carried in a 96-well plate reader. The NBD was excited at 460 nm and the emission was read at 538 nm. The 96-well plates were clear in order for the fluorescence to be read from the bottom, to prevent a reading artifact due to possible condensation of water on the lid at the highest temperatures. For lipid mixing assays, several protein to lipid ratios were tested.

Results:

Nanodisc sample was analyzed under the Transmission Electron Microscopy (TEM) and we found that the produced nanodisc were highly monodispersed and appears as a blob under the TEM. After preparing the liposomes, liposomes were checked under the dynamic light scattering and the size of the liposomes are verified and we found that the size of the liposome ~100 ± 20 nm.

After preparing nanodisc and liposomes, I started with the FRET analysis, and it indicated that the fusion between the nanodisc made of DOPS lipids and DOPS liposome is occurring spontaneously upon addition of the calcium ions to the mixture. We also found that from the FRET analysis the DOPS nanodisc and liposomes of the DOPC doesn't fuse upon addition of the calcium ions. 10 mM Ca2+ ions was used for the FRET analysis.

Participation in conferences:

1. Participated and presented a poster in the 2nd Malopolska Centre of Biotechnology, held in between May 23 – 24, Zakopane, Krakow.

2. Participated and presented a poster in the 44th FEBS Congress held in Krakow between 6th July - 11th of July, 2019.

Publications, scientific presentations as a leading author:

1. Delivering DNA origami to cells. Dhanasekaran Balakrishnan, Gerrit D Wilkens and Jonathan Heddle. Nanomedicine (Lond) 14 (7), 911-925, 2019.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Maciej Migdał	**Year of studies:** 1st

Title of the project: Tissue specific enhancers prediction based on accessibility data and DNA sequence

Name of the supervisor: Cecilia L. Winata

Affiliation: International Institute of Molecular and Cell Biology in Warsaw

AIMS of the project:

The main objective of our work is to understand the transcriptional regulatory grammar underlying the development of heart. To this end, we aim to identify and characterize key regulatory elements and transcription factors associated with them based on the analysis of high throughput sequencing and genome-wide chromatin accessibility data. The project will also generate analysis tools and methodology for ATAC-seq data which will be a valuable resource for the genomics research community. The new regulatory elements identified in our study will provide novel insights into how key genes and regulatory nodes are regulated during heart development and help us to better understand the genetic mechanism of congenital heart defects.

Methods:

To achieve our goal, we have developed a computational pipeline which was build out of three blocks, each serving specific stages of the genomics data analysis. In the first step, sequencing reads were compared to the reference genome using bowtie2 for the alignment and tools such as fastqc, cutadapt and samtools for required input and output processing. Further output of this first step was analyzed using MACS2 and its subcommands together with custom R script to predict the locations of regions enriched with sequencing reads, which corresponded to open chromatin regions. Lastly, the genomic coordinates, nucleotide sequence, and sequencing coverage information of open chromatin regions were processed in R. Downstream analysis included Motif Enrichment Discovery, Novel Motifs Discovery, Differential Analysis of Chromatin Accesibility, and Digital Genomic Footprinting. Further level of analysis included integration with RNA-seq expression data.

Results:

The created pipeline was used to identify open chromatin regions and putative regulatory elements using ATAC-seq data. In order to guide further analysis of epigenetic data, we have explored various ways of integrating it with expression data obtained by RNA-seq, in corresponding conditions. We referred regulatory information to changes in time, as well as gene expression. This allowed us to draw interesting conclusions using analyses such as Differential Analysis of Chromatin Accesibility and Gene Set Enrichment Analysis. Furthermore, we have constructed a gene regulatory network using Weighted Gene Set Correlations Analysis, allowing us to start making hypothesizes about regulatory interactions during cardiomyocytes development. These results were published in (Pawlak et. al. Genome Research. 2019). Currently, we focus our efforts to further analyze our data to identify putative transcription factors binding sites through *in silico* prediction and further integration of open chromatin data with expression data using linear models. Ultimately, we aim to elucidate the mechanism of transcriptional regulation by cardiac transcription factors and their binding events to specific regulatory elements during heart development.

Participation in conferences:

1. Human Genome Meeting, Seoul, Korea (24-26.04.2019) – Oral presentation entitled "Elucidating The Dynamics Of Transcriptional Regulation In Cardiomyocyte Development Using Integrative Computational Analyses"
2. 6th Polish Illumina Symposium, Poznań, Poland (11-12.10.2018) – Speaker in workshop entitled "Analysis, visualization and interpretation of RNA-Seq data"
3. Single Cell RNA seq, Bio&organoids, Kiev, Ukraine (28.09.2018) – Attendee.

Publications, scientific presentations as a leading author:

1. Pawlak, M., Kedzierska, K., Migdal, M., Abu Nahia, K., Ramilowski, J., Bugajski, L., Hashimoto, K., Marconi, A., Piwocka, K., Carninci, P. and Winata, C. (2019). Dynamics of cardiomyocyte transcriptome and chromatin landscape demarcates key events of heart development. *Genome Research*, 29(3), pp.506-519.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Patryk Ślusarczyk	Year of studies: 1st

Title of the project: Dissecting the impact of systemic and cellular iron content on the iron-recycling capacity of splenic red pulp macrophages

Name of the supervisor: dr Katarzyna Mleczko-Sanecka

Affiliation: International Institute of Molecular and Cell Biology in Warsaw

AIMS of the project:

Iron is an essential metal to support life thanks to its ability to exchange electrons with a variety of biomolecules. In mammals, most of the iron pool in the body is incorporated in heme, a prosthetic group of hemoglobin that binds oxygen in erythrocytes. Importantly, free iron may also become cytotoxic when electron exchange with oxygen is unrestricted. This catalyzes the production of reactive oxygen species (ROS) which accelerate aging and may cause the iron-dependent cell death. The main cells in the body that are involved in heme breakdown are red pulp macrophages (RPMs) in the spleen. By phagocytosing senescent erythrocytes in a process called erythrophagocytosis, RPMs critically maintain blood homeostasis and mediate body iron recycling. For their functions, these cells are specialized in the hemoglobin breakdown, heme catabolism, iron export, and have adapted to high rates of iron flux. Interestingly, it is still not well understood if the process of erythrophagocytosis and iron recycling capacity of RPMs might be regulated by shifts in systemic iron homeostasis. Alterations of iron levels in the body and in the splenic macrophages occur frequently under such conditions as nutritional iron deficiency and loading, genetic iron overload disorders (e.g., hemochromatosis) or physiological aging, where iron levels drop mildly in the blood, but increase in most of the organs. Thus, the main goal of our research is to determine if the dysregulated iron balance in the whole organism and intrinsically in RPMs affect the rate of erythrophagocytosis. Our studies are expected to gain insights into still elusive molecular mechanisms which may regulate the process of erythrophagocytosis. To these aims, we will employ both *in vitro* and *in vivo* approaches. The latter will include mice with dietary iron imbalances as well as aged mice, hallmarked with splenic iron overload. By using a combination of such models, we also aim to elucidate the crosstalks between iron homeostasis, aging and the control of erythrophagocytosis intensity.

Methods:

We propose a complementary research plan to dissect if and how the erythrophagocytosis process in RPMs is affected by changes in iron homeostasis. For that we planned the following tasks:
In vitro studies were carried out on cells isolated from mouse bone marrow and differentiated into macrophages (BMDMs) with the usage of the MCS-F. We treated cells with two different iron sources Fe:NTA (Nitrilotriacetic Acid-Fe(III) Complex) and FAC (Ferric Ammonium Citrate), in a dose-dependent manner. This was followed by the erythrophagocytosis assay, where BMDMs were exposed for 1,5 h in ratio 1:10 to mouse RBCs stained with a fluorescent dye PKH67. Before the assay RBCs were left unaffected (termed as fresh) or artificially stressed (opsonization by anti-RBC antibody and temperature stress at 48 ºC for 30 min). For rescue experiments, we used the iron chelator DFO (Deferoxamine) and the antioxidant NAC (N-Acetyl Cysteine), combined with the iron challenge and used as well alone. The readout of the phagocytosis of fluorescently stained RBCs was performed on a flow cytometer (BD FACSAria II) and high throughput microscope (PerkinElmer Opera Phenix).
In vivo studies were performed on C57BL/6 mice on iron-rich (IR, 2500 mg/kg of chow, between 3 and 9 weeks of age) and iron-deficient (ID, 5.9 mg/kg, between 4 and 9 weeks of age) diet. As a control, we used mice on a balanced iron diet (IB, 200 mg/kg). At the end of the experiment, we

collected the spleen, liver and blood to measure iron parameters. For erythrophagocytosis experiment mice were injected with 100 μL of temperature-stressed erythrocytes (at hematocrit 50%) for 1,5 h to investigate how the shift in systemic and splenic iron levels affect the erythrophagocytosis rate in RPMs. The readout for the erythrophagocytosis intensity was performed by flow cytometer after preparation of the single cell suspension from the spleen and liver.

Results:

To perform both *in vitro* and *in vivo* erythrophagocytosis assays according to the highest experimental standards several steps were first optimized: preparation of stressed RBCs, dose of RBCs and BMDM culture conditions, the procedure of RBCs transfusion to mice, single cell suspension preparation and the panels of antibody staining of cells for flow cytometry.
In vitro: First, we have confirmed that opsonized and temperature-stressed RBCs are phagocytosed by BMDMs more efficiently than fresh RBCs. Next, we observed dose-dependent suppression of erythrophagocytosis process (measured as a percentage of PKH67 positive cells from F4/80+/CD11b+ BMDMs) with increasing concentrations of iron. Our rescue experiments showed a slight recovery of this phenotype after DFO treatment and complete recovery after NAC. All above results were also confirmed by microscopy analysis.
In vivo: We confirmed that our dietary regimen for both IR and ID diets accordingly modified iron levels in organs (including the spleen) and in the plasma in comparison to control IB mice. Besides, ID diet led to the expected mild microcytic anemia. Changes of systemic iron parameters accordingly modified the expression of iron-regulatory genes in the liver and the spleen, altogether validating our dietary approach as the right means to modify body iron balance.
Most importantly, we discovered that exposure of mice to both IR and ID diet, leading to splenic iron loading and deficiency, respectively, modified the rate of erythrophagocytosis of transfused RBCs. Consistent with our *in vitro* data, hight systemic (and splenic) iron content mildly diminished intensity of erythrophagocytosis measured as a percentage of PKH67 positive cells from F4/80+/Trem-L4+ RPMs. By contrast, RPMs analyzed from ID mice showed an increased rate of erythrophagocytosis. Our future experiments, using both mechanistic studies in BMDMs as well as RNA sequencing of iron-loaded and iron-deficient RPMs will be aimed to elucidate the molecular mechanisms of these observed phenomenons. We will also address if systemic and macrophage iron imbalances modify the overall iron-recycling capacity of RPMs/BMDMs following digestion of RBCs.

Participation in conferences:

Young Scientists Conference on Molecular and Cell Biology, April 11, 2019, IIMCB, Warsaw

Publications, scientific presentations as a leading author: N/A

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Eugeniusz Tralle	Year of studies: 1st

Title of the project: Tracing the cell lineage evolution of the second heart field

Name of the supervisor: Cecilia Winata PhD

Affiliation: International Institute of Molecular and Cell Biology

AIMS of the project:

Early steps of zebrafish heart organogenesis rely on migration and differentiation of cells derived from two lateral heart fields. Whereas the first heart field (FHF)-associated cells migrate to the midline and fuse into a linear heart tube relatively early in embryonic development, subsequent processes rely on continuous addition of second heart field (SHF)-derived cardiac progenitors (CPs) to the venous and arterial poles of the growing heart.

Our project's main aim is to investigate the establishment of heart field pattern and subsequent clonal lineages of SHF-derived cells contributing to the heart formation. In order to do that, we use a CRISPR/Cas9-based GESTALT barcoding approach combined with single-cell sequencing.

Methods:

In order to adapt the GESTALT barcoding system approach to our needs we want to limit the barcoding events to the second heart field-derived cells. We will use the genomic regulatory regions of *isl1*, a transcription factor expressed ubiquitously, but specifically in the SHF at the moment of its establishment, to limit Cas9 expression – and therefore the barcode editing events – to SHF cell clones. To establish the regulation of *isl1* expression we analyzed publicly available ChIP-seq data and pinpointed putative *isl1* regulatory regions. We then performed a transient enhancer assay and established stable transgenic zebrafish lines in which reporter gene expression is driven by the putative enhancer sequences in the tissue of interest.

