ICGEB Research Grants Programme

Research Grants Completed in 2019
<table>
<thead>
<tr>
<th>COUNTRY</th>
<th>PRINCIPAL INVESTIGATOR</th>
<th>PROJECT TITLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARGENTINA</td>
<td>Javier Guillermo MAGADÁN</td>
<td>Mechanistic analysis of Influenza A virus glycoprotein compensation</td>
</tr>
<tr>
<td>BOSNIA AND HERZEGOVINA</td>
<td>Mirza SULJAGIC</td>
<td>Multi-targeting survival pathways in human leukemic cells by combinatorial therapy with metformin and thymoquinone</td>
</tr>
<tr>
<td>BRAZIL</td>
<td>Ronaldo DA SILVA MOHANA- BORGES</td>
<td>Deciphering the role of NS1 protein in Dengue pathogenesis</td>
</tr>
<tr>
<td>BULGARIA</td>
<td>Marina NEDELCEVA-VELEVA</td>
<td>Dynamic changes in replication complex</td>
</tr>
<tr>
<td>CAMEROON</td>
<td>Roger MOYOU–SOMO</td>
<td>Identification of host protective mechanisms against Schistosomiasis: what role for interleukin-4 receptor?</td>
</tr>
<tr>
<td>CHILE</td>
<td>Julio D. AMIGO</td>
<td>Molecular-functional dissection of pericyte contribution to sprouting angiogenesis in zebrafish</td>
</tr>
<tr>
<td>COLOMBIA</td>
<td>John BUSTAMANTE OSORNO</td>
<td>Development of a platform to interaction of cardiac cells in an <em>in-vitro</em> model to reproduce the tissue pattern behavior in the endomyocardial fibrosis</td>
</tr>
<tr>
<td>CROATIA</td>
<td>Antonija JURAK BEGONJA</td>
<td>Deciphering the role of phosphoinositol lipids in thrombopoiesis (<em>Early Career Return Grant</em>)</td>
</tr>
<tr>
<td>ECUADOR</td>
<td>Natalia BAILON MOSCOSO</td>
<td>Phytochemical study and anti-tumor activity of <em>Grias neuberthii</em> and <em>G. peruviana</em> two species of medicinal plants endemic in Ecuador (<em>Early Career Return Grant</em>)</td>
</tr>
<tr>
<td>INDIA</td>
<td>Kiran KULKARNI</td>
<td>Structural basis of Dock3-NEDD9 interactions and their role in tumour cell plasticity</td>
</tr>
<tr>
<td>IRAN</td>
<td>Sima RAFATI</td>
<td>Identification of a protective sand fly salivary vaccine</td>
</tr>
<tr>
<td>MAURITIUS</td>
<td>Joyce GOVINDEN SOULANGE</td>
<td>Bioprospecting Mauritian phytomedicines for anticancer leads</td>
</tr>
<tr>
<td>MEXICO</td>
<td>Andres MORENO-ESTRADA</td>
<td>Human functional genomics of Native American variation in Mexico and Latin America (<em>Early Career Return Grant</em>)</td>
</tr>
<tr>
<td>RUSSIA</td>
<td>Anna V. KUDRYAVTSEVA</td>
<td>Glycolysis regulation in tumour progression</td>
</tr>
<tr>
<td>SERBIA</td>
<td>Branko JOVCIC</td>
<td>Survey for antimicrobials effective against carbapenem-resistant Gram-negative bacteria</td>
</tr>
<tr>
<td>SYRIA</td>
<td>Abdul-Qader ABBADY</td>
<td>Application of nanobody technology in the diagnosis of Leishmaniasis in Syria</td>
</tr>
<tr>
<td>TUNISIA</td>
<td>Mahmoud GARGOURI</td>
<td>Characterization of Mycorrhizal symbiotic interface in prickly pear for enhancing growth and stress tolerance of economically important-Mediterranean crops (<em>Early Career Return Grant</em>)</td>
</tr>
</tbody>
</table>
TURKEY  Fatih KOCABAS  Development of hematopoietic stem cell expansion technologies (Early Career Return Grant)
Titulares: Mechanistic analysis of influenza A virus glycoprotein compensation

