ICGEB Research Grants Programme

RESEARCH GRANTS COMPLETED IN 2018
## CRP - ICGEB Research Grants Completed in 2018

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Co-expression of the *Arabidopsis AVP1* and *NHX1* to enhance salt tolerance in transgenic soybean (*Glycine max* (L.) Merrill) (Early Career Return Grant)
Title: Molecular regulation of microRNAs biogenesis and action in plants

Abstract: MicroRNAs (miRNAs) are essential components of the genetic regulatory networks in plants and animals. Through inactivation of specific mRNAs, especially those encoding transcription factors, miRNAs modulate the homeostasis, development and responses to environmental stimuli of multicellular organisms. In order to accomplish its biological functions, a spatial/temporal balance between a given miRNA and its targets has to be reached. Thus, a fine-regulation of the miRNA action is critical. As part of our research project, we characterised miRNA-deficient mutants, established their mechanisms of action and explored their relationship with other transcriptional processes. Additionally, we studied the mature miRNA duplex interaction with ARGONAUTE 1. The project was performed in Arabidopsis as a model plant, but we expect to extrapolate the acquired knowledge to other plants, especially those of local agronomical importance. Current strategies directed to improve crops in Argentina make use of manipulation of gene expression to enhance a particular plant trait. In this sense any effort to understand post-transcriptional gene silencing is beneficial for such research lines.

Objectives:

During this project, we proposed to explore the regulation of the miRNA pathway focusing on two critical steps: their production and strand sorting.

(i) To identify regulatory factors acting in the miRNA biogenesis: From a pool of miRNA-deficient mutants previously isolated in the lab we proposed to characterise some of these mutants. We aimed to identify the mutated genes causing the defective miRNA production and their mechanisms of action. Globally our objective here was to generate a more complete picture of the diversity of mechanisms controlling miRNA production in plants.

(ii) To determine the mechanisms directing miRNA strand sorting and AGO loading: During the miRNA-mediated gene silencing process, a change of AGO preference for miRNA or miRNA* could lead to dramatic changes in the sets of genes to be silenced and therefore in the physiology of the plant. We have observed that strand loading varies between different tissues of the same individual, implying developmental plasticity in the system. In this part of the project we aimed to elucidate the mechanisms involved in the differential loading of the miRNA duplex strands, using biochemical and genetic approaches.

Results Obtained:

Following the objectives of this project we first identified RCF3 as a new miRNA co-factor. Its characterisation allowed us to propose this protein as one of the first tissue specific regulator of the miRNA machinery in plants. Our results showed how RCF3, which is specifically expressed in the shoot apexes, regulates HYL1 activity by changing its phosphorylation status. Such phenomenon, directed by the interaction between RCF3 and CPL1/2, only takes place in the vegetative and reproductive apices modulating the plant development (Karlsson, et al., PNAS, 2015). We identified THO2 as a new co-factor in the miRNA pathway. This protein, which is the core of the THO/TREX complex in Arabidopsis, is not only important for miRNA accumulation but also to siRNA and tasiRNA, pointing it as a general factor participating in most small RNA pathways. Mechanistically, we found that the THO/TREX complex is not directly involved in the miRNA processing but acts delivering the neo-transcribed pri-miRNAs to the processing centres. In addition to its role in miRNA biogenesis, we also found that THO2 is essential for mRNA splicing. This discovery, links miRNA production with splicing, a link we aimed to explore (Francisco-Mangilet, et al., Plant J., 2015). An added value to our research is that the isolated THO2 mutants (we found 3 new mutants alleles) are the first THO2 mutant alleles that can be obtained as homozygous adult plants. All previous identified alleles die early at embryonic stage, which reflect the importance of the protein, but make any effort to study its function frustrating. Additional miRNA defective mutants are also currently under study in the frame of this project. MSS27 and MSS141, which present strongly reduced miRNA levels, act controlling the subcellular localisation and dynamics of the miRNA components and by post-translationally regulating key proteins in the pathway respectively. In December 2016 we published a review about miRNA biogenesis where we integrated all our discoveries with the current knowledge in the field. We focused on the dynamics and paradigms of the pathway as well as the new frontiers on this research topic (Achkar, et al., Trends Plant Sci., 2016).

We also continued studying how the phosphorylation of HYL1, key factor in the miRNA biogenesis, affects and modulates its activity. We found that phosphorylation of this protein controls its...
protein-protein interaction capacity, its ability to interact with miRNA precursors and its subcellular distribution. All these changes affect the proteins stability in a process that we found to be essential for the proper response of the plant to a reduction in the quantity or quality of the available light (Achkar, et al., manuscript submitted).

Considering the second objective of this project, we have used a novel reporter system to isolates mutant plants where the normal miRNA-strand sorting in AGO1 is switched. We have done all the basic characterisation of this novel mutants and are currently ready to proceed to whole-genome re-sequencing and polymorphisms identification. We have also studied a particular mutant from our initial screening that showed altered AGO1 activity. MSS47 is a mutant that has a general failure in the activity of miRNA, but surprisingly these molecules over-accumulate in the plants. Our studies have shown that the protein encoded by this gene control the turnover of AGO1 by preventing its degradation. Such degradation, which is tissue, light and environment dependent, is triggered by an induction of specific proteasome and autophagy genes.

Publications:
Ré, D.A., Manavella, P.A. Caught in a TrAP. 2015. elife 4, e11509
Title: Large-scale functional evaluation of microRNAs in pluripotency, self-renewal and differentiation of stem cells using high-content screening

Principal Investigator: Rodrigo Alexandre Panepucci, Laboratory of Functional Biology, Center for Cell-Based Therapy, Blood Center of Ribeirão Preto, Rua Tenente Catão Roxo 2501, CEP: 14051-140 Ribeirão Preto, SP, Brazil. Tel: +55-16-3602-2223, Fax: +55-16-2101-9309, E-mail: rapane@gmail.com
ICGB Contract No.: CRP/14/005
ICGB Reference No.: CRP/BRA14-02

Abstract: MicroRNAs (miRs) play an important role in Embryonic Stem Cells (ESCs), binding to target mRNAs transcripts, inducing translation blockage and/or transcripts degradation; however, their specific roles are largely unexplored. We investigated miR roles in pluripotency and differentiation, using quantitative automated fluorescence microscopy (High Content Screening). For this, we transfected mRNA mimics in human ESCs or embryonal carcinoma NTera-2 cells and evaluated several morphometric parameters and intensity measurements of Oct4 and Cyclin B1 in the nucleus and cytoplasm. We highlight differences between both cell models, and as compared to mouse ESCs lacking endogenous miRs, used in the only screenings published up to date. Next, hierarchical clustering of the miR-specific multiparametric phenotypic profiles allowed us to identify miRs inducing similar effects. By carrying an enrichment analysis of the predicted targets of these clustered miRs, we uncovered several potentially post-transcriptionally modulated pathways. Inhibition of Argonaut transcripts by top-expressed ESC-miRs may restrict the action of RISC and pro-differentiation miRs, without compromising pluripotency. Specifically, miR-363 targets Eomes and PSEN1 and inhibits Notch-induced differentiation; a mechanism potentially implicated in naive and primed pluripotency states.

Objectives:
(i) To explore the roles of miRNAs on pluripotency and differentiation of human pluripotent cells;
(ii) To establish a High Content Screening (HCS) assay based on quantitative automated fluorescence microscopy, capable of interrogating the effects of transfected miR mimics on the pluripotency and cell-cycle status of pluripotent human cells; specifically, the embryonal carcinoma cell line NTera-2 and the H1 human Embryonic Stem Cell line (H1 hESCs);
(iii) To carry a full library screen (2,042 miRNAs, miRBase v.19) in Ntera-2 cells;
(iv) To identify predicted targets of miRs inducing similar effects and uncovered pathways enriched for these targets (i.e. potentially post-transcriptionally modulated by them);
(v) To functionally validate targets and pathways identified.

Results Obtained:
We present the first functional screen evaluating miRNA effects on pluripotency and differentiation of human ESCs and embryonal carcinoma NTera-2 cells, using automated quantitative fluorescence microscopy (High-Content Screening). Following transfection with thirty miRNA mimics, cells were cultured for 3-4 days, stained with Hoechst/CellMask Blue (nucleus/cytoplasm), immunostained for OCT4 and Cyclin B1, and imaged. Several cellular morphometric and intensity measurements (nuclear and cytoplasmic) were grouped into miRNA-specific multiparametric phenotypic profiles and submitted to hierarchical clustering; allowing us to identify miRNAs showing similar or distinct phenotypic effects. While the comparison of the functional miR screenings carried with NT2 and H1 hESCs, revealed several similarities, we were able to identify several contrasting phenotypic effects mediated by the evaluated miRs. For instance, the miR-302-3p family (miR-302-3p/372-3p/373-3p/520-3p, sharing the seed AAGUGCU) showed conserved effects in NT2 and H1 hESCs. Similarly, miR-18a/b-5p and miR-106a-5p had an overall comparable effect in both cells. In the other hand, some miRs belonging to the miR-17~92 or the miR-106a~363 clusters had different effects in NT2 or h1 hESCs. For example, OCT4 and CycB1 levels were strongly repressed by miRs miR-19a/b-3p and miR-20a/b-5p in NT2 cells, and strongly induced in H1 cells. In turn, miR-92a-3p and miR-363-3p had opposing effects on cell size, decreasing it in NT2 cells, while increasing it in H1 hESCs. In turn, some members of the same family had opposing effects in the same cell line, despite identical seeds. For instance, miR-20a/b-5p and miR-106a-5p (which belong to the family miR-17-5p/20-5p/93-5p/106-5p/519-3p/526-3p, sharing the seed AAAGUGC) showed, respectively, pro-differentiation and pro-pluripotency effects in NT2 cells. In the other hand, miR-92-3p and miR-363-3p (which belong to the family miR-25-3p/32-5p/92-3p/363-3p/367-3p, sharing the seed AUUGCAC) had opposite effects on proliferation and nuclei size in the same cellular context, in both cell lines. These results highlight how specific targets of closely related miRs can have extremely contrasting functional effects on a given cell, and also, how the same miR can have contrasting effects depending on the cellular context. Moreover, we show important differences between our results with human pluripotent cells, with those obtained with DGCR8 KO mESCs lacking endogenous miRs. By identifying transcripts targeted by clustered miRs (i.e. similar profiles), we uncover several post-transcriptionally modulated pathways. Interestingly, by comparing all the
pathways enriched for predicted targets of the pro-pluripotency clusters to those of the pro-differentiation clusters, we identified pathways exclusively associated with pluripotency-related clusters, including, for example, the Dicer/RISC miRNA pathway and a pathway describing Presenilin action in Notch and Wnt signaling. Interestingly, we propose that generalised inhibition of all Argonaut transcript variants by top-expressed ESC-miRs of the miR-302-3p/372-3p/373-3 family may downregulate RISC activity, restricting the action of pro-differentiation miRNAs, without compromising pluripotency. In the other hand, Presenilin/Notch pathway was specifically targeted by miRs that characteristically restricted proliferation in NTERA2 and H1 hESCs. Since the Notch signaling is associated ESCs differentiation, we functionally explored the potential role of selected miRs in the inhibition of Notch signaling. Importantly, we demonstrated that miR-363 targets Notch1 and PSEN1 and inhibits Notch-induced differentiation; a mechanism that could be implicated in the naïve pluripotent state, as the expression of Notch receptors and components has been specifically associated with cells in the naïve state.