As a safeguard, we established the GESTALT transgenic lines using Tol2-mediated transposition (F0). The fish carrying the germline mutation have been incrossed in order to establish stable lines (F1). Genotyping the F1 fish to identify carriers of single barcode and Cas9-sgRNA arrays via qPCR will be conducted when F1 fish reach maturity.

Results:

1. GESTALT lines have been established
2. 5 putative *isl1* enhancer regions of varying tissue specificity have been identified and validated. The identification of the next sequences is ongoing.

Participation in conferences:

1. 78th Society for Developmental Biology annual meeting, July 26 – 30, 2019, Boston, MA, USA. Poster: E. Tralle, C. Winata: Functional analysis of *isl1* regulatory regions in zebrafish embryos.
2. Workshop: NGSchool x RNAClub workshop in basics of R and Python programming, 6 May 2019, IIMCB, Warsaw.

Publications, scientific presentations as a leading author: N/A

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Aleksandra Majewska	Year of studies: 2nd

Title of the project: Endothelial PTEN as a target for hypoxia alleviation therapy in melanoma and renal cancer

Name of the supervisor: prof. dr hab. Claudine Kieda

Affiliation: Laboratory of Molecular Oncology and Innovative Therapies, Military Institute of Medicine, Warsaw

AIMS of the project:

Hypoxia is a characteristic feature of the tumor microenvironment – it affects the activity of cancer cells and promotes pathological angiogenesis. Non-functional, leaky blood vessels contribute to metastases and failures of chemo and radiation therapy. Vessel normalization can be obtained by an allosteric effector of hemoglobin: ITPP (*inositol tris pyrophosphate*). The resulting effect of ITTP delivery is a better availability of oxygen inside the hypoxic sites of the tumor. In addition, ITPP activates the tumor suppressor molecule (PTEN – *phosphatase and tensin homolog*).

Aim of the project is to determine how the modification of PTEN activityin endothelial cells in tumor microenvironment affects the course of cancer. In this academic year, the aims were to: 1) confirm PTEN activity in cancer cells using second experimental model (melanoma), 2) investigate the effect of hypoxia and conditioned medium from cancer cells on endothelial cell function and activity, 3) check the effect of ITPP on *in vivo* tumor development in a mouse model of kidney cancer.

Methods:

In vitro: Cancer cells (murine melanoma -B16-F10, and kidney cancer -RenCa) and murine brain derived endothelial cells (MBr MEC FVB) were culture in normoxia (~ 19% pO2) and hypoxia (1% pO2). PTEN and p-PTEN levels were checked using Western Blot in total, nuclear and cytoplasmic protein fractions. Expression of genes related with hypoxia (*vegf - vascular endothelial growth factor, vhl - von Hippel-Lindau and hif-1α - hypoxia inducible factor) was* assessed by RT-PCR in total RNA. The active factors in conditioned medium (CM) from cancer cells were detected using Cytokine Membrane Array and ELISA. The *in vitro* angiogenesis capacity was assessed by tube formation assay on Matrigel, and analyzed by ImageJ. The permeability assay was performed *in vitro* on Transwell filters using Evans Blue-labeled albumin. Claudin1 levels were checked using Western Blot in total cell protein fraction.

In vivo: RenCa cells were implanted in BALB/c mice leg as subcutaneous tumors by injection of a plug constituted by 10^5 cells in 100 µl Matrigel. ITPP was injected intraperitoneally (1.5 g/kg) in saline every 5 days in two experimental groups: RenCa/ITPP1 (first ITPP dose 5 days after tumor injection) and RenCa/ITPP2 (first ITPP dose 10 days after tumor injection – when tumor were visible). After 22 days, the experiment had to be terminated due to tumor size. Cells from the tumors were analyzed after tissue dissociation by collagenase for the presence of surface and intracellular markers using flow cytometry to identify different populations of cells in the tumor mass. Protein isolation from tumor was performed. The Western Blot method was used to assess PTEN, p-PTEN, VHL and VEGFR2 (*vascular endothelial growth factor receptor 2*) levels in the tumor. Spleen cells were isolated to assess for the presence of CD45+, CD8+ and CD4+ cells and check for the state of the immune response. Part of tumors have been frozen and will undergo immunohistochemical analysis. RNA was isolated from tumors in view of next generation sequencing.

Results:

Invitro: B16 F10 melanoma cells displayed *vegf* expression and secretion induced by hypoxia (which confirms the results from the mouse model of renal cancer – RenCa obtained last year). We also confirmed the decrease in PTEN levels in low pO_2, with the simultaneous dominance of the less active isoform(s) (p-PTEN). We observed that the inactive form (p-PTEN) dominates in the cell nucleus. In response to low pO_2 cancer cells (RenCa and B16-F10) and endothelial cells (MBr MEC FVB) showed increased *vhl* gene expression and reduced *hif-1α* gene expression, which is characteristic for long term hypoxia. Conditioned medium from RenCa cells affected endothelial cells activity and function: accelerated *in vitro* angiogenesis, increased the number of junction and nodes. CM also affected vascular permeability *in vitro* and reduced the expression of Claudin1 responsible for tight junctions between cells. These changes were more pronounced with CM from RenCa cells cultured in low oxygen tension than in normal culture conditions.

Invivo: No reduction in tumor size was observed after ITPP treatment. However, there was a large heterogeneity of tumors within the groups. Analysis of spleen cells indicated that tumor-bearing mice were characterized by an influx of CD45+ cells as compared to healthy controls. This suggests the activation of the peripheral immune response. Moreover, when tumor bearing mice had been treated with ITPP mice (RenCa/ITPP1) this percentage of CD45+ cells in spleen was increased together with a concomitant elevation of the CD4+ and CD8+ lymphocyte populations. Preliminary Western Blot results indicate a relationship between ITPP treatment and the level of VHL protein and VEGFR2 in the tumor mass. However, these results must be confirmed on a larger number of samples. Western blot analysis will be also confirmed by immunohistochemical staining on tumor section. Our preliminary data suggest a strong effect of ITPP induced changes of the tumor microenvironment on the antitumor immune response.

Participation in conferences:

Personalized Oncology „Wyzwania onkologii spersonalizowanej i interdyscyplinarnej" 10-11 May 2019, Warsaw (passive participation)

6th LIA MeetingWorkshop report and evolution of the Polish-French cooperation in biotechnology "Biomarkers and Mediators of Diseases - New approaches in repair/regenerative therapies" 4-5 June 2019, Warsaw (poster session; organization of conference)

44th FEBS CONGRESS, "Form molecules to living systems" 6-11 July 2019, Krakow (poster session).

Publications, scientific presentations as a leading author:

A. Majewska, K. Brodaczewska, C. Kieda, *Intra-tumor hypoxia as a modulator of endothelial cell activity: in-vitro model of pathological angiogenesis,* 44th FEBS Congress, 6-11 July 2019, Krakow

A. Majewska, K. Brodaczewska, C. Kieda, *Effects of hypoxia on cancer PTEN activity*, 6th LIA Meeting, 4-5 June 2019, Warsaw

A. Filipiak, K. Brodaczewska, A. Majewska, C. Kieda, "*Development of an alternative drug-testing method in stem-like cells enriched melanoma model",* IV Zjazd Naukowy Polskiego Towarzystwa Biologii Medycznej „Biologia-Medycyna-Terapia", 22-25 May 2019, Szczawnica

A. Filipiak, K. Brodaczewska, A. Majewska, C. Kieda *"Enrichment in stem-like cells in melanoma provides an alternative drug-testing method"*, 6th LIA Meeting, 4-5 June 2019, Warsaw

Bouchra El Hafny-Rahbi, KlaudiaBrodaczewska, Guillaume Collet, Aleksandra Majewska, Krzysztof Klimkiewicz, Catherine Grillon, Claudine Kieda *"Inversion of tumor immunosuppression into anti-tumor immune response by angiogenesis normalization via myo-inositol-trispyrophosphate-mediated pO_2 increase"* [in review].

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Michał Janyst	Year of studies: 2nd

Title of the project: Influence of drugs and immunomodulators on T regulatory lymphocytes

Name of the supervisor: Prof. dr hab. Witold Lasek

Affiliation: Medical University of Warsaw, Department of Immunology

AIMS of the project:

T regulatory cells (Tregs) are a fraction of $CD4^+$ lymphocytes responsible for suppression of immune response. Tregs can be divided into three different subpopulations. Thymically derived T regulatory cells constitute a fraction of Tregs developed in thymus. Peripherally derived T regulatory cells develop *in vivo* during suboptimal stimulation of TCR, but require also additional factors, such as TGF-β. Third fraction of T regulatory cells are iTregs - *in vitro*-induced T regulatory cells. They are generated from conventional T cells *in vitro* in cultures supplemented with TGF-β.

The aim of the study is to investigate the effect of various immunomodulators and drugs on activity of iTregs. The influence of drugs on both phenotype and functionality of Tregs will be investgated.

It was shown that number and functionality of T regulatory cells in patients suffering from myasthenia gravis are impaired. Hence the ability of most promising immunomodulators selected from the study to improve the impaired functionality of T regulatory cells isolated from patients suffering from myasthenia gravis will be evaluated *in vitro*.

Methods:

$CD4^+$ T cells were isolated from peripheral blood mononuclear cells by a negative selection method. For Treg induction, $CD4^+$ T cells were stimulated with CD3/CD28 beads in the presence of TGF-β and one of the following agent: sodium butyrate, glatiramer acetate, inosine pranobex, rapamycin, prednisolone, atorvastatin or acetic acid. Following incubation, cells were stained with monoclonal antibodies and analysed using flow cytometry. To test iTregs' functional inhibitory activity, mixed lymphocyte culture (MLC) assay was performed. Freshly isolated allogenic PBMCs were inactivated by gamma irradiation and served as stimulator cells (Tstim). Tstim were incubated with responder CD4+ T cells (Tresp) and co-cultured with isolated iTregs from cultures with different drugs. After 5 days, cultures were pulsed with ^{3}H-thymidine for the last 18 h of the incubation and harvested with an automated cell harvester. The amount of incorporated ^{3}H-thymidine was measured.

In order to investigate if immunomodulators increase the functionality of impaired Tregs, lymphocytes from patients suffering from myasthenia gravis were isolated. In the first step of experiment, antigen specific stimulation was performed. $CD4^+$ $CD25^-$ T cells were isolated from lymphocytes by a negative selection method. Following isolation, $CD4^+CD25^-$ cells stained with CFSE were cocultured with auto-antigen-presenting cells (APC) and acetylcholine receptor (AChR)- peptides. Level of proliferation was assessed with flow cytometry analysis.

Results:

Experiments concerning the influence of drugs and immunomodulators on the number of T regulatory cells in the culture were continued. Co-culture of $CD4^+$ T lymphocytes with immunomodulators resulted in an increase of Tregs in comparison to TGF-β alone, in a dose-dependent manner. The strongest effect was observed in cultures with prednisolone concentration of 250 ng/ml, rapamycin (4 ng/ml and more), sodium butyrate (20 and 100 μM), glatiramer acetate (125 ng/ml), and inosine

pranobex (200 mg/ml). In contrast, incubation of lymphocytes with atorvastatin or acetic acid (used as a control for butyrate) did not lead to significant increase in proportion of T regulatory cells nor in Foxp3 expression. Induction of Treg cells in the presence of the tested agents was also associated with augmented expression of Foxp3. The expression of Foxp3 was the highest in $CD4^+$ T cells incubated with prednisolone and rapamycin.

$CD4^+$ T cells activated in the presence of TGF-β significantly increased number of Treg cells and most of the tested drugs/compounds enhanced this effect. Prednisolone and rapamycin seemed the most effective in this activity. However, the open question was if the induced Treg cells were functional. To test this hypothesis, activity of Treg cells generated in the presence of prednisolone or rapamycin were investigated in allogeneic mixed lymphocyte culture (MLC). Results from the experiments reported in previous year were confirmed and showed that Treg cells from cultures incubated with prednisolone or rapamycin effectively suppressed proliferation of allo-stimulated T cells in MLC. Interestingly, while Treg cells generated in cultures with rapamycin showed similar suppressive activity when compared to control cultures, prednisolone-activated Treg cells were found superior and exerted significantly stronger suppressive effect. Rapamycin-generated Treg cells exerted measurable inhibitory effect in MLC at 1:0.5 but also at 1:0.25 Tresp–to-Treg cell ratio.
The effect of immunomodulators on impaired Tregs remained to be investigated. In the first step, AChR-peptides were used to induce proliferation of autoimmune responder cells. In the initial experiments the induction of proliferation was not observed. This effect remains to be investigated in the further experiments.