Principales Investigadores: Javier G. Magadán, Centro Científico Tecnológico (CCT)-CONICET Mendoza, Instituto de Histología y Embriología de Mendoza (IHEM-CONICET), Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Avenida Ruiz Leal s/n, Parque General San Martín, Mendoza (5500), Argentina. Tel: +54-261-4494143, ext. 7008, Fax: +54-261-4494193, E-mail: jmagadan@mendoza-conicet.gob.ar

ICGEB Contract No.: CRP/15/001

ICGEB Reference No.: CRP/ARG15-01

Abstract: Antigenic drift allows influenza A viruses (IAV) to evade certain adverse conditions that may affect their proper replication and propagation, like the host immune response and specific pharmacological therapies. Despite of this apparent beneficial outcome, mutations on the viral surface glycoproteins hemagglutinin (HA) or neuraminidase (NA) might also severely compromise their normal function and, by inference, influenza virus fitness. At the end of this research project, we were able to show that drifted IAV exposed in vitro to specific anti-HA neutralising antibodies "compensates" harmful amino acid substitutions within HA by incorporating non-homologous mutations on NA that promote its misfolding and retention in the host endoplasmic reticulum (ER) or just before passing through the trans Golgi network. As a consequence of being stuck within the early secretory pathway, unfolded NAs are not properly targeted to lipid rafts but instead are poly-ubiquitinated and subjected to proteasome-dependent degradation. Overall, mutant virus particles are only able to incorporate few (if any) molecules of NA, which thus exhibit poor sialidase activity per virion. Our observations uncover a variety of mechanisms that allow drifted IAVs maximise viral fitness by compensating lost in HA function through NA misincorporation to budding viruses at the host cell surface. In addition, our data have important implications for NA biology towards identifying new therapeutic targets in influenza-related diseases.

Objectives:

(i) Characterise cellular and biochemical mechanisms by which IAVs compensate viral glycoprotein content and activity in order to maximise their replication and propagation under unfavourable conditions, i.e., exposure to anti-viral drugs and/or neutralising antibodies;

(ii) Gain insight into the basic biology of the poorly characterised IAV NA glycoprotein.

Results Obtained:

Generation of recombinant IAVs by reverse genetics

We started the proposed research program by attempting to "rescue" recombinant A/Puerto Rico/8/1934 (PR8) H1N1 IAVs with only NA harboring compensatory mutations. Although non-compensated, we thought that these new viruses might represent valuable tools for precisely dissecting the mechanism/s by which amino acid substitutions in NA solely affect influenza biology independently of its background. Optimising a reverse genetics approach, we thus successfully created several recombinant IAVs carrying point mutations on NA [H260N (CV1), V101I (JV9), K239R (KV2), G339S (SEQ8), and T198I (F10)] while all the other segments remained as PR8 versions, even HA.

Recombinant IAVs contain low number of drifted/compensated NAs per virion

By employing a variety of biochemical and fluorometric procedures, we then showed that all new recombinant IAVs are almost depleted of NA after ~48 hs post-infection of MDCK cells and, as a consequence, they exhibit very reduced (if any) sialidase activity. Importantly, we also found that mutant viral RNA levels encoding NA are similar when compared to those on wild-type (PR8) viruses. All together, these results indicate that compensatory amino acid substitutions on NA but not HA are fully responsible for the observed defects on glycoprotein incorporation into mutant IAVs.

All mutant NAs, except JV9, fail to efficiently reach the host cell surface

We then set up a number of radioactivity-based protocols in order to specifically immunoprecipitate newly-synthesised NA from recombinant IAV-infected cells. Under these experimental conditions, we observed that accumulation of all mutant but JV9 NAs on the host cell surface is slowed down if compared with wild-type (PR8) NA. We thus analysed NA maturation through the secretory pathway looking for N-linked oligosaccharides processing at the Golgi complex. Our results indicated that transport of CV1, SEQ8 and F10 NAs is delayed at the ER level, while KV2 NA just traffics through the secretory pathway like PR8 NA normally does, which suggests that mistargeting of KV2 NA occurs beyond the ER and even the Golgi complex but certainly before its plasma membrane incorporation.