Results Unforeseen in the Original Project:
Interestingly, clustering of the quantified phenotypic features across distinct miRs allowed us to identify that the median intensity levels of Oct4 and CyclinB1 (in the nucleus and cytoplasm) were highly correlated features in both cell lines used (forming a tight cluster); with pluripotency-related miRs associated with high levels of both proteins in the cell population, and differentiation-related miRs associated with low levels. Human and mouse ESCs are characterised by a high proliferation rate and a shortened G1 phase. Cyclin B levels increase during late-S across G2 and M phases, and drastically drop upon re-entry into G1, when it abruptly translocates into the nucleus (shortly before nuclear envelope breakdown), being degraded during G1. Thus, an increase in the number of pluripotent cells would be associated with a lower fraction of the population in G1 (i.e. in S, G2 and M, instead), and vice-versa. Despite the expected association, its exact biological meaning is not completely understood. Data from the literature indicated that Cyclin B1 expression could be regulated by pluripotency factors (including Lin28 and Rex1 and, likely, Oct4 and others), and vice-versa. Thus, the coordinated regulation of Cyclin B1 by Oct4 and other pluripotency factors could allow pluripotent cells to divide and progress through the cell cycle (from G2 to G1), only when adequate levels of pluripotency factors are available for daughter cells. This would imply that as cells progress through the cell cycle along G1, S and G2 phases, the increasing DNA content and Cyclin B1 levels would be paralleled by Oct4 levels at the single-cell level. To test this, we analysed the quantitative results of selected wells from our NT2 and H1 hESCs screenings, at the single-cell level. By carrying a correlation analysis between nuclear integrated intensities of Hoechst (known to correlate linearly with DNA content), Cyclin B1 and Oct4, we found that all were strikingly and statistically significantly correlated. Interestingly, in cells transfected with miR-Ctrl, Hoechst and Oct4 showed the highest correlation (~R = 0.95), followed by Oct4 and Cyclin B1 (~R = 0.87) and Hoechst and Cyclin B1 (~R = 0.75). The high correlation between Hoechst and Oct4 prompted us to take a closer look at the Oct4 and Cyclin B1 staining patterns along the cell cycle. Strikingly, it became evident that, while, during interphase Oct4 was apparently homogeneously dispersed in the nucleus; in mitotic cells (in prophase, metaphase and anaphase), Oct4 was tightly co-localised with the Hoechst-stained DNA chromatin. Of notice, we found that Oct4 apparently starts to bind to the chromatin in prophase cell in which nuclear envelope breakdown is just about to occur, as indicated by the strong nuclear localisation of Cyclin B1. As expected, cells in Metaphase and Anaphase show a marked reduction in Cyclin B1 intensity, in line with the rapid degradation that occurs before G1 phase. More importantly, Oct4 apparently starts to leave the bookmarked chromatin immediately following mitosis, when chromatin starts unpacking and Cyclin B1 levels are the lowest. Overall, the increase in Oct4 levels from G1 to G2 cell-cycle phases and its strong linear correlation with DNA content and Cyclin B1 levels; coupled to the strictly co-localisation of Oct4 with the chromatin during mitosis, would ensure that daughter cells receive equal Oct4 amounts. Together with recent data from the literature, our results corroborate the mechanisms underlying the close inter-relationship between pluripotency and cell-cycle progression in pluripotent cells.
**Title:** Role of herpes simplex virus 2 (HSV-2) replication within dendritic cells on tissue inflammation, immunity and neuron infection in the mouse model

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**ICGEB Contract No.:** CRP/14/006

**ICGEB Reference No.:** CRP/CHI14-01

**Abstract:** Herpes simplex virus 2 (HSV-2) causes neonatal encephalitis, genital ulcers and is an important catalyst of HIV infection. At present, there are no available vaccines against HSV-2. Dendritic cells (DCs) are professional antigen presenting cells that link innate and adaptive immunity and help mount effective immune responses. HSV-2 interferes with DC activation and viability leading to apoptosis and reducing their capacity to activate T cells in vitro. Here, we proposed to assess the effect of HSV-2 replication within DCs at early time points over genital tissue and neuron infection in a mouse HSV infection model. We genetically engineered HSV-2 to produce a mutant virus impaired at replicating within DCs and other hematopoietic cells, yet capable of infecting and replicating within epithelial cells, the primary target cells of HSV-2. We found that although the mutant virus was attenuated in DCs in in vitro experiments, the control virus, which was constructed with a similar strategy and should not be susceptible to any particular host miRNA was similarly attenuated in vivo, making it difficult to interpret the results obtained.

**Objectives:**

The objective of this project were:

(i) To characterise in vitro and in vivo an HSV-2 virus that is impaired at replicating within hematopoietic cells but not other non-immune cells, such as epithelial cells;

(ii) To assess the effect of HSV-2 replication within hematopoietic cells, namely DCs at early time points on neuron infection in the mouse model (days 4-5 after virus inoculation);

(iii) To assess the effect of HSV-2 replication within hematopoietic cells, namely DCs on the establishment of inflammation at the infection site at early time points (days 2-4 after virus inoculation);

(iv) To assess the effect of HSV-2 replication within hematopoietic cells, namely DCs and T cells on the activation of adaptive immune components to this virus (days 6-8 after virus inoculation).

**Results Obtained:**

We observed that the HSV-2 mutant virus that contained the specific miRNA target sequence that targets the viral genome and should block virus replication within dendritic cells by silencing the viral *ul54* gene, and which we named F-virus, was attenuated in in vitro-differentiated dendritic cells (DCs) and DCs obtained from mice (ex vivo DCs). In these cells, the F-virus displayed reduced genome replication and produced lesser virions. Furthermore, the F-virus displayed reduced expression of viral proteins within DCs, which suggested that along with hampered genome replication, the F-virus could not transcribe and translate efficiently its viral proteins in these cells indicating virus control in DCs. These results differed from those observed for the control virus (R-virus, which contains a control sequence downstream of the *ul54* gene), and suggested that the F-virus was particularly impaired in its replication capacity within DCs, which would allow assessing this specific aspect over disease outcome in an animal model.

However, when tested in vivo, both the F- and control R-virus displayed reduced virulence, likely as a consequence of the genetic modification downstream of the *ul54* gene. Both mutant viruses, the control R-virus and the F-virus were unable to induce significant neurological and epithelial disease in infected mice and were overall non-lethal to these animals. This attenuation was also observed at the level of neuron infection, as determined by qPCR and infiltration of immune cells to the infection site, among others. This result was not expected for the R-virus given its design and in vitro results. Taken together, these findings limited the capacity of determining the role of HSV-2 replication within DCs on neuron infection and pathology, as the genetic modifications in the mutant viruses may have affected their virulence, particularly in vivo because of the nucleotide sequence inserted in the 3’ untranslated region of the *ul54* gene.

**Results Unforeseen in the Original Project:**

In addition to the experiments initially proposed in this project, we worked in this project on the hypothesis that dendritic cells might play a pivotal role in defining protection to HSV in infected individuals and actually could correspond to a correlate of protection, depending on the HSV-DC interaction outcome. This idea was recently published in the *Theory and Hypothesis* section of Frontiers in Immunology and proposes that the relationship between DCs and the wild-type or different mutant HSV viruses could be a key factor in defining immunity and the fate of the host anti-herpes immune response, because these cells are central in eliciting protective immunity to viruses. Indeed, we suggest that the fate of DCs after interaction with HSV mutants could define a
new correlate of protection for this virus, an aspect that has been poorly explored with attenuated HSV mutants viruses that have been tested in animal models and proposed as potential vaccines. In some cases, attenuated mutant HSV viruses do not elicit protective immunity. Assumptions and experimental approaches for this model are proposed in the paper (Retamal-Díaz et al., A Herpes Simplex Virus Type 2 Deleted for Glycoprotein D Enables Dendritic Cells to Activate CD4+ and CD8+ T Cells Frontiers in Immunology 2017).

Publications:


Chinese Functional analysis of phospholipase in *Pseudomonas aeruginosa* type VI secretion system

**Principal Investigator:** Guowei Yang, Institute of Pathogen Biology, Chinese Academy of Medical Sciences, Rong Jing Dong Jie 6, 306, Beijing 100176, China. Tel: +86-10-67877738, Fax: +86-10-67877736, E-mail: yangguowei@hotmail.com

**ICGEB Contract No.:** CRP/14/007  
**ICGEB Reference No.:** CRP/CHN14-02

**Abstract:** The type VI secretion system (T6SS), which was first identified in the human pathogens *Pseudomonas aeruginosa* and *Vibrio cholerae*, has become recognised as an important mediator of bacterial virulence. Recently, a superfamily of bacterial phospholipase/lipase enzymes has been identified as T6SS lipase effectors (Tle), which are widely encoded in animal and plant-associated Proteobacteria. From 2015 to 2017, with the funding support from ICGEB/CRP, we identified a *P. aeruginosa* T6SS effector - TplE. It is a Tle4 phospholipase family protein which *P. aeruginosa* can use to kill competing bacteria and also it is possible that TplE may influence host cells by ER localisation by virtue of its PGAP1-like domain. This work will reinforce the argument that *P. aeruginosa* can use its T6SS not only for bacterial competition but also to influence mammalian cells (Cell Reports, 2016 Aug 9;16(6):1502-9). Besides TplE, we also confirmed the function of an Outer Membrane Phospholipase A (PldA) protein in *Shigella flexneri*. It both stabilises the bacterial membrane and is involved in bacterial infection under ordinary culture conditions. The results of this study support that PldA, which is widespread across Gram-negative bacteria, is an important factor for the bacterial life cycle, particularly in human pathogens (Open Biology, 2016 6: 160073).

**Objectives:**

The main goal of this project is to investigate PA1510, a putative T6SS phospholipase effectors, for its potential anti-eukaryotic activities. We hope to gain insights into the understanding of T6SS function and provide exciting targets for novel antimicrobial drug treatments.

In this project, we have the following objectives:

(i) To directly address the impact of PA1510 phospholipase activity in bacterial growth advantage;
(ii) To investigate the contribution of PA1510 PGAP1 domain to the interaction between bacteria and mammalian host.

**Results Obtained:**

In this study we characterised the activities of an H2-T6SS dependent phospholipase effector, TplE, which encodes a eukaryotic PGAP1-like domain. In addition to exerting a toxic effect in the periplasmic space of adjacent bacteria, this trans-Kingdom effector can also be injected into eukaryotic host cells. In mammalian cells, TplE becomes localised to the ER, whereupon it causes stress and the UPR and ultimately triggers an autophagy response.

Our observations illustrate how a single T6SS effector protein can contribute to the complex interplay among a bacterial pathogen, competing microbes, and the host cell during an infection. It will facilitate further exploration of how *P. aeruginosa* utilise its T6SSs and arsenal of effectors for manipulating the host cell and potentially lead to strategies for designing approaches to combat bacterial infections.

**Results Unforeseen in the Original Project:**

We also identified an Outer membrane phospholipase A (PldA) which is a enzyme located in the outer membrane of *Shigella flexneri*. The proteomic and transcriptomic data indicated that pldA mutant strain shows the disorganisation of the bacterial outer membrane and the periplasmic space. Furthermore, the pldA mutant strain showed decreased levels of type III secretion system expression, contributing to the reduced internalisation efficiency in host cells. PldA is widely distributed and well conserved in Gram-negative bacteria. Our results confirmed that this protein plays an important role in bacterial survival.

**Publications:**


Title: Mechanisms of muscle damage by snake venom phospholipases A2
Principal Investigator: Bruno Lomonte, Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José 11501, Costa Rica. Tel: +506-25117888, E-mail: bruno.lomonte@ucr.ac.cr
ICGEB Contract No.: CRP/13/006
ICGEB Reference No.: CRP/COS13-01

Abstract: Envenomings due to snakebites account for an estimated 500,000 cases and 30,000 deaths every year, often leaving survivors with permanent tissue damage. Major venom components causing muscle necrosis are phospholipases A2 (PLA2). Intriguingly, not all venom PLA2s are myotoxic, and their determinants of toxicity, mechanisms, and pathways to cell damage are only partially understood. Some venom PLA2 “homologues” lack enzymatic activity but exert myotoxicity. The role of phospholipolytic activity in muscle damage induced by PLA2s was characterised, revealing two mechanisms. A synergism between catalytically-inert and catalytically-active PLA2 myotoxins was demonstrated in myotubes and, for the first time, in a mouse model. Several new PLA2s were isolated and characterised, to approach structure-function relationship and evolutionary clues. The crystal structure of one revealed a putative “myotoxic cluster” for this group of enzymes. However, the ‘acceptor site’ for PLA2s remains elusive. Biophysical studies using artificial phospholipid monolayers revealed that toxic PLA2s might be distinguished from non-toxic counterparts by their resistance to lateral pressure and penetration ability. A non-hydrolysable substrate analog for PLA2s was synthesised and will be studied for possible inhibition of catalytically-active PLA2 myotoxins.