Participation in conferences:

Karolina Janyst, Michał Janyst, Marta Siernicka, Witold Lasek. Synergistic antitumor effect of histone deacetylase inhibitor scriptaid and proteasome inhibitor bortezomib in vitro against ovarian cancer cells, Molecular Biology and Immunology of Cancer – R&D perspectives: ScanBalt Forum 2019, Gdańsk, 24-25 of September 2019 - poster, in review.

Publications, scientific presentations as a leading author:

Michał Janyst, Beata Kaleta, Karolina Janyst, Radosław Zagożdżon, Witold Lasek. Comparative study of immunomodulatory agents to induce human T regulatory (Treg) cells: preferential Treg-stimulatory effect of prednisolone and rapamycin, Archivum Immunologiae et Therapiae Experimentalis (AITE)- in review.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Barbara Dymek	Year of studies: 2nd

Title of the project: Discovery and development of small-molecule chitotriosidase 1 (CHIT1) inhibitor and validation of CHIT1 as a new therapeutic target for the treatment of interstitial lung diseases

Name of the supervisors: Prof. Rafał Krenke, Dr Karolina Dzwonek

Affiliation: OncoArendi Therapeutics SA

AIMS of the project:

The project aims at the discovery and development of small-molecule chitotriosidase (CHIT1) inhibitors and the validation of CHIT1 as a new therapeutic target for the treatment of interstitial lung diseases.

Firstly, the project objectives are: the establishment of the screening methods e.g. enzymatic assays and the evaluation of the activity of the newly designed and synthesized small-molecule compounds developed at OncoArendi Therapeutics leading to the selection of the CHIT1 inhibitors with the best activity and physicochemical properties for further studies (selectivity, pharmacokinetic profile, *in vivo* efficacy studies).

Secondly, the project objectives are the translational studies aiming in the assessment of chitinolytic activity and CHIT1 level in samples from patients with various pulmonary disorders. These results should allow validation of CHIT1 as a new therapeutic target for the treatment of interstitial lung diseases and may facilitate the selection of patients that could benefit from the therapy with CHIT1 inhibitor developed by OncoArendi.

Methods:

The inhibitory activity of small-molecule compounds designed and synthesized at OncoArendi Therapeutic was assessed using established enzymatic assays utilizing recombinant human and mouse chitinases – AMCase and CHIT1 and the 4-methylumbelliferyl β-DN,N′-diacetylchitobioside substrate.

In order to assess in vitro safety of the compounds human lung fibroblasts have been treated with the increasing concentrations of the inhibitors for 72h followed by evaluation of the cell viability using CellTiter Glo assay.

CHIT1 expression and activity was assessed during monocyte-to-macrophage differentiation. Briefly, human monocytes (CD14+ cells) were negatively separated from peripheral blood mononuclear cells and were stimulated with MCSF with addition of IL-4 or IFNγ to obtain activated Mφ, M1-type or M2-type macrophages. CHIT1 activity and expression were assessed with real-time PCR and enzymatic assay.

The treatment-naïve male and female patients were recruited at the Public Central Teaching Clinical Hospital of the Medical University of Warsaw, Poland. The study was approved by the Local Bioethics Committee, at the Medical University of Warsaw, Poland, No. of approval: KB/236/2015.

CHIT1 activity in serum, induced sputum and bronchoalveolar lavage fluid (BALf) was assessed by the established enzymatic assay using 4-methylumbelliferyl β-DN,N′-diacetylchitobioside substrate. CHIT1 protein level in serum, induced sputum and BALf has been assessed using commercially available CHIT1 ELISA kits (Circulex) according to the manufacturer's protocol.

Results:

The screening cascade composed of enzymatic assays using recombinant human and mouse chitinases, cell-based assays using human lung fibroblasts and hERG channel binding assay was applied to evaluate the activity and safety of the novel series of small-molecule compounds developed at OncoArendi Therapeutics. More than 300 compounds have been screened and several active inhibitors with different selectivity profiles have been identified, among them OAT-2068 – mCHIT1 selective inhibitor with mCHIT1 IC_{50} = 29 nM, 140-fold selectivity over mAMCase and favorable pharmacokinetic profile in mice. This compound has been selected for further *in vivo* studies to assess its anti-fibrotic efficacy. The ex vivo studies using human monocyte-derived macrophages identified CHIT1 as the protein with highly induced expression during monocyte-to-macrophage differentiation. The CHIT1 activity and mRNA level were highly upregulated upon MSCF stimulation of monocytes from day 3 onwards.

Additionally, in collaboration with Department of Internal Medicine, Pulmonary Diseases and Allergy, Medical University of Warsaw, human samples, including serum, induced sputum and bronchoalveolar lavage fluid (BALf) were collected from over 100 subjects: non-diseased volunteers or patients with interstitial lung diseases like sarcoidosis, idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD). Analysis of expression and activity of chitinases by enzymatic assays and ELISA demonstrated the increased level of CHIT1 but not AMCase in the samples from diseased subjects in comparison to normal controls. The significant correlation between chitinolytic activity and CHIT1 protein level in analyzed body fluid has been demonstrated.

Conclusions:

- More then 300 compounds have been tested against human and mouse chitinases; OAT-2068 – potent and selective mCHIT1 inhibitor has been identified
- The chitinolytic activity and CHIT1 protein level were determined in the serum, induced sputum and BAL fluid demonstrating significant upregulation of CHIT1 concentration and activity in IPF, sarcoidosis and COPD patients when compared to the non-diseased control subjects

Participation in conferences:

ERS Satellites 2019 ‘Advances in precision medicine in COPD and ILD’ 21.02.2019, Warsaw

EMBO|FEBS lecture course ‘Molecular mechanisms of tissue injury, repair and fibrosis’ 23-31.05.2019, Spetses, Greece

Publications, scientific presentations as a leading author:

Mazur M., Dymek B., et al. ‘Development of dual chitinases inhibitor as potential new treatment for respiratory system diseases’ J Med Chem, 2019

Dymek B. et al. ‘Chitotriosidase (CHIT1) as a novel therapeutic target in interstitial lung diseases’ poster presentation, EMBO|FEBS lecture course, Spetses, 2019

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Gerrit Wilkens	Year of studies: 2nd

Title of the project: Design, production and imaging of a DNA origami device for capturing membrane proteins within a liposome in controlled orientations

Name of the supervisor: Jonathan Heddle

Affiliation: Malopolska Centre of Biotechnology, Jagiellonian University, Krakow

AIMS of the project:

Bottom up assembly of artificial cells is a major goal of synthetic biology. To interact and exchange material with their surrounding environment, such "cells" will require channels in their outer membrane. In nature, this function is carried out by membrane proteins, but controlled insertion of these into lipid membranes is a challenging task. This project aims at developing a novel methodology for controlled delivery of membrane proteins to lipid bilayers that can potentially be useful in work aiming at producing artificial or proto-cell systems. To this end we are using the DNA origami methodology which has emerged as a powerful tool to create self-assembling nanoscale objects build from DNA. In my research I am designing and producing a DNA origami templated liposome system. The design uses a ring-shaped DNA origami with a central cavity which is known to be able to template the formation of a lipid vesicle. Liposomes formation can be seeded by DNA-lipid conjugated strands that are facing towards the cavity. I am recapitulating this approach, but with addition of detergent stabilized membrane protein that is conjugated to a ssDNA oligonucleotide and complementary ssDNA capture strands on one face of the DNA origami, which allows attachment of the protein on the DNA origami, and subsequent integration into the lipid vesicle in a controlled fashion. Finally, the small protein containing lipid vesicle that is constraint in the DNA origami is supposed to be fused with a large giant unilamellar vesicle (GUV) in order to deliver the membrane protein. GUVs could potentially serve as a protocell for the construction of artificial cells.

Methods:

Specific research steps and methods used are as following: i) in silico design of DNA origami using conventional DNA origami design and modelling software (i.e. CaDNAno, CanDO), ii) assembly and imaging of DNA origami structure using TEM/ AFM. iii) Production and purification of a model membrane protein (bacteriorhodopsin, bR) under denaturing conditions, renaturing the protein into its active form and conjugation of the protein to a single stranded DNA oligonucleotide that allows binding the protein to the DNA origami (copper free click chemistry). iv) Generation of DNA origami constraint liposomes and optimization of liposome DNA origami interaction (dialysis step and assessment of the formed product using AFM/TEM).

Results:

The design of the DNA origami ring that was produced during the last reporting period was improved to remove problems related with aggregation and stability of produced DNA origami structures. Improvement included a rerouting of staple strands and decreasing the total number of helixes that form the structure to enhance folding of the DNA origami rings. The DNA origami rings having the new design could formed in a significantly shortened annealing time compared to older structures (36 h v 72 h). The assembly was verified using optical techniques (AFM/ TEM) as well as agarose gel shift assays.

Bacteriorhodopsin (bR) was chosen as a model membrane protein for DNA origami attachment trials because of its well characterised properties. The protein was expressed as a fusion protein to the membrane integrating protein Mistic that has been shown to enhance GPCR (G protein-coupled

receptor) membrane protein expression. To be able to attach the membrane protein to the DNA origami structure we produced a bR-DNA chimera. To this end we incorporated an amber stop codon at the N-terminal side of the bR gene and used an amber stop codon suppression approach to integrate an azide containing amino acid at the amber stop position into the protein. Using copper free click chemistry, we conjugated the azide group of the protein with an DBCO modified DNA oligonucleotide in denaturing conditions. The DNA modified protein was subsequently refolded into its active state into lipid/ detergent (DMPC/CHAPS) micelles following commonly known procedures for refolding of bR. Initial experiments to attach the thus modified membrane protein-DNA chimera to the DNA origami ring structure have been conducted. While some membrane proteins attaching to the DNA origami could be observed in TEM, the degree of attachment is rather low. Conditions for the protein attachment are being optimized now (protein: DNA origami ration, temperature and detergent concentrations).
To be able to deliver DNA origami constraint lipid vesicles containing inserted membrane protein to larger cell sized liposomes (giant unilamellar vesicles) we additionally modified the DNA origami as following: single stranded 'leg' strands were placed on the opposite the membrane protein capture strands. By placing cholesterol modified DNA strands into the lipid membrane of GUVs (which were complementary to the 'leg' strands) we could show the attachment of bare DNA origami rings on the surface of such GUVs mediated by the hybridisation between the single stranded 'leg' extensions on the origami and complementary DNA on the GV surface. Experiments using liposome filled DNA origami structures in order to fuse the lipid contents of GUVs and DNA origami constraint lipid vesicles are now ongoing.

Participation in conferences:

- 2nd summer school of the Malopolska Centre of Biotechnology, poster presentation
- 3rd interdisciplinary FNP conference, poster presentation
- FEBS Congress 2019 "From Molecules to Living Systems", oral presentation

Publications, scientific presentations as a leading author:

- Dhanasekaran Balakrishnan*, Gerrit D. Wilkens*, and Jonathan G. Heddle. "Delivering DNA origami to cells." Nanomedicine (2019), * equal contribution
- DNA origami as a tool for orienting membrane proteins within lipid bilayers,oral presentation, FEBS Congress 2019

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Zuzanna Pakosz	Year of studies: 2nd

Title of the project: Understanding the apicoplast gyrases

Name of the supervisor: Prof. Jonathan Heddle

Affiliation: Malopolska Centre of Biotechnology, Jagiellonian University, Krakow

AIMS of the project:

Apicoplast gyrases are interesting proteins in context of drug discovery. Apicoplast is a nonphotosythetic organelle present in human eukaryotic single cell parasites such as *Plasmodium falciparum* (Pf) or *Toxoplasma gondii* (Tg). Inside this organelle DNA gyrase can be found. DNA gyrase is mainly present in bacteria where it plays an important role in supercoiling and relaxing bacterial DNA during replication. Functional enzyme consists of two A subunits and two B subunits, which creates A_2B_2 heterodimer. It is also a good drug target for antibacterial drugs like fluoroquinolones (due to enzyme absence in humans).

The aim of this project is to better describe apicoplast gyrases, mostly PfGyr and TgGyr, as their nature still is not fully understood. Because of the difficulties in production PfGyA full length protein we focused on separate functional domains to characterize the enzyme. To achieve this goal, biochemical and structural biology methods are being used.

Moreover, we have discovered a compound which exhibits inhibitory potential against PfGyrB, which may prove useful in intelligent desing of novel drugs. The goal is to investigate which functions of the protein are inhibited and what is the mechanism of action, including finding the potential docking place.

Methods:

Genes were cloned to pET28a+ vector with N-terminal histidine tag for purification. For protein expression strain *E. coli* BL21 GOLD was used. After transformation cells were grown at 37 ^{o}C till OD_{600} reached 0.6 and protein expression was started with addition of 0.25 mM IPTG and continued overnight at 18 ^{o}C. Proteins were purified using three steps of chromatography – affinity (Histrap column), ion-exchange (MonoQ 16/10) and finally size exclusion (Superdex200 16/600).