Objectives:
The major objective was to determine the mechanisms of muscle damage caused by snake venom myotoxic phospholipases A2. Specifically:
(i) To characterise the role of phospholipolytic activity of the PLA2 myotoxins in muscle damage;
(ii) To determine structure/function relationships, determinants of toxicity, and molecular targets of PLA2 myotoxins;
(iii) To characterise biophysical alterations induced by PLA2 myotoxins, or their syntetic peptides, in artificial phospholipid mono- or bilayers or model membranes;
(iv) To evaluate if immunisation with recombinant and cross-linked PLA2 myotoxins results in an enhancement of the antibody response to these toxins in experimental animals.

Results obtained:
New findings of general significance were obtained which have increased our understanding of the mechanisms by which snake venom phospholipases A2 lead to skeletal muscle necrosis. Phospholipid hydrolysis in biologically relevant targets was characterised using sensitive mass spectrometry techniques, showing two distinct mechanisms of membrane and cell damage. A synergism between catalytically-active and catalytically-inert PLA2 myotoxins was demonstrated in cultured myotubes and in mice. The former (Mt-I) was inferred to hydrolyse phospholipids on the external monolayer of the sarcolemma, while the latter (Mt-II) did not induce phospholipid hydrolysis, thus excluding the activation of endogenous PLA2s in its mechanism of toxicity. The catalytic activity was shown to be essential for the myotoxic mechanism of Asp49 PLA2s such as Mt-I, clarifying some controversial reports from earlier studies. Comparisons in the structural and functional characteristics of several PLA2s were made aiming to disclose relevant clues for identifying their determinants of toxicity. Furthermore, the successful crystallisation of one of these toxins, Mt-I, revealed a putative cluster of amino acids that could be responsible for the myotoxic effect of this family of venom proteins, a hypothesis that needs future experimental verification. Attempts to identify a protein acceptor in the plasma membrane of myogenic cells or mature muscle were unsuccessful, leaving still open the challenging question on what structures do myotoxic PLA2s recognise on their target cells. Imaging of myotoxic PLA2s by fluorescent methods clearly showed binding to the sarcolemma of skeletal muscle fibers, rather than internalisation, in agreement with the proposed mode of action at the level of plasma membrane. Artificial phospholipid monolayers were used to assess the hydrolytic activity of the PLA2 enzymes under conditions of variable lateral pressures. Results suggest that toxic PLA2s have a resistance to higher lateral pressure, and thus a stronger penetration ability, in contrast to non-toxic PLA2s which cease their hydrolytic activity when lateral pressure is increased.

Results unforeseen in the original project:
Novel PLA2 toxins were isolated from various snake venoms, whose structural and functional characterisation may provide valuable clues towards understanding the modes of toxicity exerted by this family of enzymes, as well as enlighten the evolution of this family of toxins in snake venoms. Also, a novel form of synergism was discovered between snake venom PLA2s that are devoid of direct toxicity, and snake venom metalloproteinases that affect endothelial cells. This
unforeseen phenomenon needs to be characterised in more detail, since it is apparently not dependent on the enzymatic activity of the PL\textsubscript{A2}.

**Publications:**


Lomonte, B., Mora-Obando, D., Fernández, J., Sanz, L., Pla, D., Gutiérrez, J.M., Calvete, J.J. First crotoxin-like phospholipase A\textsubscript{2} complex from a non-rattlesnake venom: nigroviriditoxin, from the arboreal Central American snake Bothriechis nigroviridis. 2015. Toxicon 93, 144-154


Title: Role of Activating Transcription Factor 3 and related molecules in the activation of spinal cord endogenous stem/progenitor cells

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Abstract: In this project two different in vitro spinal cord (SC) models were used to study SC stem/progenitor cells (SPCs) and molecular basis of neuronal regeneration. The effort has been made to characterise molecular changes occurring in rat neonatal SC tissue in vitro and to identify the factors that regulate the activation of spinal endogenous SPCs using proteomics. Our results have shown that: (i) the rat neonatal SC tissue in culture in the first 24h does not undergo massive cell death or neurodegenerative processes and mostly continue normal cellular metabolism, but that prolonged culturing induces dysfunction of the spinal networks due to the loss of inhibitory synapses; (ii) there is the upregulation of the heat-shock proteins in the cultured tissue, which play a pivotal role in neuroprotection after injury; (iii) The protocols has been developed for proteomic studies of the rat and opossum SC tissue to allow the comprehensive protein profiling related to neuronal regeneration, as well as the protocols for the isolation of the cerebrospinal fluid from the adult and neonatal opossums to allow the analysis of the exosomes which may contain the molecules controlling the spinal ependymal SPCs. The financial support given by the ICGEB through this grant has enabled the beginning of the research with the final goal to understand the factors controlling the activity of the spinal SPCs in mammals.

Objectives:

(i) Cloning of ATF3 transcript(s) expressed in quiescent and activated spinal SPCs and analysis of their spatial and temporal distribution in spinal and brain tissue of regenerating and non-regenerating animals (rat and opossum);
(ii) Identification of the molecules differentially expressed in quiescent and mobilised spinal SPCs;
(iii) Analysis of the cerebrospinal fluid of neonatal rats or opossums to identify the molecules important for the control of the spinal SPCs;
(iv) Analysis of the ATF3 and other molecules of interest found to be differentially expressed in the quiescent and activated spinal stem cells and their role in neuroprotection or in SPCs control.

Results Obtained:
The effort has been made to characterise the molecular and cellular changes occurring in the rat neonatal spinal cord tissue cultured in vitro and to identify the factors that regulate the quiescence, activation and migration of spinal endogenous stem cells using proteomics. During the in vitro studies, we found it necessary to extend the survival of the culture of the neonatal rat spinal cord tissue up to 3 days. During these studies, we noticed the dysfunction of the spinal networks underlying walking due to the loss of inhibitory synapses (see the Unforeseen studies). We have used the proteomic studies to reveal the proteins differentially expressed: 1) in the rat neonatal spinal cord tissue kept in culture, compared to fresh tissue, to reveal the proteins related to spinal ependymal SPCs activation and migration and 2) in the opossum spinal cord tissue that can or cannot regenerate, to detect the proteins controlling neuroregeneration. The relevant protocols have been developed and the preliminary results shown that: 1) the rat neonatal spinal cord tissue in culture in the first 24h does not undergo massive cell death or neurodegenerative processes and mostly continue normal cellular metabolism; 2) there is the abundance of the heat-shock proteins in the rat cultured neonatal spinal cord tissue and these results prompted us to study their involvement in the neuroprotection after spinal cord injury. We have performed unforeseen proteomic, electrophysiological and immunofluorescence studies with the neuroprotective compound Celastrol, known to increase the expression of the relevant heat-shock proteins. 3) The protocols have been developed to study opossum spinal cord tissue that can or cannot regenerate, giving the limited number of the proteins (400-800/sample). Thus, in the future, different proteomics approaches will be performed to allow the comprehensive protein profiling of the neuronal tissue that can regenerate and compare it to the tissue that has lost this capacity.

The attempt to clone ATF3 specific sequences from the polyA RNA extracts from the fresh rat and opossum neonatal spinal cord tissue and from the rat tissue that has been kept in culture for 24h (to allow the activation of the ependymal stem cells and the expression of the alternative ATF3 isoforms) has been made, using Rapid Amplification of cDNA Ends (RACE) procedure (with different primers specific for the ATF3 gene, covering all the regions of the full-length sequence) and standard molecular biology techniques. The Atf3 transcript has been cloned (5’ end part) from the rat spinal cord tissue kept in culture for 24h (where the ependymal SPCs are activated), but not
from the rat or opossum fresh tissue, indicating: (i) that the opossum Atf3 sequence(s) is considerably different from the predicted one or from the rat sequence; (ii) that in the fresh rat tissue (quiescent ependymal SPCs) there is no detectable level of the Atf3 transcript. The further attempts will be made to elucidate the expression, distribution and diversity of the Atf3 transcripts in the rat and opossum spinal cord tissue.

Additionally, the protocols for the anesthesia and the isolation of the cerebrospinal fluid (CSF) from the adult and neonatal opossums have been developing, to allow the analysis of the CSF exosomes, which may contain the molecules controlling the activation of the spinal ependymal SPCs.

**Results Unforeseen in the Original Project:**
The results have shown that the prolonged culturing of the neonatal rat spinal cord tissue in culture (up to 72h) to study the migration and activation of the spinal endogenous stem/progenitor cells, induces the dysfunction of locomotor networks underlying walking, due to the time-dependent loss of inhibitory synapses.

The proteomics results have revealed abundance and difference in the expression of the heat-shock proteins during *in vitro* culturing of the neonatal rat spinal cord tissue in culture. Thus, the further studies on neuroprotective role of the Heat Shock protein 70 (HSP-70) has been performed, revealing the neuroprotective effect of the Celastrol (compound able to increase the expression of the induced HSP-70 due to the nuclear translocation of the transcription factor Heat Shock Factor 1) after experimentally induced spinal cord injury in both rats and opossums, but at different temperatures (rat 37°C, opossum 30°C, corresponding to their body temperatures).

**Publications:**


**Mladinic, M., Nistri, A., Dekanic, A.** How the discovery of neuronal stem cells have changed neuroscience and perspective for the therapy for central nervous system illnesses. 2015. Period. Biol. **117**, 185-192


Title: Genetic factors for development and therapy of colorectal cancer

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ICGEB Reference No.: CRP/MAC14-01

Abstract: This project helped in the introduction, training and validation of NGS technologies in the analysis of rare inherited malignancies in Macedonia and provided through information on the molecular defects responsible for the familial colorectal cancer in 103 unrelated families as a basis for establishment of national registry for this disease. Overall, the molecular defects were detected in 60 (58%) families, VUSs in 28 (27%) families and in 16 families (15%) no potentially pathogenic mutation were detected. In addition to mutations in genes that have been previously described previous, a high frequency of mutations were found in genes belonging to the DNA repair pathway. The pharmacogenetic part of the project included the evaluation of the effect of various inherited and somatic changes with the efficacy and toxicity of capecitabine therapy in adjuvant setting of patients with locally advanced disease. The data obtained showed that MSI and KRAS mutations are strong predictive factors for DFC and OS after adjuvant capecitabine monotherapy. Unlike the somatic defects, the studied polymorphisms associated with variable 5-FU metabolism, showed only a marginal significance.

Objectives:
(i) To evaluate the genetic predisposition to colorectal cancer in Macedonia including: molecular characterisation of high penetrance alleles (divided into 2 panels of genes: polyposis and nonpolyposis colorectal cancer) and a case/control study of low penetrance genes as factors for CRC development;
(ii) To identify new common and rare sequence variants (including indels, splice site and single nucleotide variations) in genes involved in the pharmacokinetics and pharmacodynamics of capecitabine as predictive markers of toxicity and/or efficacy of capecitabine adjuvant monotherapy.

Results Obtained:
Overall, in the group of 65 families with HNPCC, we were able to detect molecular defects in 51 (80%) families of which 35 (55%) with clearly pathogenic variants and 16 (25%) with VUS. Majority (85%) of mutations in patients with MSI+ tumours were distributed in genes from the MMR pathway, whereas mutations in these genes were rare, but still present with significant frequency (22%) in patients with MSI- tumours. Fourteen different mutations were detected in 15 different genes in 50% of HNPCC patients, pointing to the extreme heterogeneity of the molecular basis of HNPCC in Macedonia. However, we have identified 3 mutations, 2 in MLH1 [(NM_000249.3: c.392C>G or NP_000240.1:p.(Ser131Ter), and NM_000249.3:c.244A>G or NP_000240.1:p.(Thr82Ala)] and 1 in MSH2 [NM_000251.2:c.2211C>T or NM_000251.2:c.2211C>T or ], were found with high frequency in Lynch patients with Macedonian ancestry, which enabled us to design specific tests and thus facilitate the screening process of Lynch patients in the future. Of the mutations present in other genes, the majority (>70%) were detected in genes belonging to the DNA repair pathway (DRP), strengthening the notion that DNA repair defects are primarily affected in patients with CRC. Where appropriate, functional studies on RNA samples from tumour and normal tissues were also performed for mutations affecting splicing sites.