Activity of the purified proteins was tested using gyrase assay(DNA supercoiling assay or DNA cleavage assays), where gyrase complex is incubated with relaxed DNA and results are resolved on agarose gel. There changes in DNA topoisomers are visible. In supercoiling assays increase of supercoiled DNA band can be observed, while in cleavage assays the main focus is linear DNA.

Compound-protein interactions were checked using electrophoretic mobility shift assay (EMSA), where differences in DNA binding capabilities were tested in the presence and absence of the compound. Proteins (single or complexed) were incubated for 0.5 h with short DNA fragment and the compound. Mixture was resolved on acrylamide gel and stained with ethidium bromide to visualise DNA strands.

Gyrase B subunit possess ATPase activity domain. To measure this activity ATP hydrolysis was assessed in an assay by the measure of the conversion of NADH to NAD+ which can be measured by a change in absorbance at 340 nm.

Results:

After screening the library of the small molecules against PfGyrB two hits were found. Next the properties of the compounds were tested using various gyrase assays: supercoiling activity, cleavage

activity and EMSAs. As the production and purification of PfGyrA subunit is still impossible, we used hybrid $EcGyrA_2PfGyrB_2$ which act as a functional complex.

One of the analyzed compounds inhibited supercoiling proprieties of the hybrid complex, while the EcGyr complex was not affected. Also the compound promoted DNA cleavage, which was reversible with EDTA addition. The same experiment was also conducted with part of the PfGyrA, PfGyrA_NTD, which is a N-terminal part of the protein responsible for DNA cleavage. Complex $PfGyrB_2PfGyrA\_NTD_2$ tested in the presence of the compound also exhibited increased amounts of linear DNA in cleavage assay. This means that the compound stabilized complex of cleaved DNA with enzyme thus inhibiting religation.

To narrow down position of binding site the ATPase activity in the presence of the compound was checked. Results showed that PfGyrB ATPase activity was not disrupted in the compound presence, which may suggest that is not a binding place.

Moreover, the DNA binding capabilities were tested in the presence of the compound using EMSA. This assay revealed that the binding of the $PfGyrB_2EcGyrA_2$ hybrid complex was disrupted in the presence of the compound, but binding of $EcGyrB_2EcGyrA_2$ was not affected. This result suggests that this area takes part in protein-compound interactions.

Within this research the properties of the novel compound against PfGyrB alone as well as complexed with EcGyrA were investigated. This knowledge may be used in further drug development studies. The interaction spot, responsible for the drugs mechanism of action was narrowed in context of whole protein, however still thorough investigation is needed to aid further anti-malaria drug research and development.

Participation in conferences:

1. Bionano 2018 Workshop – poster, 17-18.09.2018, Krakow, Poland
2. 2nd Summer School of the Malopolska Centre of Biotechnology - oral presentation, 23-25.05.2019 Zakopane, Poland
3. 13th Biophysics Symposium & NanoTemper Technologies Workshop – workshop, 13-15.06.2019 Lublin, Poland
4. The 44th FEBS congress – poster, 6-11.07.2019 Krakow, Poland
5. Prot-XRD – workshop, poster, 12-16.07 Krakow, Poland
6. EMBO workshop DNA topology and topoisomerases in genome dynamics – poster, 16-20.09.2019 Les Diablerets, Switzerland

Publications, scientific presentations as a leading author:

Result presented on conferences listed above (in previous part):

Ad. 1. Poster presentation – Z. Pakosz, E. Michalczyk, J. Heddle 'Understanding apicoplast gyrases'
Ad. 2. Oral presentation – Z. Pakosz, J. Heddle 'A new approach to characterize apicoplast gyrases'
Ad. 4. Poster presentation – Z. Pakosz, T-Y Lin, E. Michalczyk, J. Heddle 'A new approach to characterize apicoplast gyrases'
Ad. 5. Poster presentation – Z. Pakosz, T-Y Lin, E. Michalczyk, J. Heddle 'A new approach to characterize apicoplast gyrases'
Ad. 6. Poster presentation – Z. Pakosz, T-Y Lin, E. Michalczyk, J. Heddle 'A novel small molecule compound with inhibitory potential against *Plasmodium falciparum* gyrase'

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Agata Braniewska	Year of studies: 3rd

Title of the project: Investigation of hemoglobin fate in carrier and recipient cells in new anticancer drug delivery system

Name of the supervisor: dr Tomasz Rygiel

Affiliation: Department of Immunology, Medical University of Warsaw

AIMS of the project:

The aim of our project is to develop a new drug delivery system to the tumour. The potential therapy consists of live cell carriers that migrate into the tumour site and transfer anticancer drug in complex with iron-binding proteins: ferritin and hemoglobin. Such approach is intended to increase uptake of drug by tumour cells and to decrease side effects of anticancer therapy. We focused on hemoglobin (Hb), which biochemical properties and easy availability provide a tremendous advantage for using it as a drug transporter. In the potential therapy setup, Hb can be isolated the from patient's own blood and used for further modifications. We observed that macrophages uptake and transfer Hb to the neighboring cancer cells. It is completely new and undescribed process that we are currently investigating to characterize its molecular mechanism.

The aim of the study is to investigate the mechanism of Hb transfer from carrier cells to the recipient cancer cells and to determine intracellular processing of Hb in carrier and recipient cells.

Methods:

Macrophages: murine bone marrow-derived macrophages, RAW 264.7, THP-1 differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA), human monocyte-derived macrophages
Cancer cell lines: Skov3, MDA-MB 231, CT26, 4T1
Methods: flow cytometry, confocal microscopy

Results:

We confirmed that Hb transfer between macrophages and cancer cells occurs in both murine and human in vitro models. On contrary, a control protein - albumin is taken up from medium but is not transferred from macrophages to cancer cells, which suggests that this phenomenon is protein-specific. The precise mechanism of Hb transfer between macrophages and cancer cells is still unknown. Up till now, we determined that Hb transfer requires direct cell-cell contact and is independent of membrane receptor on recipient cells. Intracellular vesicle transport is involved in the transfer of Hb. Disruption of actin cytoskeleton inhibits the transfer of Hb, however microtubules are of minor significance. Interestingly, inhibiton of ROCK kinase increases Hb transfer. Using confocal microscopy, we visualized membrane connections between macrophages and cancer cells. These connections contain actin and are resemble to tunneling nanotubes (TNT). This manner of intercellular transport will be investigated more deeply.

Publications, scientific presentations as a leading author:

Pilch Z, Tonecka K, Skorzynski M, Sas Z, Braniewska A, Kryczka T, Boon L, Golab J, Meyaard L, Rygiel T 2019. The pro-tumor effect of CD200 expression is not mimicked by agonistic CD200R antibodies. PLoS One. 2018

Braniewska A, Sas Z, Tulodziecka K, Bialasek M, Pilch Z, Skorzynski M, Miaczynska M, Krol M, Rygiel T. Transfer of hemoglobin from macrophages to cancer cells as potential new anticancer drug delivery system. 10th EFIS-EJI South Eastern European Immunology School, Yerevan, Armenia 2018

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Salwador Cyranowski	Year of studies: 1st

Title of the project: Evaluation of the role of CHI3L1 (YKL-40) in pathobiology of gliomas

Name of the supervisor: Prof. Bożena Kamińska, PhD

Affiliation: Nencki Institute of Experimental Biology PAS, Warsaw, Poland

AIMS of the project:

Chitinase 3-like 1 (CHI3L1) is a secreted glycoprotein that mediates inflammation, macrophage polarization, apoptosis, and carcinogenesis. The expression of CHI3L1 is strongly increased by various inflammatory conditions, including rheumatoid arthritis, multiple sclerosis, Alzheimer's disease, and in several cancers. However, its physiological and pathophysiological roles in the development of cancer and neurodegenerative and inflammatory diseases remain unclear. Several studies have reported that CHI3L1 promotes cancer proliferation, inflammatory cytokine production, and microglial activation, and that multiple receptors, such as advanced glycation end product, syndecan-1/αVβ3, and IL-13Rα2, are involved.

The aim of the project is to evaluate the role of CHI3L1 (YKL-40) in pathobiology of glioblastoma (GBM), the most malignant brain tumor, in order to pave the way for novel therapy against it. In accordance with 1st year plans, the aim of the project was to perform transient knock-down (KD) and (if possible) stable knock-out (KO) of CHI3L1 in human U87-MG cells by siRNA and CRISPR/Cas9, respectively. The derivative cell lines with CHI3L1 KD or KO were to be used in matrigel invasion assays against human immortalized microglia and to produce glioma conditioned medium (GCM) to stimulate primary microglia and astrocytes cultures. We would like to examine a putative impact of CHI3L1 KO/KD on the invasiveness of U87-MG glioma cells and an activation state of primary microglia cultures.

Methods:

Transient silencing of CHI3L1 in U87-MG was performed using *CHI3L1*-specific siRNA introduced to the cells via electroporation; as a control non-targeting siRNA was used. Cells were then cultured for 48 and 72 h to establish the timepoint when cells express least CHI3L1 after siRNA introduction. Then culture media from transfected cells were collected and frozen with a protease inhibitor PMSF in -80C, while cells were harvested, pelleted and lysed. Culture media were subjected to CHI3L1-specific ELISA, while cell lysates were subjected to RNA isolation and RT-qPCR analysis with primers specific to the *CHI3L1* transcript.

In order to assess putative impact of CHI3L1 KD on U87-MG invasiveness, U87-MG cells were seeded onto matrigel-coated inserts, which were then placed in a multi-well plate with or without human immortalized microglia HM-SV40 at the bottom of the well. Co-cultures were performed in microglia media with 2% FBS as high FBS level is known to induce U87-MG invasiveness itself. Matrigel coating imitated tissue extracellular matrix that U87-MG cells would have to invade through towards microglia-secreted chemokines, while porous membrane of the inserts allowed the cells to freely migrate on the other side where they were fixed, stained with DAPI, captured with fluorescent microscope and counted. Controls for the experiment included U87-MG WT (unaltered), U87-MG mock (subjected to electroporation but without any external siRNA), U87-MG siCTR (electroporated with an unspecific siRNA) and U87-MG siCHI3L1 (electroporated with a specific siRNA). Each condition was co-cultured with or without human SV40 immortalized microglial cells (+HM-SV40 and -HM-SV40). Results were quantified using ImageJ software and given as a fold change of control for each condition to observe potential differences over basal invasiveness of U87-MG. A portion of U87-MG cells after electroporation with siRNA was cultured on the side and then harvested, pelleted and lysed to isolate RNA and perform RT-qPCR analysis with a primer set for epithelial-mesenchymal transition (EMT) genes that could be up- or down-regulated after siRNA treatment.

U87-MG cells with siRNA-based KD of CHI3L1 were also used to generate GCM that was used to stimulate primary murine microglia cultures obtained from murine pups. Microglia were seeded onto chamber slides, exposed to GCM from U87-MG WT, siCTR and siCHI3L1 for 48 h, then fixed, stained with anti-Iba1-FITC antibody and visualized using fluorescent microscope. FITC signal was then quantified using thresholding and particle analysis using ImageJ software.

Results:

The expression of *CHI3L1* was evaluated in several human glioma cell lines (both established and patient derived) and its level was the highest U87-MG glioma cells that were taken for further analysis. In cells transfected with control or specific siRNA, qPCR analysis revealed successful knockdown of *CHI3L1* expression after 48 h. After 72 h the level of *CHI3L1* transcript was going back to the control level, hence 48 h time-point was selected for further analysis. ELISA results confirmed the finding, however at the protein level the silencing was efficient up to around 50% after 48 h.

Matrigel invasion assay demonstrated that U87-MG WT invasiveness was significantly increased in the presence of microglia, while invasiveness of U87-MG with CHI3L1 KD is significantly decreased as compared to the -HM-SV40 condition. No significant difference was observed for mock and siCTR variants against their individual -HM-SV40 controls. However, comparison between experimental groups revealed that electroporation itself increased basal invasiveness of U87-MG cells. HM-SV40 cells did not to express *CHI3L1* as demonstrated by RT-qPCR analysis, so U87-MG were the only source of the protein in this experiment.

RT-qPCR analysis on RNA isolated from U87-MG cells that were used in matrigel experiments with primers specific for EMT genes did not reveal any significant differences for siCTR and siCHI3L1 conditions in EMT gene expression; the levels of *TGF-B1, TGFBR1, ZEB1, SMAD3, SNAIL2, S100A4, ACVR1 and CDH2* were determined.