Twelve of the 15 patients with classical polyposis exhibited various defects of the APC gene. We found a strikingly high frequency (30%) of large deletions responsible for this condition in our country that had a clustered 5’ and 3’breakpoints. A frame shift mutation was detected in folliculin gene in a family with this condition, which has previously been described only in a single family. The molecular defect in patients with oligopolyposis was extremely heterogeneous. Overall, we were able to determine gene variants in 20 (83%) of patients with this condition of which 13 (54%) clearly pathogenic mutations and 7 (29%) VUS. The pathogenic variants were detected in 6 different genes in 13 patients (2 in APC, 4 in MUTYH, 3 in NTHL1, and 1 each BMPR1A, BLM, FH and CHEK2), whereas the VUS were found in 4 genes in 7 different families (3 in MRE11, 2 in ATM, and 1 each in POLE and PALB2). A novel molecular signature was detected in patients with NTHL1 mutation, a frequent deletion breakpoint site was detected in the APC gene and initial data were collected on association of genotype/phenotype alterations which strengthened the notion that patients with mutations in this gene belong to a specific new polyposis syndrome.

The data obtained showed that mutations in the KRAS gene are a strong negative predictive factor for disease-free survival and overall survival after adjuvant capecitabine monotherapy, particularly in male patients. BRAF V600E mutation is probably a negative predictive factor for survival in capecitabine therapy, but only in patients with microsatellite stable colon cancer. Excellent
prognosis of patients with MSI-H genotype does not change depending on the BRAF mutational status. Patients with KRAS or BRAF mutated MSS tumours had poorer survival outcomes of capecitabine adjuvant monotherapy. Five-year survival of patients in this group was 36.0% versus patients in the other two groups (88.2% and 72.2% for group 1 and group 3 respectively). Based on these findings, routine testing of patients with colon cancer for MSI, KRAS and BRAF can give significant contribution to decision making regarding the treatment strategies by identifying patients with a highly aggressive disease and poor survival irrespective of the capecitabine adjuvant monotherapy. Unlike the somatic defects, the inherited intervariability regarding the studied polymorphisms associated with variable 5-FU metabolism, showed a marginal significance and probably cannot be used in prediction of the effect or the therapeutic failure of the capecitabine monotherapy.

**Publications:**

Title: In vivo enzymology in genome integrity

Principal Investigator: Beata G. Vertessy, Budapest University of Technology and Economics, Faculty of Molecular and Cell Biology. We aimed at designing specific mutant species of M. smegmatis and D. melanogaster dUTPases and provide their detailed in vitro characterisation with respect to protein folding, enzyme kinetics, as well as interaction with the bacterial Stl repressor. Our aim was also to determine the high-resolution 3D structure of the mutant dUTPase species using X-ray crystallography and provide the structural background for the differences observed in the in vitro characteristics. We also wished to assess the phenotypic effects of mutant dUTPases in several models. For a highly specific and relevant method of perturbation of dUTPase action, we aimed to use the proteinaceous dUTPase inhibitor, Stl (a repressor protein from Staphylococcus aureus).

Abstract: Stable preservation and faithful transmission of genetic information are crucial for all living organisms. In addition to the numerous dedicated DNA damage recognition and repair pathways that repair the mistakes that have already occurred, mechanisms for preventive care of DNA integrity are also present. Among these, the right balance of nucleotide pools is of utmost importance. The enzyme family of dUTPases, which hydrolyze dUTP into dUMP and inorganic pyrophosphate play an important role in maintaining the correct low ratio of dUTP to dTTP, thereby preventing uracil incorporation into DNA. dUTPase is presumed to be essential for viability and its inhibition is a promising strategy to fight infectious microbes as well as fast-dividing cancer cells. In our project, we discovered that a protein inhibitor of phage dUTPase is also effective against mycobacterial, human and Drosophila dUTPases and characterized the corresponding protein complexes. dUTPase inhibition results in a characteristic phenotype of dUTP pool expansion and increase in uracil-DNA levels. Application of our newly developed uracil-DNA detection method led to key insights of DNA metabolism from bacteria to human cells.

Objectives:
Objectives of our project were focused on the mechanism of dUTPase action and its physiological role in diverse organisms using a complex set of experimental approaches from structural to molecular and cell biology. We aimed at designing specific mutant species of M. smegmatis and D. melanogaster dUTPases and provide their detailed in vitro characterisation with respect to protein folding, enzyme kinetics, as well as interaction with the bacterial Stl repressor. Our aim was also to determine the high-resolution 3D structure of the mutant dUTPase species using X-ray crystallography and provide the structural background for the differences observed in the in vitro characteristics. We also wished to assess the phenotypic effects of mutant dUTPases in several models. For a highly specific and relevant method of perturbation of dUTPase action, we aimed to use the proteinaceous dUTPase inhibitor, Stl (a repressor protein from Staphylococcus aureus).

Results Obtained:
We discovered that a Staphylococcus pathogenicity island repressor protein called StlSaPIbov1 (Stl) exhibits potent dUTPase inhibition in Mycobacteria. To our knowledge, this is the first indication of a cross-species inhibitor protein for any dUTPase. We demonstrate that the Staphylococcus aureus Stl and the Mycobacterium tuberculosis dUTPase form a stable complex and that in this complex the enzymatic activity of dUTPase is strongly inhibited. We also found that the expression of the Stl protein in Mycobacterium smegmatis led to highly increased cellular dUTP levels in the mycobacterial cell, this effect being in agreement with its dUTPase inhibitory role. Therefore, we propose that Stl can be considered to be a cross-species dUTPase inhibitor and may be used as an important reagent in dUTPase inhibition experiments either in vitro/in situ or in vivo. In agreement with this suggestion, we found that Stl is also an efficient inhibitor of Drosophila and human dUTPases.

Results Unforeseen in the Original Project:
Based on a bioinformatic high-level genome analysis of all available prokaryotic species, we also showed that the dUTPase lacking genotype occurs quite frequently, despite earlier suggestions for this enzyme being ubiquitous. This observation has far-reaching consequences for genome integrity and nucleotide pool imbalances in numerous prokaryotic species, which are under further investigations. The dut– genotype is present in diverse bacterial phyla indicating that loss of this (or these) gene(s) has occurred multiple times during evolution. We discussed potential survival strategies in lack of dUTPases, such as simultaneous lack or inhibition of UNG and possession of exogenous or alternate metabolic enzymes involved in uracil-DNA metabolism. The potential that genes previously not associated with dUTPase activity may still encode enzymes capable of hydrolysing dUTP is also discussed. Our data indicate that several unicellular microorganisms may efficiently cope with a dut– genotype lacking all of the previously described dUTPase genes, and potentially leading to an unusual uracil-enrichment in their genomic DNA. Accordingly, Using the highly sensitive and high-throughput assay for determination of the uracil content in genomic DNA, we could successfully show that the level of uracil in DNA is surprisingly high in wild type Staphylococcus aureus.

Using our uracil-DNA detection method also led to pioneearing observations in tumours with elevated APOBEC3B expression correlated with a kataegic pattern of localised hypermutation. We
assessed the cellular phenotypes associated with high-level APOBEC3B expression and the influence of p53 status on these phenotypes using an isogenic system.

In a collaborative study, we used RNA interference of p53 in cells with inducible APOBEC3B and assessed DNA damage response (DDR) biomarkers. We showed that although APOBEC3B expression increased the incorporation of genomic uracil, induced DDR biomarkers and caused cell cycle arrest, inactivation of p53 circumvented APOBEC3B-induced cell cycle arrest without reversing the increase in genomic uracil or DDR biomarkers. The continued expression of APOBEC3B in p53-defective cells not only caused a kataegic mutational signature but also caused hypersensitivity to small-molecule DDR inhibitors (ATR, CHEK1, CHEK2, PARP, WEE1 inhibitors) as well as cisplatin/ATR inhibitor and ATR/PARP inhibitor combinations. We have concluded that loss of p53 might allow tumour cells to tolerate elevated APOBEC3B expression, however, continued expression of APOBEC3B may lead to therapeutic vulnerabilities in tumour cells.

Publications:


Benedek, A., Pölöskei, I., Ozohanicus, O., Vékey, K., Vertessy, B.G. The Stl repressor from Staphylococcus aureus is an efficient inhibitor of the eukaryotic fruitfly dUTPase. 2017. FEBS Open Bio. 8(2), 158-167


Papp-Kádár, V., Szabó, J.E., Nyíri, K., Vertessy, B.G. In vitro analysis of predicted DNA-binding sites for the Stl repressor of the Staphylococcus aureus SaPBoV1 pathogenicity island. 2016. PLoS One. 11(7), e0158793


Title: Functional characterisation of gut microbial communities and their fitness effects during dietary switches in butterflies

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Abstract: Bacteria that live within insects can do wonders, helping with host digestion and affecting host behaviour and reproduction. What factors make or break such insect-bacterial associations? How do insects acquire and maintain bacterial communities across generations? We addressed these questions using butterflies. Butterfly larvae eat leaves, whereas adults feed on fluids (e.g. nectar). We expected that this major dietary shift would generate large differences in gut bacterial communities. Surprisingly, we found that in most butterflies, larvae acquire microbes through their diet, and larval and adult bacterial communities are very similar. Thus, dietary and developmental changes have almost no impact on butterfly-associated bacteria. When we experimentally altered bacterial communities, we found that larval growth and survival was unchanged. These results suggest that most butterflies have not evolved mutualistic relationships with their gut bacteria. However, we found that an intracellular bacterium, Wolbachia sp., causes a strongly female-biased sex ratio in one of our focal butterflies. Although Wolbachia is known to manipulate host sex ratio in other insects, its impact and the underlying mechanism are unknown for this butterfly. Thus, our work suggests that butterfly ecology and evolution is more strongly affected by intracellular rather than gut-associated bacteria.

Objectives:
(i) To test whether host-associated microbes affect butterfly fitness;
(ii) To determine gut microbiomes of host species as a function of diet and developmental stage;
(iii) To functionally characterise microbes of focal species with divergent microbiomes;
(iv) To determine the timeline of microbial colonisation of host species.

Results Obtained:
We analysed bacterial communities of larvae, pupae and adults of 12 butterfly species that use a wide range of resources. Surprisingly, very few species showed significant developmental transitions in bacterial communities, suggesting weak impacts of dietary transitions across butterfly development. On the other hand, bacterial communities were strongly influenced by butterfly species identity and dietary variation across species. Larvae of most butterfly species largely mirrored bacterial community composition of their diets, suggesting passive acquisition rather than active selection. Overall, our results suggest that although butterflies harbour distinct microbiomes across taxonomic groups and dietary guilds, the dramatic dietary shifts that occur during development do not impose strong selection to maintain distinct bacterial communities in all butterflies. Our study is one of the first investigations of bacterial communities in a diverse set of wild-caught butterfly species, across developmental stages and larval diets. Our work highlights the importance of comparative analyses across multiple species within an insect group, because focusing on any one butterfly species may have led to different conclusions.

Next, we tested whether extracellular and intracellular bacteria affect butterfly fitness, by measuring different fitness proxies such as host development rate, host weight, digestion efficiency and duration of metamorphosis for two butterfly species. We found that eliminating dietary microbes had very weak and variable fitness impacts, indicating that butterflies do not have a strong beneficial association with their gut microbes. This may be due to the difficulty of consistently transmitting beneficial bacteria across the distinct life stages and dietary habits.