Homeostatic microglia tend to have elongated morphology with 2-3 pseudopodia and relatively small cytoplasm area. Activated microglia on the other hand exhibit hypertrophy and few cytoplasmic protrusions. U87-MG are known to promote microglia activation so the idea behind the experiment was to assess the level of microglia activation after CHI3L1 transient silencing. Experiments with GCM from U87-MG siCHI3L1 revealed no effect of CHI3L1 silencing on murine microglia activation as assessed by morphology (hypertrophy). As expected, U87-MG WT induced microglia activation (around 150% in comparison to the unstimulated control). Other tested conditions for the experiment were GCM from LN229 – glioma cells that express low CHI3L1 levels (via RT-qPCR and ELISA), IPIN 0420 –GBM patient derived primary cell culture with cells expressing high CHI3L1 levels (via RT-qPCR and ELISA) and media with a recombinant human CHI3L1 protein (hCHI3L1) at the concentration mimicking CHI3L1 serum level in mice with implanted U87-MG tumours. Upon stimulation with LN229 GCM microglia retained their homeostatic morphology. Upon stimulation with IPIN 0420 GCM microglia showed a tendency towards hypertrophy – however, the results were not statistically significant because of high standard deviation due to the variability of different primary microglia cultures. Stimulation with hCHI3L1 alone revealed the lack of activation, even silencing of microglia, which suggests that either CHI3L1 is withholding microglia activation or it is simply toxic to cells in high concentrations.

Participation in conferences:

10th Anniversary International Conference of Contemporary Oncology, Poznań, Poland – poster

Publications, scientific presentations as a leading author:

Bozena Kaminska, Salwador Cyranowski· Recent advances in understanding mechanism of TGF beta signaling and its role in glioma pathogenesis. Glioma Signaling, chapter 9, 2019, Springer.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Joanna Domagała (Stachura)	Year of studies: 3rd

Title of the project: The influence of the metabolic changes characteristic for tumor environment on the NK cells' activity

Name of the supervisor: Dr hab. n. med. Magdalena Winiarska

Affiliation: Department of Immunology, Medical University of Warsaw

AIMS of the project:

Adoptive therapy, like other immunotherapies based on immune checkpoint inhibitors, relies on the activity of immune effector cells, such as NK cells. Unfortunately, the efficacy of currently utilized immunotherapies is limited, which is partially associated with immunosuppressive properties of the tumor microenvironment. Within the tumor microenvironment, various cytokines and checkpoint molecules coordinate the activity of immune cells. Moreover, preclinical studies show that metabolic changes also affect the functions of immune cells. Metabolites secreted by cancer cells can block the activity of immune effector cells. However, the exact mechanisms of this inhibition still remain elusive. Therefore, this project aims to identify the molecular mechanisms responsible for the inhibition of NK cells' activity by cancer cells metabolites present in the tumor microenvironment. Moreover, the identification of new metabolites involved in the regulation of NK cells' activity can potentially help to improve the effectiveness of anti-tumor adoptive therapies.

Methods:

We performed experiments evaluating the influence of the tumor-conditioned medium on NK cells' activity. As a source of tumor metabolites, we have used conditioned medium collected from Raji lymphoma cells culture. Briefly, Raji cells were seeded at 1,2 mln/ml density in 10 ml RPMI 11640 medium supplemented with FBS and penicillin and streptomycin. After 48h of culture, Raji cells were centrifuged and the conditioned medium was collected. We performed standard natural cytotoxicity and ADCC assay with NK cells treated for 4 hours with conditioned medium followed by propidium iodide staining. In order to evaluate whether inhibition of NK cells' cytotoxicity results from impaired degranulation, surface expression of CD107a, a widely-used marker of degranulation, was evaluated. To further identify the potential inhibiting factors, we filtered the conditioned medium through membranes with 3 kDa cut-off limit. Briefly, after 48h of culture, Raji cells were centrifuged, and the conditioned medium was subjected to centrifugal filtration. The flow-through fraction was used in the cytotoxicity assay.

Results:

In our preliminary studies, we have investigated the impact of the conditioned medium on the NK cells natural cytotoxicity against K562 cells. We observed thatconditioned medium almost completely inhibited NK cell natural cytotoxicity and their ability to kill target tumor cells, while it did not influence the viability of NK cells. . By performing assay with the conditioned medium and rituximab, an anti-CD20 monoclonal antibody, we have shown that metabolites present in the conditioned medium abolish NK cells' activity and their potential to kill target cancer cells in ADCC-dependent mechanism. Moreover, the results of the experiments demonstrate that the inhibition of NK cells' activity may result from the impairment of degranulation process. We also observed that small-molecule metabolites present in the conditioned medium inhibit NK cells' activity without impact on their viability.

Participation in conferences:

1. Keystone Symposia – Tumor metabolism, February 24-28, 2019

Publications, scientific presentations as a leading author:

1. Sasi BK, Martines C, Xerxa E, Porro F, Kalkan H, Fazio R, Turkalj S, Bojnik E, Pyrzynska B, Stachura J, Zerrouqi A, Bobrowicz M, Winiarska M, Priebe V, Bertoni F, Mansouri L, Rosenquist R, Efremov DG. Inhibition of SYK or BTK augments venetoclax sensitivity in SHP1-negative/BCL-2-positive diffuse large B-cell lymphoma. Leukemia. 2019 Mar 14.

2. Bojarczuk K, Wienand K, Ryan JA, Chen L, Villalobos-Ortiz M, Mandato E, Stachura J, Letai A, Lawton LN, Chapuy B, Shipp MA. (2019). Targeted inhibition of PI3Kα/δ is synergistic with BCL-2 blockade in genetically defined subtypes of DLBCL. Blood. 133(1):70-80

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Klaudyna Fidyt	Year of studies: 3rd

Title of the project: Evaluation of the thioredoxin system as a new therapeutic target against B cell acute lymphoblastic leukemia

Name of the supervisor: Dr Malgorzata Firczuk, Dr Magdalena Winiarska

Affiliation: Department of Immunology, Medical University of Warsaw

AIMS of the project:

B cell acute lymphoblastic leukemia (B-ALL) is highly heterogeneous, immature B cell-derived malignancy. In the recent decade the cure rates of B-ALL have improved significantly, especially for pediatric patients, however there are still particular subgroups difficult to treat, mainly due to development of drugs-resistance or relapse. Recently, we showed that targeting deregulated redox homeostasis could be a potent strategy against B-ALL. We observed that leukemic cells upregulate enzymes of the antioxidant thioredoxin (TXN) system that allow them to neutralize the excessive levels of highly toxic reactive oxygen species (ROS). Moreover, we presented that TXN system inhibitors (auranofin, adenanthin) are effective against B-ALL *in vitro* and *in vivo* and we hypothesized that combining them with other targeted drugs could be even more beneficial. During the 3rd year of my project I focused mostly on B-ALL with *mixed lineage leukemia* (*MLL*) gene rearrangements – MLLr B-ALL, which belongs to high-risk group, with event free survival not exceeding 40%. For this academic year my main tasks were:

-determination of the efficacy of thioredoxin system inhibitors in combination with a selected targeted drug (venetoclax) against MLLr B-ALL *in vitro* and *in vivo*,

-elucidation of the mechanism responsible for synergistic effect of auranofin and venetoclax combination using MLLr B-ALL cell lines model.

Methods:

Cell line and primary cells: We used SEM and RS4;11 cell lines representing B-ALL with MLL-AF4 fusion. Primary blasts were isolated from the bone marrow of pediatric and adult patients diagnosed with B-ALL, following appropriate patient consent.

Primograft cells generation: Primograft B-ALL cells were generated through transplantation of primary B-ALL samples to NSG immune-deficient mice. The whole blood staining was performed to evaluate the engraftment of leukemic cells (staining with anti-mCD45, anti-hCD45, anti-hCD19 antibodies). Once engrafted, primograft B-ALL cells were collected from spleens of leukemic mice.

Drugs cytotoxicity: Cytostatic/cytotoxic effects of TXN-family enzymes inhibitors (adenanthin (ADE) and auranofin (AUR)) on normal peripheral blood mononuclear cells (PBMC) were determined by flow cytometry using anti-CD19, anti-CD3 antibodies and dead cell marker 7-aminoactinomycin D (7AAD). The efficacy of AUR and BCL2 specific inhibitor – venetoclax (VEN) was assessed by MTT viability assay and by propidium iodide (PI) staining using flow cytometry.

Drug treatment in the B-ALL-MSC co-culture model: B-ALL primografts were co-cultured with BM-MSC for 24h and exposed to AUR, VEN or their combination for additional 5 days. The number of viable cells was evaluated by flow cytometry, using anti-CD19 antibody and cell counting beads.

In vivo studies: Cryopreserved primograft sample derived from MLLr B-ALL patient was thawed and transplanted through tail vein injections to NSG mice. The mice were treated with AUR (10mg/kg, i.p), VEN (100mg/kg, o.g) or their combination for 3 weeks and the leukemia progression was measured by peripheral blood staining with specific antibodies, as described above.

CRISPR-Cas9 system: We employed lentiCRISPR v2 plasmid to produce lentiviral vectors encoding *PMAIP1*-specific sgRNA and Cas-9 and used them to generate SEM cells with *PMAIP1* genomic deletion (*PMAIP1* encodes NOXA protein).

Results:

Considering more selective and safe profile of auranofin (AUR) in comparison to adenanthin (ADE), as determined by cytotoxicity tests on normal peripheral blood mononuclear cells (PBMC), for further *in vitro* and *in vivo* combination studies we chose only AUR. Based on MTT and PI-staining assays performed on MLLr B-ALL cell lines, we selected BCL2 specific inhibitor, venetoclax (VEN), as the most effective MLLr B-ALL targeted drug potentiating AUR activity. Further, we tested AUR and VEN combination in various MLLr B-ALL primografts in monoculture and co-culture with BM-MSC and observed strong synergistic effect of this combination. Following these results, we wanted to determine the combination efficacy *in vivo.* Using patient derived xenograft model of MLLr B-ALL we observed that combination of AUR and VEN diminished the progression of leukemia during 3 weeks-long treatment more effectively than any of the single drugs, and also prolonged the survival of NSG mice. Next, we focused on determination of mechanism responsible for synergistic action of AUR and VEN. Following our initial observation showing that combination treatment induced apoptotic cell death as indicated by caspases activation in SEM cells, we also evaluated its impact on apoptotic signaling. We tested the levels of major anti-apoptotic (BCL2, BCL-XL, MCL-1) and pro-apoptotic proteins (NOXA, Bim, Bak and Bax) and observed changes only in BCL-XL, MCL-1 and NOXA proteins. Importantly, we observed strong upregulation of pro-apoptotic NOXA at protein level upon AUR and AUR+VEN treatment, showing that it could be the driver of combination-induced mitochondrial apoptosis. To further prove this hypothesis we generated SEM cells with genomic knockout of NOXA using CRISPR-Cas9 system. As determined by MTT assay and PI-staining, we observed that NOXA knockout reduced the combination treatment efficacy, demonstrating AUR+VEN dependence on NOXA expression. Lastly, as we observed that AUR single treatment induced NOXA at transcriptional level and considering the fact that MLL-AF4 fusion protein is involved in epigenetic regulation of many genes, during the next year of my project I want to evaluate whether AUR-mediated NOXA upregulation is regulated through epigenetic processes. For this reason I plan to perform chromatin immunoprecipitation with sequencing in the collaboration with Prof. Thomas Milne from University of Oxford.

Participation in conferences:

1. 20th International Summer School on Immunology - Immune System: Genes, Receptors and Regulation, Hvar, Croatia, 21-28 September 2019.
2. 24th Congress of European Hematology Association, Amsterdam, The Netherlands, 13-16 June 2019.
3. Young Scientists Conference on Molecular and Cell Biology, IIMCB, Warsaw, 11 April 2019.
4. 10th EFIS-EJI South Eastern European Immunology School (SEEIS2018), Yerevan, Armenia, October 2018.