Results Unforeseen in the Original Project:
Given previous work with other insects, we had expected to find strong signatures of evolved host-bacterial associations in butterflies, manifesting as developmental and dietary variation in microbiomes. Thus, two of our objectives were aimed at understanding the functional role of such potentially beneficial microbes, and determining how these microbes were acquired and maintained across generations. Unexpectedly, we did not find the expected patterns of microbiome variation, and our manipulative experiments also indicated a lack of host dependence on associated bacteria. Hence, we did not pursue the last two objectives of our project, and instead decided to test whether intracellular bacteria (rather than gut-associated, extracellular bacteria) may impact host fitness.

While conducting butterfly samples in the field, we noticed that we found mostly females of a specific butterfly, whereas the expected sex ratio is 1:1. Our microbiome analyses showed that these butterflies harboured a large number of Wolbachia, a bacterial genus that is a well-known parasite of many insects. Conducting manipulative experiments, we found that Wolbachia is
responsible for the highly female-biased adult sex ratio in our focal butterfly species, and we are now trying to understand the mechanism of such *Wolbachia-* induced female bias.

**Publications:**

Title: Diagnosis of treatable inborn metabolic disorders of intellectual disability

Principal Investigator: Fazli Rabbi Awan, Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Jhang Road, P.O. Box 577, Faisalabad, Pakistan.
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ICGEB Contract No.: CRP/14/012
ICGEB Reference No.: CRP/PAK14-02

Abstract: Inherited metabolic disorders (aka IEM) are rare diseases but grave health problem worldwide. In Pakistan, this problem is compounded due to other common diseases, lack of financial resources and technical expertise. Many IEM cause intellectual disability (ID) but about 90 of them are potentially treatable if diagnosed early. So, in this project we worked on capacity building for the diagnoses and characterisation of such IEM causing ID.

We collected biofluid samples from ID and healthy children, found significant changes in liver and lipid profiles in patients. We optimised HPLC assay for aminoacidopathies and found high levels of glutamic acid and ornithine in 90 of 409 patients. Additionally, in 2 families Phenylketonuria was identified. Once confirmed, these results will provide novel data.

We also developed low-cost PCR assays for Wilson disease and screened 19 patients (in 14 families). Interestingly, mutation in one family was confirmed, where one patient had overt pathology but another was yet asymptomatic. Paediatricians started early treatment of this patient to prevent pathological damages. This is indeed translational outcome of this project. We hope to get more exciting findings once we characterise all samples. This study will provide data for prevalent IEM in Pakistan and thus lay foundation for developing a national level new-born screening program.

Objectives:
(i) To identify the type and prevalence of inborn errors of metabolism (IEM) disorders in patients with intellectual disabilities in Punjab, Pakistan;
(ii) Setting up chromatography and mass spectrometry (HPLC, GC-MS and LC-MS/MS) and genetic (PCR) based screening/diagnostic assays for such disorders.

Results Obtained:
(i) We collected 800 samples from ID-IEM (n=409) and healthy controls (n=391) children;
(ii) Biochemical parameters showed changes in liver and lipid profiles of patients;
(iii) We developed HPLC based assay for screening of 14 amino acids in one chromatogram;
(iv) We screened all plasma samples for amino acid changes and found high levels of Glutamic acid and Ornithine in 90 out of 409 patients. Moreover, upon request of paediatrician we analysed two families suspected for aminoacidopathies and identified Phenylketonuria. Confirmation of these results is in progress;
(v) We developed in-house low-cost ARMS-PCR assays for 12 common mutations in ATP7B gene of Wilson disease and screened 14 families with 19 Wilson disease patients;
(vi) We identified mutation (Cys271Trp c.813 C>A) in one Wilson disease patient by our in-house developed ARMS-PCR assay and screened the whole family. In this family one asymptomatic sister of the patient was found to harbour same mutation. Based on this result, paediatricians initiated treatment of this child to prevent pathological damage. All family members were screened and offered them genetic counselling for this disease;
(vii) We have optimised PCR assays for mutational hotspot regions for Phenylketonuria and N-Acetylglutamate synthase deficiency. Screening of patient samples is in progress;
(viii) In our samples, we have identified 16 families with more than one patient where in most cases parents are consanguineous and we suspect strong genetic contribution for the disease. Samples have been collected from these families for further genetics work (e.g. next generation sequencing, exome sequencing) for characterisation of disease;
(ix) We are pursuing our efforts to characterise all patient samples using advance biochemical and genetic techniques by developing/optimising assays for them;
(x) The most important outcome of this research is that our efforts in Inherited Metabolic Disorders field are being recognised by scientific and medical community and we have started receiving requests from doctors for disease identification in such patients.


**Title:** Search for self-destruction signals in *Plasmodium falciparum* parasites

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ICGEB Contract No.: CRP/14/013

ICGEB Reference No.: CRP/PAN14-01

**Abstract:** *Plasmodium falciparum* will grow to a maximum number in *in vitro* culture, even when nutrients and erythrocytes are available. Based on this behaviour, it is possible that *P. falciparum* parasites have self-regulatory signalling mechanisms among the members of a shared niche, finite in living space and resources, like in a laboratory culture setting. These signals would be activated in response to density stress. Previous reports have demonstrated that extracellular vesicles (EVs) shed into the media by infected erythrocytes serve as means of communication between the parasitic populations. Therefore, we set to isolate EVs from cultures with high parasitemia and used them to challenge low parasitised cultures, which resulted on a diminished parasitemia when compared to controls. We observed that parasites were dying and found that this death was caused by apoptosis. The molecules found in the cargo of the EVs should could be carriers of a death command induced when there is a high number of infected erythrocytes, and they should be able to produce apoptosis in a new culture furnished with plenty of nutrients and erythrocytes to invade. Next, we set to examine the components of these EVs to analyse which of them could be responsible for inducing apoptosis in low parasitised cultures. We have used several types of apoptosis markers after challenging parasites with EVs from high-parasitised cultures. In addition, we have used mass spectrometry and cellular biology techniques to test our hypothesis and identify the probable molecules that carry *P. falciparum* suicidal signals associated to EVs.

**Objectives:**

(i) To isolate and characterise *P. falciparum* extracellular vesicles and analyse their content;
(ii) To challenge *P. falciparum* low parasitemia cultures with high parasitemia culture-shed extracellular vesicles;
(iii) To identify death signals in the content of high parasitemia culture-shed extracellular vesicles.

**Results Obtained:**

In the course of the project we found evidence that supports apoptosis as the main regulated death mechanism when *P. falciparum* is under population stress. Preliminary results point to apoptosis being induced by *P. falciparum* lactate dehydrogenase (PfLDH), which was the most expressed EV-associated protein upon differential proteomic analysis. PfLDH is different enough from human LDH that its presence is currently used in rapid malaria diagnostic kits. We found that EVs from high parasitemia cultures can trigger the activation of Panspases as well as other apoptosis markers such as phosphatidyl serine translocation and DNA fragmentation. In addition, these EVs produce high levels of reactive oxygen species when added to low parasitised culture. On the other hand, gossypol (a PfLDH inhibitor) was able to reverse the apoptosis induction by PfLDH and, thus, confirm this cell death pathway. In summary, although more studies remain to be performed, PfLDH could be a promising molecule to be used in the future not only as drug target but also as an alternative treatment against the parasite.

**Results Unforeseen in the Original Project:**

We performed a headspace solid-phase micro-extraction/gas chromatography-mass spectrometry (HSPME GC-MS) analysis of the volatile organic composition (VOC) of extracellular vesicles (EVs) and supernatants of ultracentrifugation. Although we could not find any significant VOC within the EVs, we identified hexanal as an important compound associated only to supernatants from infected cultures. Interestingly, hexanal was identified as a powerful mosquito attractant in plants. The production of hexanal could be advantageous to the parasite transmission capabilities although other studies are needed to test hexanal production *in vivo* in order to determine whether it plays a significant role in attracting mosquitoes to malaria-infected patients.

**Publications:**


Title: MiRNAs as a potential biomarkers in prostate cancer

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ICGEB Contract No.: CRP/13/024
ICGEB Reference No.: CRP/POL13-01

Abstract: MicroRNAs are important regulators of all hallmarks of cancer (cell growth, cell cycle control, evasion of apoptosis, tissue invasion, metastasis, angiogenesis and unlimited replicative potential). Some miRNAs may act as tumour suppressor genes while others – oncogenic miRNAs – promote tumour development. High-throughput technologists (miRNA microarrays, RNA-seq) allow to evaluate the diagnostics potential of broad miRNAs profiling in tissue as well in biofluids. Interestingly, circulating cancer-associated miRNAs seems to play a crucial role as non-invasive biomarkers for managing different cancer types. We are interested in miRNA role as prognostic indicators in prostate cancer (PCA).

The first goal of this study was to systematically compare miRNA profiling in prostate cancer cell line, which differ in perinuclear compartment prevalence. The perinucleolar compartment (PNC) is a unique subnuclear structure that forms in cancer cells. The more cells that have these structures in the primary tumours, the higher the chance patients will develop metastasis. The prevalence of PNCs has been found to be correlated with disease progression in breast cancer and in tumours from other tissues, including prostate, colon, ovary, and endometrium. PNC prevalence increases with the degree of malignancy and reaches nearly 100% in distant metastases.

In this study, we used miRNA microarray to evaluate miRNA profile in prostate cancer cell lines with high PNC prevalence (PC3M LN4: 92% PC3M: 83% and PC3M Pro with 72%) versus low PNC prevalence (PC3: 5.3%). Secondly, we made a comparison between miRNAs detected in prostate cell line and circulating miRNA analysed in prostate cancer patients’ biofluids (plasma). Thirdly, we choose 36 miRNAs to validate them (qPCR) on plasma collected from a large cohort of prostate cancer patients. Finally, the most promising miRNAs were evaluated on ddPCR and we correlated miRNA expression versus clinical information and observed statistically significant differences between clinical groups (p-value ≤0.01; p-value ≤ 0.05). Our miRNA profiling brings a new insight into potential blood-based miRNA biomarkers in prostate cancer.

Objectives:
The following objectives were proposed:
(i) to evaluate changes in miRNA level in cancer cell lines with high/low PNC prevalence by high-throughput technology (miRNA microarrays);
(ii) to evaluate changes in miRNA level in biofluids (plasma) collected from prostate cancer patients with different cancer aggressiveness (qPCR);
(iii) to validate selected miRNAs on larger cohort (qPCR);
(iv) to correlate miRNA expression data with clinical information.

Results Obtained:
In this study we used prostate cancer cell line model and miRNA expression arrays to profile the expression of 453 miRNA found in all studied cell types. Based on microarrays data we selected 22 miRNAs that diversified PC3M, PC3MLN4 and PC3PRO, as well 19 miRNA that diversified PC3MLN4 and PC3MPRO. As miRNAs in biofluids is a promising non-invasive tumour biomarker - we also performed broad miRNA expression analysis (179 miRNAs in assay) on a few plasma samples collected from prostate cancer patients and controls - the average number of detected miRNAs was 124. Firstly we selected 69 miRNAs, which well-distinguished patients with different clinical status (without or with metastasis), secondly we narrowed up to 19 miRNAs, which were at least 2-fold differentially expressed between patients.

Our next step was to analyse miRNA expression in plasma on cohort of 187 patients diagnosed with benign prostatic hyperplasia or prostate cancer without/with metastasis. For this purpose we selected 19 miRNAs - based on broad miRNA profiling from plasma, 6 miRNAs were selected based on cell line only and 9 miRNAs had a very distinct expression profile (statistically significant) and overlap with microarrays data on prostate cancer cell lines and plasma miRNA expression profiling. Last but not least we added 2 miRNAs as a plasma specific controls and 6 reference miRNA for normalisation.

We characterised cohort of 187 patients with prostate cancer or benign prostatic hyperplasia and found a few miRNAs which had statistically significant different expression in advance prostate cancer. Our miRNAs profiling brings a new insight into potential blood-based miRNA biomarkers in prostate cancer.