Publications, scientific presentations as a leading author:

1. Fidyt K., Pastorczak A., Goral A., Szczygiel K., Fendler W., Muchowicz A., (...) Firczuk M. (2019) Targeting the thioredoxin system as a novel strategy against B cell acute lymphoblastic leukemia. *Molecular Oncology*, Vol. 13:5, 1180-1195, IF=5.497.
2. Graczyk-Jarzynka A., Goral A., Muchowicz A., Zagozdzon R., Winiarska M., Bajor M., Trzeciecka A, Fidyt K., (...) Firczuk M. (2019) Inhibition of thioredoxin-dependent H2O2 removal sensitizes malignant B-cells to pharmacological ascorbate. *Redox Biology*, 21, 101062, IF=7.613
3. Fidyt K., Pastorczak A., Goral A., Muchowicz A., (…) Firczuk M. Thioredoxin system inhibitors are effective against B cell acute lymphoblastic leukemia *in vitro* and *in vivo.* Poster presented at 24th Congress of European Hematology Association, Amsterdam, The Netherlands, 2019.
4. Fidyt K., Pastorczak A., Szczygiel K., Muchowicz A., Goral A., Madzio J., ... Firczuk M. Targeting thioredoxin system as a novel strategy against B cell acute lymphoblastic leukemia. Poster presented at 10th EFIS-EJI South Eastern European Immunology School (SEEIS2018), Yerevan, Armenia, 2018.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Grzegorz Gula	Year of studies:4th

Title of the project: The potential of embryo-derived cardiac tissue macrophages

Name of the supervisor: Prof. Anna Ratajska

Affiliation: Department of Pathology, Medical University of Warsaw

AIMS of the project:

The aim of my PhD-research is to characterize the embryo-derived cardiac tissue macrophages (cTMs) by studying their phenotypes and in situ location. The main hypotheses are that among embryonic macrophages there are subpopulations that take part in regulating of new vessels formation and play a role as niche cells for extracellular matrix remodeling. Moreover, based on new reports and our own studies including the proepicardial organ and epicardial-derived cells we also assume that cTMs could play a mediatory role in EMT (the epithelial-mesenchymal transition) during heart development.
We could observe some kind of confusion in the classification and general change in perception of macrophages. Increasingly they are regarded as organ-specific cells and not only part of immune system. Following that trend, we took aim to characterize cardiac tissue macrophages during heart development. We would multi-methodically specify their phenotypes focusing on their putative function.

Methods:

Hearts derived from embryonic stages E12-15 and E17.5 and from adults were studied. The study consists of the following methods:
1. confocal microscope analysis of heart specimens immunolabeled with various macrophage markers (cryosections and the whole-mount-specimens)
2. flow cytometry analysis (Canto II) and sorting of macrophage populations (FACS Aria III)
3. qPCR analysis of mRNA expression of selected genes in sorted macrophage populations. T
hese techniques helped identify macrophage subpopulations based on their phenotypes, quantity, and location within the heart. The selected genes are possibly related to angiogenesis/ lymphangiogenesis regulation and extracellular matrix remodeling.

Results:

Embryonic cTMs consist of several subpopulations of different phenotypes. Using combination of different methods we were able to distinguish three main cTM populations differing in terms of mRNA expression levels of certain genes and the stage of development. The *in situ* studies on cryosections revealed a significant number of macrophages that are scattered in the subepicardial area. We used the following markers of cTMS: CD45, CD11b, CD64, CD68, F4/80, CD206, Lyve1, CD163. In comparison to adult murine heart, macrophages are only occasionally found in the myocardial wall of fetal heart. These cells are situated close to newly developed blood and lymphatic vessels, which are marked by CD31, Prox1, Lyve1, VEGFR2, VEGFR3. Some subpopulations of cTMS (GSI^+,$CD68^+$) were detected with a higher density in the place of massive apoptosis i.e. remodeling of valves and shortening of cardiac outflow tract, where they occurred as main phagocytes. The use of whole-mount immunostaining gave us an opportunity to better visualize co-localization of cTMs and vessel walls and to estimate macrophage shapes, i.e., whether they form protrusions. cTMs not only adhere to the walls of initial capillaries but also take specific shapes, what confirms their direct cell-to-cell function in angiogenesis and lymphangiogenesis. Moreover, whole-mount technique helped to recognize subpopulations among the subepicardialy located macrophages.

Based on the in situ data, FACS was used to distinguish, count and sorted subpopulations of embryonic cTMs: P1 - $CD45^+$ $CD11b^+$ $F4/80^+$ $CD64^{low}$; P2 - $CD45^+CD11b^+$ $F4/80^+$ $CD64^{high}$ $CD206^+$ and P3- $CD45^+$ $CD11b^+$ $F4/80^+$ $CD64^{+\ high}$ $CD206^-$. The RT-PCR studies and their results reveal differences in the level of mRNA expression for various genes among these three subpopulations and between two stages of development: 14dpc and 17dpc. All subpopulations express detectable levels of VEGFa (mainly P3 at 17 dpc) and only P1 expressed mRNA for VEGFc. Since VEGFC is a major lymphangiogenic factor, therefore, $CD64^{low}$ population could play a role in lymphangiogenesis. The expression of mRNA for MMP13 was higher in P3 than P2 and was absent in P1 (at 14dpc and 17dpc). P3 has significantly higher levels of mRNA for IGF1 than P1, P2 at both stages. The expression of mRNA for Retlna, Chill3, FGF2 was detected only at 17dpc and mainly in the P1 subpopulation.

During academic year 2018/2019 I was analyzing all previous data, summarized results and I was preparing manuscript as a main author.

Participation in conferences:

- SOE 2019, Congress of European Society of the Ophthalmology, 13-16 June 2019, Nice, France

Publications, scientific presentations as a leading author:

- Publication: *Proepicardium: Current Understanding of its Structure, Induction, and Fate.* Niderla-Bielińska J, Jankowska-Steifer E, Flaht-Zabost A, Gula G, Czarnowska E, Ratajska A. Anat Rec (Hoboken). 2018 Nov 12; DOI: 10.1002/ar.24028
- Presentation: *Implantation of iris-claw IOL in patients with coexisting glaucoma.* Gula G, Kaźmierczak R, Róg G, Filipiak E, Sempińska-Szewczyk J; SOE 2019, Congress of European Society of the Ophthalmology, 13-16 June 2019, Nice, France.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Łukasz Komorowski	Year of studies: 1st

Title of the project: Studies of the role of thioredoxin family antioxidant enzymes in B cell acute lymphoblastic leukemia with BCR-ABL1 translocation

Name of the supervisor: Małgorzata Firczuk, PhD

Affiliation: Department of Immunology, Medical University of Warsaw

AIMS of the project:

B cell acute lymphoblastic leukemia (B-ALL) is a cancer characterized by the overproduction and accumulation of immature lymphocytes, which occurs both in adults and in children. Currently the main therapeutic approach of B-ALL is chemotherapy that results in response to induction therapy exceeding 90%, although about 20% of treated pediatric patients experience relapse, being the principal cause of treatment failure. In adults B-ALL is rare in comparison to children, but is much more difficult to treat, as 50% of patients develop drug resistant disease. Therefore, novel therapeutic strategies allowing more successful treatment of resistant B-ALL are needed. One of the genetic subtypes of B-ALL is Philadelphia chromosome positive B-ALL (Ph+ B-ALL), which expresses BCR-ABL1 fusion protein and has poor survival prognosis. This subtype occurs predominantly in adults.

Research has shown increased levels of ROS and increased expression of thioredoxin (TXN) family antioxidant enzymes in B-ALL cell lines and primary cells. Particular dysregulation of redox homeostasis was observed in Ph+ B-ALL. One of its elements that is commonly upregulated in Ph+ B-ALL is peroxiredoxin 1 (PRDX1), a cytosolic enzyme which reduces hydrogen peroxide.

Aim of the project is to elucidate TXN system elements' role in Ph+ B-ALL, focusing mainly on PRDX1, and access its potential as novel therapy target.

Methods:

Cell lines and primary cells: We used BV173 chronic myeloid leukemia in lymphoid crysis cell line expressing BCR-ABL1 protein, characterized by similar phenotype to Ph+ B-ALL cells and expressing high levels of PRDX1. We also used SUP-B15 cell line which was established from Ph+ B-ALL patient. Primary cells were obtained from patients bone marrow, following their consent.

mRNA level measurement: TXN system elements mRNA levels in primary patient material were assessed by performing qPCR using SybrGreen and primers for PRDX1, thioredoxin 1, thiredoxin reductase 1 and RPL29 as a reference. Measurement was performed by LightCycler 480 instrument.

CRISPR-Cas9 gene silencing: BV173 cells with PRDX1 knockout were obtained by generating lentiviral particles in HEK293-T cells encoding Cas9 and sgRNA targeting PRDX1. Knockout was confirmed by Western Blotting and immunodetection.

Drug cytotoxicity: Cytotoxic effect of TXN system inhibitor, auranofin (AUR) and tyrosine kinase inhibitors imatinib (IMA) and dasatinib (DASA) were assessed using MTT viability assay. Percentage of dead cells was defined using propidium iodide staining and flow cytometry. Combination index was calculated in CompuSyn software.

Evaluation of cells' clonogenic potential: BV173 wt and sgPRDX1 cells' clonogenic potential was assessed in clonogenic assay, in which cells were seeded onto plates in semi-fluid methylcellulose medium with addition of IMA and counted after 6 days.

Results:

Our initial experiments showed increased expression of TXN family antioxidant enzymes in primary B-ALL cells. A particular dysregulation of redox homeostasis was observed in Ph+ B-ALL, especially upregulated levels of PRDX1 mRNA comparing to chronic myeloid leukemia primary cells expressing BCR-ABL1, showing that elevated PRDX1 expression is characteristic trait of Ph+ B-ALL cells and not only dependent on BCR-ABL1 expression.

Moreover, CRISPR-cas9-mediated knockout of PRDX1 significantly reduced BV173 cell proliferation, which was ROS dependent and could be rescued with ROS scavengers, catalase and pyruvate.

PRDX1 knockout also sensitized the cells to IMA, as shown in both MTT and PI assays. We checked whether this effect is ROS dependent by treating cells with IMA with addition of ROS scavengers and we observed that increased IMA sensitivity is only partially ROS dependent, indicating that PRDX1 may play a role in apoptotic signaling. It has been proven that prolonged exposure to elevated ROS levels can lead to selection of IMA resistant clones (Nieborowska-Skorska et al, *Leukemia* 2013), which could be troublesome in case of PRDX1 targeting therapy. To test it we performed 6 days clonogenic assay without scavengers, showing that PRDX1 knockout did not lead to increased IMA resistance over this period of time. We also performed initial experiments with DASA showing that PRDX1 ko cells are also more sensitive to second-line TKIs.

It has been shown that p38 phosphorylation is crucial for IMA cytotoxic effect (Parmar et al, *Journal of Biological Chemistry* 2004). Moreover, there is evidence that PRDX1 indirectly inhibits p38 phosphorylation in ROS dependent manner (Turner-Ivey et al, *Oncogene* 2013). In our results we show that PRDX1 knockout enhances p38 phosphorylation during imatinib treatment, indicating that PRDX1 is an important signaling molecule during the treatment. We also investigated changes in JNK1 and ERK 1/2 phosphorylation levels, which are also involved in apoptotic signaling after IMA treatment. Our initial Western Blot analysis showed that lack of PRDX1 causes higher and prolonged phosphorylation of both proteins in response to IMA administration.

As for now we are during a process of optimization of SUB-B15 transduction protocol and we are planning to knock out PRDX1 in them and access its effect on cell proliferation and sensitivity to therapeutics. In the next year we are also planning to further look into role of PRDX1 in signaling Ph+ B-ALL cells. Moreover we want to optimize culturing conditions for Ph+ B-ALL primary cells to obtain new model for therapy evaluation. If cytotoxic effect of drugs combination in primary culture will prove to be effective, we want to move to *in vivo* studies on mice injected with leukemia cells.

Participation in conferences:

1. FEBS 2019 Advanced Course - 20th International Summer School on Immunology. Immune System: Genes, Receptors and Regulation
2. XIV First Medical Department Scientific Conference.

Publications, scientific presentations as a leading author:

1. FEBS 2019 Advanced Course - 20th International Summer School on Immunology. Immune System: Genes, Receptors and Regulation, 2019 September 21-28, Croatia.
   Poster: L. Komorowski, K. Szczygieł, K. Fidyt, A. Pastorczak, T. Stokłosa, A. Muchowicz, A. Góral, E. Patkowska, J. Gołąb, M. Firczuk. Role of thioredoxin family antioxidant enzymes in B cell acute lymphoblastic leukemia with BCR-ABL1 translocation

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Agata Mikołajczyk	Year of studies: 2nd

Title of the project: Development of innovative small-molecule inhibitor to target both tumor cells and tumor microenvironment

Name of the supervisor: prof. Bożena Kamińska, dr Aleksandra Stańczak

Affiliation: Celon Pharma S.A.

AIMS of the project:

The main goal of research project is the selection and further preclinical development of innovative molecules inhibiting kinases from TAM tyrosine receptor family. Under normal physiological conditions, proteins from this family are expressed, e.g. on epithelial cells, macrophages, platelets, playing an important role in maintaining homeostasis, controlling cell survival, stabilizing blood clots and others. In cancer diseases, overexpression or ectopic expression of TAMs and their ligands are often observed. In addition to tumor cells, TAM proteins are also expressed in the tumor microenvironment. Inhibition of TAM proteins, both in tumor cells and in tumor microenvironment, can bring many benefits, besides inhibiting tumor growth or metastasis reduction - stimulation of anti-tumor immunity. Active compounds should also have drug-like properties such as high oral bioavailability, low toxicity, and an appropriate pharmacokinetic profile. Satisfactory parameters, combined with antitumor activity and stimulation of the antitumor immune response *in vivo* will provide the basis for further clinical development.

Methods:

The activity of all newly synthesized compounds was tested using kinase assay and in cell-based assays for selected compounds. Compounds' activity and selectivity within the TAM family were determined using kinase assay ADP-Glo™. In addition, kinase selectivity of selected compounds was tested in scanELECT panel of 100 kinases (Eurofins DiscoverX). Cell-based studies were performed on the following lines: BaF3 line with activated MER kinase and cell lines with confirmed expression of proteins from the TAM family, i.e. G361 (melanoma) or H1299 (NSCLC). The influence of the compounds on the phosphorylation of TAM family members and downstream proteins was determined using Western blot. Cells were starved and then stimulated with agonist anti-MER antibody before inhibitors treatment. To assess the effect of compounds on selected cell lines, proliferation and viability tests were performed using commercially available tests: ATPlite and CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT). In parallel, physicochemical and ADMET properties of compounds were conducted by Physicochemical Research Laboratory and ADMET Laboratory (Celon Pharma).

Results:

Based on obtained data, Celon Pharma's Medical Chemistry Department has synthesized 200 new small molecule compounds since last year. The activity of all compounds was tested toward MER kinase, for selected compounds also toward other kinases from the TAM family. Several compounds have subnanomolar IC_{50} toward MER kinase and varied activity towards other family members. Compounds with diverse activity were selected based on the result of studies on other kinases. External analysis in a panel of 100 kinases (scanELECT), suggested correlation between compound selectivity profiles and chemical structures. Furthermore, the kinase selectivity panel allowed the initial determination of off-target kinases.

Setting the stimulation method with MER-agonist antibody enabled the detection of the phosphorylated form of MER protein. Compounds treatment of cell lines expressing TAM kinases resulted in a dose-dependent decrease in phosphorylation, including MER protein and downstream proteins i.e. AKT. Physicochemical and ADMET studies allowed the selection of compounds with the desired parameters, i.e. high kinetic solubility or stability on human and mouse microsomes.

Based on the results obtained this year, selection of the compounds for *in vivo* tests and subsequent analysis of the results will be performed. Moreover the results will also be used for further *in vitro* tests, including studies related to the cancer microenvironment.

Participation in conferences:

10th Inhibitors of Protein Kinases Conference "Challenges in Molecular Biology, Biophysics and Biomedicine" (upcoming), 14^{th}-19^{th} of September, Warsaw, Poland

Publications, scientific presentations as a leading author:

1) Mikolajczyk A., Yamani A., Olejkowska P., Piorkowska N., Naitana M., Maliszewski P., Dubiel K., Pieczykolan J., Wieczorek M., Stanczak A. "Rational-based drug design of Novel, highly potent MER inhibitors as potential treatment of cancers"; poster session at 30th EORTC/AACR/NCI symposium, November 13-16, Dublin, Ireland
2) Kozlowska D., Górnicka A., Stypik B., Mroczkiewicz M., Mikolajczyk A., Hucz-Kalitowska J., Szwalbe A., Mulewski K., Smuga D., Dubiel K., Pieczykolan J., Wieczorek M., Stanczak A. "CPL-410-005, a novel ubiquitin-activating enzyme (UAE) inhibitor in preclinical evaluation as an anticancer treatment for solid"; poster session at 30th EORTC/AACR/NCI symposium, November 13-16, Dublin, Ireland
3) Hucz-Kalitowska J., Teska-Kamińska M., Stańczak A., Skupińska M., Mikołajczyk A., Pieczykolan J., Wieczorek M. "Efficacy study of Celon Pharma FGFR kinase inhibitor in two Patient-Derived Tumour Xenograft (PDTX) Models"; poster session at VI Conference: animals in scientific research), September 9-11, Warsaw, Poland

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Piotr Pankiewicz	Year of studies: 2nd

Title of the project: Development of novel, small molecule TrkB agonists as potential therapeutic in treatment of central nervous system disorders.

Name of the supervisor: dr hab. Katarzyna Kalita-Bykowska, dr Jerzy Pieczykolan

Affiliation: Nencki Institute of Experimental Biology PAS; Celon Pharma S.A.

AIMS of the project:

Amongst CNS disorders depression is the most prevalent mental disorder worldwide. The therapeutic effects of the currently available antidepressants are observed after a few weeks of regular dosage, they are coupled with many side effect manifestations and many patients do not respond to the therapy. Due to that there is a huge medical need to develop a new therapies.

TrkB is a receptor with the tyrosine kinase activity and is modulated upon BDNF binding. BDNF (brain derived neurotrophic factor) is a member of the neurotrophin family and acts as a key regulator of many neuronal processes. BDNF triggers TrkB dimerization in canonical activation event that leads to autophosphorylation of tyrosine residues in its intracellular domain. As a consequence, three intracellular signaling pathways related to Akt, PLCγ and ERK kinases, become activated. It is already known that BDNF is implicated in pathophysiology of many neuropsychiatric diseases. For instance, the reduced level of the BDNF protein in brain tissue, in particular in the hippocampus, is observed in patients suffering from depression. Targeting the BDNF-TrkB pathway by the small molecular compounds may have antidepressant and procognitive effects.

Aims of the study.

I. Identification of active and selective molecules within the library of structures which display affinity to TrkB receptor
II. Selection of a lead candidate with the antidepressant and procognitive properties
III. Characterization of selected compounds *in in vitro* and in vivo models – pharmacological verification of mechanism of action and compounds' properties

Methods:

Design of structures library: compounds library was designed based on published data of the known natural and small molecular TrkB receptor agonists which was delivered by Celon Pharma Medicinal Chemistry department.

Affinity Screen and dimerization assessment: The affinity to the TrkB receptor was determined biophysically by using thermophoresis method (Microscale Thermophoresis). The ability of compound to provoke TrkB receptor dimerization was analyzed by native electrophoresis.

In vitro Screening: The SN56 T48 cell line with stable TrkB receptor expression and differentiated SH-SY5Y cell line were used for testing of compound activity *in vitro* in orthosteric and positive allosteric mode. Activation of the TrkB receptor and its effector proteins in these cell lines was evaluated by the Western blot and ELFI (Enzyme-linked fixed cell immunoassay method). Preliminary experiments of compound specificity were performed using K252a inhibitor and ANA-12 antagonist.

Results:

Compound libraries of TrkB receptor agonists contained more than 800 that have beed screened. Primary screen for interaction of compound with TrkB revealed 65 structures with satisfactory affinity. Obtained results from the microscale thermophoresis were used for the structure affinity relationship study.

Secondary screening used cell line models for search orthosteric and positive allosteric modulators of TrkB receptor. By using ELFI method several derivates of DMAQ-B1 compounds were identified as a TrkB orthosteric activators. The compounds activity was also confirmed by examination of downstream protein phosphorylation (Akt1, $ERK_{1/2}$) in SN56T48 and differentiated SH-SY5Y models. Then, selected compounds were tested for receptor specificity *in vitro* by using K252a inhibitor and ANA-12 antagonist. Finally, the ability to provoke TrkB receptor dimerization by selected compound was examined by native electrophoresis.

Conclusions;

- Microscale thermophoresis combined with the *in vitro* models can be applied for screening TrkB receptor orthosteric agonist and allosteric modulators. 4 molecules from 860 screened compounds act as a orthosteric agonist, one of them also positively modulates BDNF activity.
- K252a and ANA-12 are useful tools for preliminary examination of compound specificity. Pretreatment with ANA-12 and K252a significantly decreases TrkB activity and downstream protein phosphorylation after TrkB agonist treatment.
- Native electrophoresis can be used as a method for monitoring TrkB dimerization. None of small molecular agonists provoke receptor dimerization. However, receptor dimerization is not necessary for achieving molecular activity by small molecular TrackB agonists [*Zahavi et al. 2018*].

Participation in conferences:

- 31st ECNP Congress, 6-9 October 2018, Barcelona, Spain
- 7th Mediterranean Neuroscience Conference, 23-27 June 2019 - Marrakech, Morocco.

Publications, scientific presentations as a leading author:

Pankiewicz et al. "*Development of compound screening platform for discovery small molecular TrkB receptor agonist".* 7th MNS, 23-27 June 2019 - Marrakech, Morocco.

Pankiewicz et al. "*CPL-500-036 – novel and highly bioavailable pde10a inhibitor activates cyclic nucleotides dependent signaling in rat striatum*". 31st ECNP Congress, 6-9 October 2018, Barcelona, Spain.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Zuzanna Sas	Year of studies: 3rd

Title of the project: Use of the cellular tumor drug-delivery system to modulate tumor microenvironment

Name of the supervisor: dr Tomasz Rygiel

Affiliation: Department of Immunology, Medical University of Warsaw

AIMS of the project:

The main goal of this project is development of more effective carriers of anticancer drugs to increase their tissue delivery and uptake by tumor cells. Up to now several protein drug-carriers for drug delivery system have been developed. In our project we exploit the properties - of iron-binding proteins. So far we have been using various ferritin constructs in order to deliver cavity encapsulated anticancer drugs to tumor tissues. Last year we focused on other iron- binding protein- haemoglobin (Hb). We have observed unexpected biodistribution of intravenously injected free Hb. The aim of this part of the project was to observe *in vivo* biodistribution of Hb in mice and to characterize the cell population of liver cells responsible for uptake of free Hb from blood.

Methods:

Optimization of Hb-AF750 dose for intravenous injection and measurement of its *in vivo* biodistribution

Hb was isolated from Balb/c mouse blood and conjugated with Alexa Fluor 750 dye (AF-750). Fluorescently labeled Hb (Hb-AF750) was injected intravenously in different doses. 20h post injection mice were sacrificed and total fluorescence of Hb-AF750 was measured in isolated organs including: lungs, liver, spleen, kidneys and heart using Bruker *in vivo* Imaging System.

Biodistribution of Hb-AF750 after clodronate treatment

Clodronate liposomes were injected intravenously. One day later mice were injected intravenously with 0.2 mg of Hb-AF750. 20h post injection mice were sacrificed and total fluorescence of Hb-AF750 was measured in isolated organs including: lungs, liver, spleen, kidneys and heart using Bruker *in vivo* Imaging System.

Characterization of liver cells populations using Flow cytometry

Isolated, perfused mice livers were digested into single cell suspension using Collagenase/DNAse solution and mechanically dissociated. Populations of cells in liver were visualized and quantified using monoclonal antibody staining and flow cytometry. The following cellular markers were used: CD45, F4/80, Stab2 and CD146.

Results:

To check the role of macrophages in Hb uptake, we depleted these cells in mice using clodronate liposomes. Interestingly Hb uptake increased in liver after macrophages depletion. Using flow cytometry we confirmed that macrophages ($CD45^+CD11b^+F4/80^+$) are not the main population responsible for Hb uptake. We identified Liver Sinusoidal Endothelial Cells ($CD45^+CD146^+Stab2^+$) asthe population that accumulated Hb with the highest efficiency.

Publications, scientific presentations as a leading author:

1.Pilch Z, Tonecka K, Braniewska A, Sas Z, Skorzynski M, Boon L, Golab J, Meyaard L, Rygiel T 2018. Antitumor activity of TLR7 is potentiated by CD200R antibody leading to changes in the tumor microenvironment. Cancer Immunology Research.
2.Taciak B, Białasek M, Braniewska A, Sas Z, Sawicka P, Kiraga Ł, Rygiel T, Król M 2018. Evaluation of phenotypic and functional stability of RAW 264.7 cell line through serial passages. PLoS One.
3.Pilch Z, Tonecka K, Skorzynski M, Sas Z, Braniewska A, Kryczka T, Boon L, Golab J, Meyaard L, Rygiel T 2019. The pro-tumor effect of CD200 expression is not mimicked by agonistic CD200R antibodies. PLoS One.
4.Sas Z, Braniewska A, Pilch Z, Pingwara R, Taciak B, Krol M, Rygiel T. Use of the cellular tumor drug- delivery system to modulate tumor microenvironment. 10th EFIS-EJI South Eastern European Immunology School, Yerevan, Armenia 2018.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Karolina Soroczyńska	Year of studies: 1st

Title of the project: Molecular profile of exosomes in the bronchoalveolar lavage fluid as a new biomarker for impaired immune status in non-small cell lung cancer

Name of the supervisor: Małgorzata Czystowska-Kuźmicz, PhD

Affiliation: Medical University of Warsaw

AIMS of the project:

Numerous studies provided evidence that tumor cells – including lung cancer – develop various suppressive mechanisms to protect against immune eradication. Tumor-derived exosomes (TEX) have been recently indicated as negative modulators of the anti-tumor immune response, enabling the tumor to develop a systemic immunosuppression and thus interfering with existing immune therapies. The general aim of the project is an in-depth molecular and functional analysis of the immune-inhibitory profile of exosomes present in bronchoalveolar lavage fluid (BALF) in reference to the immune status within the tumor microenvironment of non-small cell lung cancer (NSCLC) patients.