Results Unforeseen in the Original Project:
As the digital droplet PCR technology became more available during CRP grant performance we confirmed qPCR results for the most promising miRNA using ddPCR and the same set of primers. As expected, selected miRNAs expressions were consistent by both methods but from diagnostic view ddPCR seems to have better performance. We observed statistically significant differences between clinical groups (p-value ≤ 0.01; p-value ≤ 0.05).
Incorporates the role of Na,K-ATPase glutathionylation in cells at hypoxia. The main reason for Na,K-ATPase inhibition under hypoxia is regulatory S-glutathionylation of alpha subunit. We identified the intracellular parameters, which influence on this glutathionylation, and elucidated the role of glutathionylation of constitutive alpha1 and tissue specific alpha2 and alpha3 subunits of Na,K-ATPase in the adaptation of cardiomyocytes and neurons to hypoxia. We have found that efficiency of alpha subunit glutathionylation depends on enzyme conformation; increases with decreasing pH value of the medium and decreases in the presence of ATP. We have shown that redox changes under hypoxia induced glutathionylation of alpha1, 2, 3 subunits of the Na,K-ATPase in cardiomyocytes and neurons and alpha2 and alpha3 isoforms are more sensitive to glutathionylation than alpha1. Drop in ATP and decrease of pH under severe hypoxia increase efficiency of glutathionylation. We have found that selective glutathionylation of alpha2 in cardiomyocytes and alpha3 in neurons by ethyl glutathione and nitrosoglutathione results in improved contractility of cardiomyocytes and better survival of cardiomyocytes and neurons. We revealed basal glutathionylation of alpha subunit which depend on redox status of cells during folding of Na,K-ATPase.

Objectives:
(i) Using Na,K-ATPase from duck salt gland, establish dependency of the effectiveness of glutathionylation of the Na,K-ATPase alpha1 subunit and the concentration of sodium, potassium, phosphate, ADP, ATP, and pH of the medium; evaluate basal glutathionylation of Na,K-ATPase alpha subunit;
(ii) To identify the relationship between changes in the intracellular parameters (level of Na, ATP, pH), the redox status of cells, and the glutathionylation level of Na,K-ATPase alpha isoforms in cardiomyocytes and neurons during hypoxia of varying severity; analyse X-ray structures of the Na,K-ATPase alpha-subunit for determination of basal glutathionylated residues localisation;
(iii) Determine the role of glutathionylation of Na,K-ATPase isoforms in the survival of neurons and cardiomyocytes under conditions of hypoxia.

Results Obtained:
Na,K-ATPase is a principal ion-transporting protein of the cytoplasmic membrane of animal cells, creating a gradient of sodium and potassium ions, which is necessary for maintaining the transmembrane potential, cell volume and transport of ions and metabolites. Na,K-ATPase activity depends on the redox-status of cells and varies easily with changes in oxygen concentration. During hypoxia, one of the earliest and most critical events for cell viability is the inhibition of Na,K-ATPase activity. Early we have shown that regulatory S-glutathionylation of catalytic alpha subunit is a major determinant of Na,K-ATPase redox sensitivity. Four cysteines of Na,K-ATPase alpha subunit can undergo regulatory glutathionylation in case of GSSG increase, which leads to reversible inactivation of the enzyme, preventing exhaustion of ATP in the cells under oxidative stress and hypoxia. Brain and heart tissues are most susceptible to damage during hypoxia, but the question of changes in the glutathionylation levels of the Na,K-ATPase tissue-specific isoforms of alpha subunit, and of its impact on the functioning of the enzyme and cell adaptation to hypoxia were unclear.

We have studied influence of different concentration of sodium, potassium, phosphate, ADP, ATP, and pH value of the medium on effectiveness of glutathionylation of the Na,K-ATPase alpha1 subunit. Using purified Na,K-ATPase form duck salt gland we have shown that E2 state of enzyme induced by K+ is more sensitive for glutathionylation and inhibition by oxidised glutathione (GSSG) than E1 state of enzyme induced by Na+. At the transition of the enzyme to the E2P conformation, the efficiency of its glutathionylation decreases strongly and became lower that in E1 and E2 state. Thus the groups undergoing regulatory glutathionylation (Cys-454, -458, -459, and -244) are less accessible to glutathione in E2P conformation of the enzyme. Efficiency of alpha subunit glutathionylation and inhibition of enzyme increases with decreasing pH value of the medium. ATP is most effectively prevents regulatory glutathionylation of enzyme than AMP and ADP. We have found that Na,K-ATPase alpha-subunit in addition to regulatory glutathionylation has a basal glutathionylation which is not abrogated by reducing agent. In contrast to regulatory glutathionylation, which increases under acute hypoxia, basal glutathionylation of alpha subunit increases under prolonged hypoxia only.
Cardiomyocytes actively express the alpha2 isoform of Na,K-ATPase which plays an important role in the regulation of calcium levels and cardiomyocyte contractility. Neuronal cells express alpha2 isoform (glial cells) and alpha3 isoforms (neurons). It was found that acute hypoxia causes a change in the redox status of cells (increase of reactive oxygen level, change of glutathione level and decrease of NO level) both in neuronal cells and cardiomyocytes. Redox changes under hypoxia induce glutathionylation of alpha1, alpha2 and alpha3 isoforms of the Na,K-ATPase catalytic subunit in cardiomyocytes and neuronal cells. It was found that the greatest efficiency of alpha subunits glutathionylation in neuronal cells and cardiomyocytes is observed in conditions of severe hypoxia (less than 1% pO2), accompanied by a drop in ATP, and the acidification of the intracellular environment. It was revealed that alpha2 and alpha3 isoforms are more sensitive to glutathionylation than alpha1 isoform. Analysis of the X-ray structures of the Na,K-ATPase has shown that the unresolved density in the structure can be occupied by glutathione associated with cysteine residues. So we defined the localization of basal glutathionylated cystein residues. Since basally glutathionylated residues are not accessible to the deglutathionylating agents in native enzyme, the pattern of basal glutathionylation reflects the redox status of the cell at the point of protein synthesis. We suggest that basal glutathionylation is important for folding of Na,K-ATPase.

We have been studied the role of glutathionylation of the constitutive (alpha1) and tissue specific (alpha2 and alpha3) isoforms of Na,K-ATPase in cardiomyocytes and neurons. For induction of glutathionylation we have used N-acetylcysteine (NAC), ethyl glutathione (et-GSH), nitrosoglutathione (GSNO), oxidised glutathione (GSSG) and penetrating analogue of oxidised GSSG (tet-GSSG). We have found that preincubation of cardiomyocytes with these substances prolongs the time of normal functioning of Ca2+ transport systems and cell viability by 4-5 times in comparison with hypoxic control. The effective concentrations of et-GSH, GSSG, and tet-GSSG (0.5 mM) were significantly lower than that for NAC (10-20mM). The lowest effective concentration (0.1 mM) was observed for GSNO. The greatest similarity of Ca2+ peaks with the control peaks under the conditions of normoxia was observed in the presence of et-GSH and GSNO. It was shown that alpha2 and alpha3 subunits are more sensitive to glutathionylation than alpha1. Selective glutathionylation of alpha2 in cardiomyocytes and alpha3 isoforms in neurons with et-GSH and GSNO results in improved contractility of cardiomyocytes and better survival of cardiomyocytes and neurons. At the same time excess glutathionylation and subsequent inhibition of constitutive and tissue-specific isoforms with the help of the penetrating analogue of GSSG (tet-GSSG), which as we have shown is a more effective glutathionylation agent, lead to an increase in the basal level of sodium and calcium. As the result the survival of cells with glutathionylation of both constitutive and tissue specific isoforms under hypoxia is worse than in the case of glutathionylation of tissue-specific isoforms alone.

Thus, regulatory glutathionylation of tissue-specific alpha2 and alpha3 isoforms of the catalytic subunit of Na,K-ATPase contributes to the adaptation of cardiomyocytes and neurons to acute hypoxia. Basal glutathionylation of Na,K-ATPase is increasing in conditions of chronic hypoxia and appears to influence on folding of the protein.

**Publications:**


Title: Dss1’s roles in genome integrity and protein oxidative damage control

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Abstract: The first question we were interested in was whether Dss1 might serve to link BRCA2 and the DNA recombinational repair system with the DNA-damage-transducing system or other important cellular functions like DNA replication or chromosome segregation. Therefore, to identify new genes important in the BRCA2 pathway we planned to perform a genome wide screen for bypass suppressors of dss1 mutants. A genome wide screen for such suppressors was performed and 26 candidates were isolated. Upon sequence analysis of the BRH2 gene we found that not a single one candidate contained a mutation in this gene. After integrating back the Dss1 gene ectopically we found that 3 of the suppressors were sensitive to UV or MMS with a differential from wild type large enough to enable selection for complementation resistance to these genotoxic agents. The gene corresponding to Sup8 mutation was cloned from U. maydis genomic DNA library by functional complementation of the UV sensitivity of the mutant. The sequence analysis revealed that Sup8 was a mutant form of a gene that encodes Psf3, a member of the GINS complex, an essential component in DNA replication. The allele we identified has a single amino acid change near the N-terminus. Our model to account for the genetic findings is that Psf3 in the GINS complex serves as the landing site for Brh2 when it is called in to repair a stalled or collapsed replication fork. In the absence of Dss1, Brh2 assumes a different conformation that can no longer interact with Psf3. However, by mutation in Psf3 a counterbalancing conformational change now can accommodate Brh2 lacking bound Dss1. Consequently, the genetic suppression was observed. When Dss1 was overexpressed in Sup14 we found that the more Dss1 is supplied in the mutant the more complete suppression is achieved. This finding made the suppressor Sup14 very interesting. Namely, the finding is suggestive of likely possibility that the relevant mutation is in the gene coding for a cellular factor that partners with Dss1 in enabling the effective DNA repair. Given that DSS1 is involved on the one hand in DNA repair and on the other in oxidative stress response, we next sought to determine which key amino acids are involved in these processes. In order to isolate “separation-of-function” mutants we started by substituting those highly conserved amino acids in Dss1 protein that are involved in recognition of BRCA2 molecules. The site-directed mutagenesis was performed using Dss1 gene to substitute 8 highly conserved amino acid residues with alanine. Severe loss of DNA repair function results when D16, D17, F19 or W43 was altered by point mutation. Expression of the DSS1 gene with substitution mutations at positions L56 or L60 dramatically abrogated the capacity of U. maydis cells to recover from H2O2-induced oxidative stress. We have extended these observations further by showing that certain of the above substitutions are also affecting sporulation. The substitutions D16, F19, W39, W43 and L60, compared with wild type, all showed reduced levels of the production of teliospores whereas D16, like dss1, failed to do so at all. As the failure of dss1 mutant to induce tumours and to sporulate could be explained by the suggested role of Dss1 in the detection and clearing of oxidatively damaged proteins we addressed this possibility by overexpression of the Catalase2 i.e. the enzyme that catalyses the decomposition of hydrogen peroxide. It was found that although the Catalase2 overexpression could substantially improve survival of dss1 mutant on complete medium containing peroxide no enhancement in tumour formation was observed. We extended these findings to suggest the role of Psf3 in the phyto-pathogenic development is other than simply contributing to overcoming the oxidative pressure imposed by the innate immune system of the plant host. The screens and the genetic readouts for mutants sensitive to hydroxyl free radical were performed under two different conditions: (i) under condition permissive for cell growth and (ii) under conditions where lack of nutrients precludes growth. Efforts to isolate mutants unable to recover from oxidative damage induced by peroxide treatment in the cycling cells resulted in identification of 7 mutants that exhibited different levels of sensitivity to H2O2. Namely, four of these mcr mutants (mcr2, mcr3, mcr5 and mcr6) showed a 5-log reduction in survival on agar medium containing 4.25 mM H2O2 and all of them could be complemented by a single intact ORF encoding Catalase2. Thus, the expression of the enzyme that catalyses the decomposition of hydrogen peroxide has a central role in resistance to killing by hydrogen peroxide. In the case of mcr7 a pronounced sensitivity to HU was found indicating that at least some of the components of the cellular machinery involved in the combating of oxidative insults also function in some important and overlapping fashion in the response to DNA damage and replication stress. Testing the post-treatment viability of H2O2-stressed U. maydis cells has shown remarkably complete recovery if the cells were incubated for extended times in nutrient-free medium prior to...
plating onto reach medium. The effect is referred to as liquid holding recovery (LHR). However, when inhibitors of DNA synthesis (hydroxyurea), RNA synthesis (thiolutin), or protein synthesis (geneticin) were added to the LH cell suspensions, recovery after damage was effectively blocked in each case. This indicates that new synthesis of DNA, RNA and protein is required for LHR. Also, we have shown that Coenzyme A is an essential factor in LHR. We devised a screen for mutants defective in LHR from peroxide induced damage. We mutagenised wild-type cells, allowed them to form colonies, and tested these individually for loss of ability to recover under LH conditions. Of 1200 colonies examined, 4 exhibited defects in LHR after peroxide treatment. Since the conditions for LHR were similar to those for growth we tested whether the restitution of viability was due to intracellular repair of the peroxide damage or the result of multiplication of the undamaged cells. As a result, we determined that the enhanced viability seen after the absorption of massive damage and following the incubation of the treated suspensions of U. maydis cells for a prolonged period prior to plating is achieved through cell multiplication by feeding on the intracellular compounds leaked from the damaged cells. Analysis of the effect of the leaked material on the growth of undamaged cells revealed opposing biological activity, indicating that U. maydis must possess cellular mechanisms involved not only in reabsorption of the released compounds from external environment but also in contending with their treatment-induced toxicity. Starting from lower concentrations of peroxide the released material supported growth but at the higher concentrations the released material exerted inhibitory effect. Therefore, the LHR mutants are actually defective in the cellular machinery involved in reabsorption and processing of the leaked cellular material. By functional complementation of the mutants we cloned and identified all four genes (adr1, did4, kel1, tbp1) that contribute to the process. The mutants in did4, kel1, tbp1 exhibited sensitivity to different genotoxic agents implying that the gene products are in some overlapping fashion involved in the protection of genome integrity. The identified gene functions indicate roles in transcription, protein turnover, growth regulation, and cytoskeleton structure. These findings would emphasise the need to recognise the importance of the cellular mechanism required for the recycling of dead cells as an important determinant of population rescue from very severe stress. The value of this cellular function for successful population dynamics of U. maydis cells would be particularly pronounced in an environment with limited nutrients and under competition from other microorganisms. Clearly, the mechanism can be seen as an adaptation that broadens fitness since it provides U. maydis a capability of sustained growth particularly under massive environmental stress that would cause elimination if the facility were not present.