Specific aims of the project are:

1) to determine the levels and molecular profiles of exosomes isolated from two BALF-compartments of the same patient: taken from the cancerous lung and from the opposite healthy lung as an internal control and to compare the identified profiles with exosome profiles from peripheral blood (PB);
2) to correlate the identified exosome cargo of inhibitory molecules with the immune profile of immune cells in the cancerous/healthy lung;
3) to elucidate the effects of exosomes from lung cancer patients' BALF on immune cells in *in vitro* studies.

Methods:

The first step of the study was to optimize the experimental methods used in aim 1 and 2, which included: setting up the isolation protocol for BALF exosomes, the analysis of exosomes by nanoparticle tracking analysis (NTA) method (NanoSight NS300, Malvern), the phenotyping of exosomes and immune cells from BALF and PB by flow cytometry (BD *FACS Lyric*s). Bronchoalveolar lavage fluid from the lung affected by cancer (cBALF as the local environment), compared to the opposite 'healthy' lung (hBALF as the internal control) and the peripheral blood (PB reflecting the systemic changes) were collected from 10 patients with confirmed lung cancer. Bronchoalveolar lavage was performed during routine bronchofiberoscopy at the Institute of Tuberculosis and Lung Diseases in Warsaw, after o*btaining patients' informed consent.* Tumor-derived exosomes were isolated from the lung cancer patients' samples using ultracentrifugation (BALF TEX) or size exclusion chromatography (PB TEX). The size and concentration of TEX was determined by measuring protein concentration (Bradford assay) and NTA method. The expression of several inhibitory molecules which might play a role in the tumor microenvironment (PD-L1, TGF-β, CTLA-4, LAG-3, FasL) and selected tumor markers of lung cancer (EGFR, MAGE-A3, EpCAM, NY-ESO-1) on TEX was determined by Western blotting and on-bead flow cytometry. The immune profile of the immune cells isolated from the same BALF and blood samples, like absolute number of CD4+, CD8+T lymphocytes, T regulatory (Treg) lymphocytes and the expression of molecules modulating their functions, like CTLA-4, PD-1, LAG-3, Tim3 as well as the percentage of apoptotic cells and the activation status, were determined by multiparameter flow cytometry.

Results:

We detected exosomes in both lung compartments, whereby the mean concentration was slightly higher in the hBALF in comparison to cBALF as meaured by NTA (11,08 +/-5,15 vs. 9,28+/-5,75). These exosomes contained some common tumor markers (EGFR, EpCAM, NY-ESO-1), typical exosomal markers (CD63, CD9, CD81, Tsg101) and in some cases immune-inhibitory molecules, like PD-L1, FasL, TGF-β, as shown by Western blotting. The immunological profile of the BALF immune cells, which represent immune cells from the tumor microenvironment, was different from the profile of the circulating immune cells and varied among analyzed patients. The CD4+/CD8+ ratio and the proportion of naïve CD8+ and CD4+ cells and effector CD8+ cells were significantly decreased in BALF whereas the proportion of peripheral memory CD8+ and CD4+ cells was increased. Nearly all CD4+ and CD8+ BALF cells were highly activated comparing to the corresponding PB lymphocytes based on the expression of an early cell surface activation marker CD69. At the same time, the BALF-derived lymphocytes were more apoptotic (both early and late apoptotic), than those from the PB. Also, the expression PD-1 on T cells was higher in BALF than in PB. In some patients, we observed also a higher expression of LAG-3 and Tim-3 on BALF lymphocytes. Furthermore, there was a significantly higher percentage of Tregs in BALF in comparison to PB. Based on these results, we can conclude, that there is a substantial immunosuppression of T-cells within the tumor-microenviroment represented by the BALF. Interestingly, no significant differences were found in the analyzed T cell subpopulations between the cBALF and hBALF. Furthermore, we provide the first evidence for the presence of the immunosuppressive molecules on the surface of BALF exosomes. Within this small cohort of analyzed patients, we already were able to notice a trend pointing at a connection between the immunosuppressive profile of T cells and the molecular cargo of exosomes within the BALF. Due to the heterogeneity of detected exosome and T-cell profiles, more patients' samples need to be analyzed to be able to evaluate significant relations between the exosome cargo of inhibitory molecules and the immune profile of immune cells in the cancerous/healthy lung. The next step will be the detailed analysis of exosome cargo by Western blotting and flow cytometry and the correlation of those results with the immune profile and clinical data of the patients.

Participation in conferences:

1. XIV Conference of the First Faculty of Medicine, 7th April 2019, Medical University of Warsaw, Warsaw.

Publications, scientific presentations as a leading author:

1. Czystowska-Kuzmicz M, Sosnowska A, Nowis D, Ramji K, Szajnik M, Chlebowska-Tuz J, Wolinska E, Gaj P, Grazul M, Pilch Z, Zerrouqi A, Graczyk-Jarzynka A, Soroczynska K, Cierniak S, Koktysz R, Elishaev E, Gruca S, Stefanowicz A, Blaszczyk R, Borek B, Gzik A, Whiteside T, Golab J. Small extracellular vesicles containing arginase-1 suppress T-cell responses and promote tumor growth in ovarian carcinoma. *Nat Commun*. 2019 Jul 5;10(1):3000.
2. Czystowska-Kuźmicz M, Soroczyńska K, Polubiec-Kownacka M, Domagała-Kulawik J. Molecular profile of exosomes in the bronchoalveolar lavage fluid as a new biomarker for impaired immune status in non-small cell lung cancer. FEBS Advanced Courses. 20th International Summer School on Immunology Immune System: Genes, Receptors and Regulation. 21 – 28th September 2019, Hvar, Croatia. Oral/poster presentation – still to come, already registered.

Report on the scientific progress in the academic year 2018/2019

Name of the PhD student: Anna Sosnowska	**Year of studies:** 3rd

Title of the project: Investigating antitumor activity and mechanisms of action of arginase inhibitors in cancer immunotherapy

Name of the supervisor: Prof. Jakub Gołąb

Affiliation: Department of Immunology, Medical University of Warsaw

AIMS of the project:

An increasing number of observations indicate that arginase (ARG) is involved in mitigating antitumor effector mechanisms of the immune response. High ARG expression or activity in the tumor microenvironment correlate with poor clinical outcomes of cancer patients. Therefore, we investigated ARG as a potential immunotherapy target and whether a novel ARG inhibitor OAT1746 can exert antitumor effects in mice.

Methods:

- $0.5x10^6$ of Lewis Lung Carcinoma (LLC) cells were inoculated subcutaneously into C57BL/6 mice (n=6) on day 0, 7 and 14 of experiment and isolated, CTV stained, OT-I $CD8^+$ lymphocytes were transferred intravenously on day 17. Antigen specific proliferation was triggered with ovalbumin protein injected subcutaneously in tumor area on day 18. Tumor draining lymph nodes were harvested on day 21 and proliferation of OT-I T cells as well as CD3ζ expression were evaluated by flow cytometry. L-arginine and L-ornithine concentrations in plasma were assessed by mass spectrometry.
- LLC cells were inoculated subcutaneously in induced by tamoxifen ARG knock out (KO) mice called ARGfloxROSA and wild type (WT) controls C57BL/6 (n=6-7) and tumor growth was measured with digital calipers starting from day 8 of experiment. In control groups peanut oil (tamoxifen diluent) was administered orally from day 7 until day 11. To confirm ARG KO, L-arginine concentration in plasma was assessed by mass spectrometry. Tumors were isolated, weighted, processed, stained with fluorochrome-conjugated antibodies and tumor infiltrating lymphocytes were analyzed by flow cytometry.
- LLC cell line was modified by lentiviral transduction with ARG1 or a control plasmid pLVX. Transduction efficiency was confirmed at the protein level by Western blotting and in enzymatic assay that measures ARG activity. LLC tumors overexpressing ARG1 at different levels (LLC-pLVX-ARG1) and control cell lines (LLC WT, LLC-pLVX) were inoculated in vivo (n=7-8) and tumor growth was monitored in C57BL/6 and RAG2 KO mice. Selected groups were treated with ARG inhibitor OAT-1746 by intraperitoneal route twice daily for 14 days starting from the next day after tumor cells inoculation. Additionally, tumors were isolated and mass was weighted during harvest.

Results:

- On harvest day 21 mice with LLC tumors inoculated on day 0, 7 and 14 had big, intermediate and small tumors, respectively, reflected as tumor mass and volume. In mice with small and intermediate tumors a drop of plasma L-arginine concentrations was observed (Mean±SD: 109.1 μM ± 10.48 μM; 100.7 μM ±15.32 μM, respectively) in comparison with control non-tumor bearing mice (147.8 μM ± 24.33 μM). Importantly plasma L-arginine was the lowest in mice with the biggest tumors (26.15 μM ± 9.34 μM) whereas L-ornithine concentrations

were the highest. OT-I proliferation was impaired in big tumors (63.58% ± 21.43%) in comparison with small (89.53%±5.48%) and intermediate (83.57% ± 5.15%) tumors. Also CD3ζ expression was downregulated in group of mice with big tumors (MFI= 719.3 ± 104.6 vs. 964.5 ± 171.6 in small and 1143 ± 64.11 in intermediate tumors).

- ARG KO was confirmed as ARGfloxROSA mice treated with tamoxifen showed accumulation of plasma L-arginine (458.3 μM ± 141.8 μM) in comparison with ARGfloxROSA control mice treated with peanut oil (45.86 μM ± 11.39 μM). ARG KO inhibited tumor growth ($p < 0.0001$; (day 19: 1354 mm^3 ± 164.1 mm^3 vs. 2431 mm^3 ± 482.4 mm^3, respectively). Furthermore, tamoxifen administered to C57BL/6 mice did not show antitumor efficacy in LLC lung cancer model. Both the percentage as well as absolute numbers of tumor-infiltrating lymphocytes $CD3^+$ were increased in ARG KO mice as compared to ARGfloxROSA control mice (1.967% ± 0.216% vs. 1.200% ± 0.585% and 1.920e+006 ± 256355 vs. 1.043e+006 ± 317272, respectively).
- Transductions were successful as the generated cell lines overexpressed ARG1 enzyme that was actively converting substrate L-arginine into urea. In vivo, ARG1 overexpression by LLC tumor cells resulted in accelerated progression of tumor growth that was dependent on the level of ARG1 overexpression. These effect was not seen in RAG2 KO that lacks T and B cells suggesting the mechanism dependent on lymphocytes presence. Moreover, treatment with ARG inhibitor strongly inhibited the tumor growth in both LLC WT as well as LLC-pLVX-ARG1 implicating that OAT1746 not only can block the activity of ARG in tumor microenvironment but also those overexpressed by tumor cells.

Conclusion:

- To conclude, these data demonstrate that ARG is relevant target in cancer immunotherapy and that inhibition of ARG represents a promising approach among other anti-tumor therapies.

Publications:

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2. Anna Sosnowska, Malgorzata Czystowska-Kuzmicz, Jakub Golab. Extracellular vesicles released by ovarian carcinoma contain arginase 1 that mitigates antitumor immune response. Manuscript accepted in OncoImmunology
3. Stelmaszczyk-Emmel A, Sosnowska A, Kurkowiak J, Sagała M, Zawadzka-Głos L, Pyrżak B, Demkow U. Utility of leucocyte antigens in distinguishing between bacterial and viral infection in children. Cent Eur J Immunol. 2018;43(3):262-269
4. Paweł Matryba, Anna Sosnowska, Artur Wolny, Lukasz Bozycki, Alan Greig, Jakub Grzybowski, Marzena Stefaniuk, Dominika Nowis, Jakub Gołąb. Systematic evaluation of chemically distinct tissue optical clearing techniques in murine lymph nodes. Manuscript under review submitted to J Immunol.