Publications:
Milisavljevic, M., Petkovic, J., Samardzic, J., Kojic, M. Bioavailability of nutritional resources from cells killed by oxidation supports expansion of survivors in Ustilago maydis populations. 2018. Front. Microbiol. 9, 990
**Title:** Designed single-chain polypeptide- and DNA-based polyhedral nanostructures  
**Principal Investigator:** Helena Gradišar, Department of Synthetic Biology and Immunology, National Institute of Chemistry, Hajdrihova 19, SI-1000 Ljubljana, Slovenia. Tel: +386-1-4760333, Fax: +386-1-4760300, E-mail: helena.gradisar@ki.si  
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ICGEB Reference No.: CRP/SLO14-03  
**Abstract:** Polypeptides and polynucleotides are natural programmable biopolymers that self-assemble into complex tertiary structures. In this project we designed new modular polypeptide- and DNA-based nanostructures with a great potential for the application. We assumed that single-strand DNA nanostructures may serve as a fast prototyping tool to design folding pathways of single-chain polypeptide nanostructures. We demonstrated the principles to guide the folding pathway and defined the rules for efficient and rapid folding into the target structure. This finding suggested the possibility of designing modular proteins that self-assemble under physiological conditions. First, we designed and characterised a wide range of coiled coil-forming peptide pairs that serve as modules. Further, we developed a computational platform for design of polyhedral protein cages. We designed recombinantly produced and characterised several variants of different polyhedral structures. We also demonstrated the efficient in vivo folding of tetrahedral structure in bacteria, mammalian cells and in mice without evidence of inflammation.  
**Objectives:**  
In this project we investigated designed, topologically constrained folding of single-chain protein- and DNA-based nanostructures, a process that more closely mimics the way of natural biomolecules fold. The advantage of a modularly composed single-chain design is that each module (coiled coil-forming peptide or short DNA sequence) folds independently of others (Fig. 1). Designing the folding pathway represented a major challenge, both for DNA- and protein-based nanostructures. Our approach for new protein-based polyhedral nanostructures employed orthogonal dimerising coiled-coil segments as interacting modules. When concatenated into a single chain in defined order, they self-assembled into a 3D structure defined by topology of interacting modules within the chain. First, the topological analysis of possible polyhedra has to be performed. Then design, production and characterisation of the structures have to be executed.  

**Results Obtained:**  
(i) First, we defined rules to guide the folding of highly knotted single-chain DNA nanostructures (Fig. 1 left) based on defining the order of formation of duplex modules according to their stability [2]. A ‘free-end’ rule states that at each folding step the favourable folding design must involve at least one pairing segment with a free end, allowing its threading through the performed structure. The findings of study of a single-chain DNA nanostructure were included into a topological analysis and design of novel polyhedral protein nanostructures [3].  
(ii) We have shown before (Gradišar et al., Nature Chem. Biol., 2013) that coiled-coil dimers can be used as modular building blocks to form the edges of polyhedral cages. We developed a set of de novo designed coiled-coil heterodimers where we modulated a dimer stability through surface amino acid residues while preserving binding interface [4]. Using this strategy we produced coiled-coil peptide pairs that maintain their binding specificity and orthogonality. Some of these peptides were able to form silver nanoparticles and showed the antimicrobial activity [5].  
(iii) To automate the design of coiled coil-based polyhedral structures (tetrahedron, square pyramid, trigonal prism, bipyramid) we developed a computational platform CoCoPOD (Coiled-coil protein origami, https://github.com/NIC-SBI/CC_protein_origami) (Fig. 2a). We investigated the design of a range of modules and linkers of different polyhedral variants. Self-assembled...
nanostructures and their properties were analysed by measuring CD (Fig. 2b), DLS, SEC-MALS and SF. The agreement of the isolated protein particles with the design was confirmed by SAXS (Fig. 2c) and EM (Fig. 2d). We demonstrated that protein origami folds also in mammalian cells and in mice (Fig. 2e) and does not induce the immune response [6].

Figure 2: Design of coiled-coil protein origami tetrahedral cage that self-assembles in vitro and in vivo [6]. (a) Coiled-coil protein-origami design platform (CoCoPOD) was developed for polyhedral cages construction. (b) CD measurements confirmed a high helical content and high thermal stability of the tetrahedral structure. SAXS (c) and TEM (d) analyses confirmed the size and shape, including a cavity inside the structure. (d) Tetrahedral structure is formed also in mammalian cells and in mice.

Publications:
**Title:** Local, sustained non-viral RNAi delivery for heart failure therapy  
**Principal Investigator:** Neil Davies, Cardiovascular Research Unit, Surgery, Health Faculty, University of Cape Town, Anzio Rd, Observatory, Cape Town, South Africa. Tel: +27-21-4066613, Fax: +27-21-4485935, E-mail: neil.davies@uct.ac.za  
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ICGEB Reference No.: CRP/ZAF14-01

**Abstract:** Heart failure is a major complication for up to one third of patients that suffer myocardial infarction. RNA interference strategies are presently being explored at the pre-clinical small animal model stage as a therapeutic approach for limiting the progression of myocardial infarction towards heart failure. An issue that is presently not explored in RNA interference for myocardial infarction is the development of more readily translatable delivery modalities. It is generally considered that the use of repeated high doses of naked RNA or viral vectors will be difficult to move into the clinic. Polymer based nanoparticle carriers for RNA interference oligonucleotides are being extensively researched as they allow for both protection from RNases and also improved transfection efficiency. However, an aspect of delivery of these polymer nanoparticles that is under researched is the use of a biomaterial to entrap and thus localise delivery of these particles. It is believed that tissue localised and sustained release of RNA interference nanoparticles will reduce off target effects and also aid in prolonging the desired RNA interference.

**Objectives:**

(i) To establish and characterise a range of siRNA nanoparticles;  
(ii) To assess the controlled release and transfection efficiency of siRNA nanoparticles encapsulated in injectable hydrogels with novel 3D cell culture assays;  
(iii) To establish a small animal model for assessing injectable siRNA nanoparticle containing hydrogels.

**Results Obtained:**

A range of siRNA nanoparticles were produced in the laboratory either from cationic polymers synthesised on-site, the PEGylated, lipid modified G4 dendrimer (MD); purchased branched 25 kDa polyethylenimine (PEI) and commercially available liposomal preparations Lipofectamine RNAiMAX and Invivofectamine. All particles efficiently knocked down green fluorescent protein (GFP) translation in an HT1080 cell line stably transfected with GFP. Knockdown ranged from 40%-80% with increasing levels from MD<PEI<Invivofectamine<Lipofectamine. Protection from RNase in serum was identified as a potentially critical parameter in a hydrogel based controlled release approach. PEI, MD and Invivofectamine were found to protect greater than 40% of their siRNA cargo for at least 10 days. RNase protection afforded by Lipofectamine was minimal but the carrier was included in downstream analysis due to its high efficacy in RNA interference.

Two types of hydrogel were investigated as potential nanoparticle scaffolds, a synthetic engineered enzymatically degradable polyethylene glycol hydrogel (PEG) and a modified natural polymer, pegylated fibrin (pFb). These polymers were synthesised in house and shown to have biomechanical characteristics suitable for purpose. Both hydrogels effectively encapsulated all nanoparticles as assessed by confocal microscopy indicating fluorescently labelled nanoparticles dispersed throughout polymerised hydrogels. Release studies showed that nanoparticles were completely entrapped in PEG hydrogels with no release observed over 14 days. pFb encapsulated MD and Invivofectamine particles were released with a small initial burst at day 1 and then at a linear rate over the remaining days. Lipofectamine showed a far more pronounced burst release in the first day. Bioactivity was retained for released particles except PEI but only Invivofectamine had similar levels of RNA interference efficacy after release. The loss of activity for PEI was shown to be due to aggregation. PEG hydrogels significantly improved RNase protection after encapsulation.

Two novel 3D cell invasion assays were developed for analysing transfection efficacy within hydrogels. An overarching theme in the design of the assays was that cells were compartmentalised away from nanoparticle containing hydrogels prior to cellular invasion. It was observed in preliminary 3D cell invasion assays whereby hydrogels were polymerised with a mixture of nanoparticles and cells, an approach used in studies reported by others, that very high levels of knockdown were achieved. This was surmised to be due to nanoparticles at a high concentration being able to readily diffuse into contact with cells during the polymerisation period. The assays developed, encapsulated cells in one gel that was placed in direct contact with a neighbouring gel containing cells. In one model carried out using a modified transwell assay, cell/hydrogels were polymerised on top of nanoparticle/hydrogels. Cells were assessed for transfection after passing through the nanoparticle layer. This assay was suitable for fibrin-based hydrogels that permitted relatively rapid invasion. Similar levels of knockdown to that previously observed for 2D transfections (see above) were found for MD, Lipofectamine and Invivofectamine.
A second model that surrounds a contracted collagen gel containing cells with a nanoparticle hydrogel was developed based on the “nested matrix” assay. Preliminary data showed significant but low levels of transfection in PEG hydrogels for PEI nanoparticles. This assay, which maintains cells in a 3D environment for the duration of the experiment is presently being utilised to assay MD, Lipofectamine and Invivofectamine.

An animal model that allows for assay of injectable hydrogels was developed. The model is based on the injection into the tibialis anterior muscle of the mouse. A homozygote GFP expressing mouse strain was bred out of a heterozygote strain available in house. Injection protocols were established that achieve insudation of hydrogel throughout the complete muscle. An extensive in vivo hydrogel degradation study was carried out. Degradation of hydrogels could be controlled for PEG and pFb hydrogels such that total pFb degradation occurred over 7 days and PEG, through titration of the enzymatically cleavable sites with hydrolytically cleavable sites, occurred from 10-30 days. The assessment of the efficacy of in vivo RNA interference by Invivofectamine containing scaffolds is presently underway.

**Results Unforeseen in the Original Project:**
The assays developed for the project assisted in the analysis of serum derived exosomes RNA delivery capability and their inherent stability. These naturally occurring nanoparticles will also be investigated in the context of controlled localised hydrogel-based delivery.

The funding has contributed significantly towards the training of 3 PhD students and 1 MSc student.

**Publications:**

Title: The thioredoxin-fold diversity in trypanosomatids and flatworms  
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ICGEB Reference No.: CRP/URU14-01  

Abstract: The identification and characterisation of specific targets from pathogenic protozoan and flatworms deems essential to fight the devastating neglected diseases they cause in humans and animals. Focusing on the unique thiol-dependent pathways of Trypanosomes and flatworms, the project shed light on the functional divergence of thioredoxin (Trx)-fold proteins in these clinically relevant lineages.

The 3D structures of glutaredoxins (Grx) and Trx from Trypanosoma brucei and Echinococcus granulosus were determined by NMR spectroscopy and analysed to atomic detail for the first time. Key insights into the structural adaptations, and their biochemical consequences, that several Trx-like proteins underwent to fulfill their biological tasks were disclosed.

Novel protein partners of the major redoxins and class II Grx were identified, providing new clues to redox-regulated cellular processes. A novel class of tapeworm-specific Trx able to bind iron-sulfur clusters (ISC) was characterised, identifying ISC metabolism as a putative target. In vivo experiments proved that African trypanosomes rely on a trypanothione-specific redoxins for infectivity, while they can fully dispense on redox-active Grx and selenocysteine-based Trx-like proteins.

Finally, Uruguayan scientists were trained on NMR spectroscopy of macromolecules, a discipline undeveloped in our country.

Objectives:
(i) In silico exploration of the genome from pathogenic and free-living species of each lineage;  
(ii) Biochemical, structural and biological characterisation of novel Trx-fold proteins from trypanosomatids and worms;  
(iii) Identification of the redox interactome from a subset of Trx-fold proteins;  
(iv) Training of Uruguayan researchers on protein NMR spectroscopy at the partner laboratory in Italy.

Results Obtained:
Eight major subfamilies of protein harbouring a Trx-fold were identified in the genome of Trypanosomatids and flatworm parasites. No lineage-specific thioredoxin-fold was found for both parasite groups. However, extensive analysis of genomes and sequences revealed that EgTrxCP4 (later named IsTRP, for Iron-sulfur thioredoxin-related protein) is a cestode lineage-specific thioredoxin absent not only other flatworms, but also in other lineages, revealing this protein as a key pharmacological target for cestode parasites. In contrast, the genome of trypanosomatids harbour a lineage-specific redoxin (named KSRP) for which not homologs were identified outside the Kinetoplastid Genus.

The NMR structures of the following proteins were solved: T. brucei ICGRx1, 2CGRx1 and 1CGRx3, EgISTRP. A full structural and biochemical characterisation of these proteins and point-mutants thereof, revealed the role of several residues and regions in protein function.

Novel protein partners involved in key metabolic or cellular processes were identified for the thioldisulfide oxidoreductase tryparedoxin (TXN) from T. brucei and for EgTrx1, EgTrx2 and EgTrx3 from E. granulosus.

Mouse infection experiments performed with RNAi or knockout cell lines affecting the function of different Trx-fold proteins (dithiol glutaredoxin I and 2, TXN and selenocysteine metabolism) demonstrated that TXN is, so far, the only redoxin indispensable for in vivo survival of African trypanosomes.

Results Unforeseen in the Original Project:
(i) For the major redoxin of T. brucei, namely tryparedoxin, we would have expected to identify several of the protein partners described in a study performed with the homolog protein from T. cruzi. This was not the case and most of the covalent partners that tryparedoxin has in T. brucei represents new interactors.
(ii) Dithiol glutaredoxins participate in the maintenance of redox homeostasis in different organisms, and the T. brucei proteins are indeed redox active contributing to protein (de)glutathionylation. Thus, it was unexpected to find that African trypanosomes lacking both genes for dithiol glutaredoxins were fully infective to mice. This led us to conclude that dithiol glutaredoxins can be disregarded as drug target candidates.
(iii) The biochemical and structural characterisation of EgISTRP shed light on the Trx fold evolutionary path from a thioldisulfide oxidoreductase to ISC-binding.

Publications:


Salinas, G., Comini, M.A. Alternative thiol-based redox systems. 2018. Antioxid. Redox Signal. 28(6), 407-409

Title: Co-expression of the Arabidopsis AVP1 and NHX1 to enhance salt tolerance in transgenic soybean (Glycine max (L.) Merrill)

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Abstract: Salinity has been known as one of the main causes of yield loss in agricultural crop production in many parts of the world. Majority of the attempts to improve salt tolerance in plants using genetic engineering have been reported with positive results in Arabidopsis, XXX. In this study, we investigated the transgenic soybean co-overexpressing Arabidopsis AtAVP1 and AtNHX1 and their combination for salt tolerance. AtAVP1 and AtNHX1 were assembled under control of 3SS promoter to constitutively drive gene expression in the soybean plants. The transgenic plants overexpressing either individual or both AtAVP1 and AtNHX1 had normal growth under non-stress conditions. Under salinity stress of 100 mM, transgenic plants had higher plant height, shoot and root dry weight and maintain better leaf greenness, and survive longer, compared to the non-transgenic control. For the purpose of investigating the seed yield response, the plants were grown in soil with salinity stress at 7 ds/m. At this stress, soybean could produce 20% of seed yield, compared to that of non-stress condition. However, no significant difference in seed yields was observed among transgenic events and the WT, suggesting that more genes conferring to salt tolerance mechanisms might be needed for application in genetic engineering of soybean to cope with the stress.

Objectives:
(i) To develop transgenic plants overexpressing both AtNHX1 and AtAVP1 under constitutive 3SS promoter for the Vietnamese soybean variety DT26;
(ii) To evaluate salt tolerance of the transgenic plants in greenhouse conditions for salt tolerance at vegetative growth and seed yield.

Results Obtained:
(i) Development of binary vectors and transgenic plants overexpressing AtAVP1 and AtNHX1. Expression cassettes of AtAVP1 and AtNHX1 were assembled under control of 3SS promoter. The final binary vectors are designated pSalt10 and pSalt11 respectively, which were delivered into Agrobacterium strain EHA101. The presence of binary vector in the bacterium was verified using restriction analyses. The plasmids were sequenced to show that the cloned sequences were identical to the submitted DNA entries in the NCBI databases (NM_101437 and NM_122597, respectively). DT26, a recently introduced variety for winter and spring cropping in Northern Vietnam, was used for transformation. Due to low transformation efficiency in our laboratory conditions, more than 7,000 cotyledons of DT26 were used to generate 25 transgenic events for the 2 gene constructs. Among the soybean transgenic events derived, 8 transgenic events were PCR positive and verified by southern analyses. We selected two events from each gene for phenotypic characterisation. These are referred to as A2 (AtAVP1-T0.2-T1.2), A3 (AtAVP1-T0.3-T1.7), N1 (AtNHX1-T0.1-T1.34), and N2 (AtNHX1-T0.3-T1.12), respectively for AtAVP1 and AtNHX1. The transgenic showed good expression of the genes (Figure 3) was further used in development of transgenic plants overexpressing both genes by crossing two events from each gene construct (AN1: A2 x N1, and AN2 (A3xN2). The F1 and F2 generations were verified by PCR using primers 3SS-F, AtAVP1-MR and AtNHX1-MR.

(ii) Co-expression of AtAVP1 and AtNHX1 confers greater salt tolerance to transgenic soybean than expression of individual genes at vegetative growth stages.

In this study, the transgenic lines and wild-type plants were tested for salt tolerance in greenhouse. Two-week seedlings were tested for vegetative growth performance by subjecting the uniformly grown seedlings to 0 mM (~2.3 ds/m) and 100 mM NaCl (~10ds/m) stress until difference was observed among the transgenic and wild type plants. Under normal conditions (0 mM NaCl), all transgenic events and WT plants exhibited vigorous growth for plant height, shoot dry weight and root dry weight. Under 100 mM NaCl stress, significant difference was noticed among transgenic and the wild-type plants. The transgenic plants had better plant height and biomass (both shoot and root) than the WT. On average, the transgenic plants had 27% plant height, 65% shoot biomass and 50% root biomass higher than the WT. The transgenic plants co-expressing two genes, however, did not have higher plant height and biomass than those having expression of a single gene AtAVP1 or AtNHX1. Leaf maintenance is important to maintain biomass accumulation and ultimately maintain yield under salt stress. It clearly showed that the transgenic plants had lower leaf scotching rate, higher...
photosynthesis rate, higher cell membrane stability (estimated from electrode leakage analyses), and higher chlorophyll content, than the control DT26. Among transgenic events, the co-expressing events had substantially lower leaf scotching rate, higher photosynthesis rate, higher cell membrane stability (estimated from electrode leakage analyses), and higher chlorophyll content, than both AtAVP1 and AtNHX1 events. This observation clearly shows that the two genes were able to protect the soybean plants from deleterious effect of the Na stress. The tolerance was better when the two genes work together, suggesting that they performed their expected functions to sequester Na+ to vacuum to maintain cell functioning, at least at 100 mM. Transgenic plants did not have better tolerance to NaCl in leaf discs assay. The leaf disc assay of wild-type and transgenic plants were performed for estimation of salt tolerance potential. One-centimeter diameter leaf discs from 3-week wild-type and transgenic lines from plants growing under non-stress condition were submerged on NaCl solutions of 0, 100, 200 and 300 mM NaCl for 96 h to investigate the effect of overexpression of AtNHX1, AtAVP1 in preventing the toxic effect of NaCl. Observations were taken every day and the chlorophyll measurements were taken at the end of experiment. It shows clearly that NaCl stresses have significant effect on the greenness and cell structure of the leaf discs. In the present study, apparently, higher salt concentration had more damage to leaf and the greenness lost much faster than the lower NaCl concentration treatments. However, we did not find differences between transgenic and control plants for both visualisation evaluation and chlorophyll concentration measurements using chlorophyll extracts.

AtAVP1/AtNHX1 overexpression regulates tissue Na, K and Cl distribution in transgenic plants. Tissues of roots and the third leaf from top were collected for chemical analyses of Na, K, and Cl concentrations, which will help predict roles of the two genes in salt tolerance in soybean. Figure 3 shows that under salinity stress, transgenic plants accumulate more Na, K and Cl- than the non-transgenic plants. The experiment was repeated for two years 2016 and 2017 with consistent results. Overexpression of the two genes increases ion content in the tissues. The better tolerance of transgenic plants, therefore, involves tolerate higher concentration of both Na and Cl- in the tissues. The Na+ export from cytosol might help plants cope better to the high salt in the tissues.

(iii) Transgenic plants did not outperform the wild type in long-term growth and yield.

To investigate whether the advantage of leaf health maintenance, better photosynthesis and biomass accumulation of the transgenic over the WT can result in higher seed yield under salinity conditions, we kept a set of plants for further investigation of seed yields. After five weeks of salt treatments at 100 mM NaCl, however, all plants died, suggesting that this concentration of NaCl was too high for the soybean plants to survive and produce seeds. Therefore, this given advantage of growth within few weeks could not translate to seed yield. This observation led us to lower the salt concentration in the media to 7.5 dS/m electrical conductance, which is close to the threshold (5dS/m) above which soybean has a reduction slope of 20% of its yield. At this lower concentration, we could grow the soybean to maturity with substantially fewer pods, compared to non-stress plants (Figure 4). Average biomass and seed yield of salt treated plants were 50% and 20% of the non-salted plants, respectively. We did not observe any difference on growth, biomass or seed yields between the transgenic and WT plants under this stress conditions.