CV1, KV2, SEQ8, and F10 NAs are intracellularly retained as misfolded/poly-ubiquitinated species

Using a combination of conformation-dependent and -independent anti-NA antibodies that recognise fully folded, oligomerised vs. immature NA, respectively, we were able to shown by confocal immunofluorescence microscopy and radioactive pulse-chase analysis that misfolded CV1, SEQ8 and F10 are heavily retained in the ER, while KV2 NA, on the other hand, is mistargeted to a
peripheral, round-shaped, intracellular compartment where it accumulates as unfolded species. Since all mutant but JV9 NAs are retained within the host cell as a consequence of their misfolding, we therefore assessed NA ubiquitination. Our results clearly shown that both ER retained and KV2 NAs are heavily modified with multiple lysine 48-linked ubiquitin chains, which further implies the involvement of certain proteasome-dependent quality control machineries in modulating NA stability.

Neither mutant NAs properly accumulate within lipid rafts

IAV envelope glycoproteins progressively accumulate on lipid microdomains within the host plasma membrane often referred as "rafts", which cluster at the trans-Golgi network (TGN) to mediate the sorting of lipids, as well as proteins, into secretory vesicles. Based on their resistance to solubilisation by mild detergents, we purified lipid rafts from recombinant influenza-infected cells and found that incorporation of all mutant NAs is severely impaired, which is expected for CV1, SEQ8 and F10 NAs due to their retention in the ER, but not for KV2 and JV9 NAs, whose trafficking through the biosynthetic pathway appears to be similar to that of PR8 NA. All together, these results indicate that an important fraction of KV2 NA is mistargeted to yet uncharacterised non-lipid raft membranes, where presumably undergoes misfolding/poly-ubiquitination and, as a consequence, recognition by certain quality control system/s operating at a pre-TGN level. On the other hand, JV9 NA misincorporation to other membrane sites but not lipid rafts might further affect glycoprotein structure/stability on the host plasma membrane itself or once it is laying on the nascent viral progeny.

NA depletion on mutant IAVs affects viral fitness in a time-dependent manner

We hypothesised that virion release from infected cells might be severely compromised even at early time points, mainly due for NA misincorporation to viral assembly-competent domains laying on the host plasma membrane. We have to put this notion to the test by examining how fast newly-synthesised IAVs are released in a quantitative, radioactive pulse-chase setup. Our results indicate that while JV9, KV2 and SEQ8 viruses are released following similar kinetics as shown by wild-type (PR8) IAV, mutant CV1 and F10 are less efficient for reaching the extracellular media. We reason that the presence of any cellular sialidase or even traces of viral NA at the host plasma membrane is sufficient to efficiently promote viral spread from infected cells. However, we favor the notion that a progressive depletion of NA after continuous rounds of viral assembling and release will ultimately affect virus titers not only in vitro but also in vivo, where this phenotype should be greatly enhanced due to the implicit impairment of influenza tropism through the host respiratory tract.
Title: Multi-targeting survival pathways in human leukemic cells by combinatorial therapy with metformin and thymoquinone

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ICGEB Contract No.: CRP/15/002

ICGEB Reference No.: CRP/BIH15-05

Abstract: Numerous studies have shown that metformin decreases cancer risk in diabetic patients. These have led to clinical trials exploring anticancer effects of metformin as monotherapy or in combination with different agents in non-diabetic cancer patients. However, characterisations of molecular targets in leukemic cells remained controversial and needed to be further elucidated. Our research efforts focused particularly on determining how combination of metformin and thymoquinone (TQ), the predominant bioactive constituent of black seed oil, affect intracellular signaling events and apoptosis regulatory mechanisms in leukemia and lymphoma cell lines. We have shown that treatment with metformin and TQ inhibited proliferation and metabolic activity of CML cell lines, particularly those resistant to current therapy by TKIs. Western blot analysis revealed metformin capacity to inhibit the activity of Akt, ERK and NFκB, demonstrated in two ABC type of DLBCL and CML cells as well as in primary leukemic cells. Importantly, metformin exhibited additional inhibitory potential in combination with ibrutinib and idelalisib, significantly lowering their IC values and further sensitising cells for apoptosis through the inhibition of the key pro-survival kinases, commonly upregulated in hematological malignancies. Inhibitory effects of metformin on metabolic activity through a multi-targeted inhibition of NFκB, Akt and ERK intracellular signaling, provide an added rationale for its therapeutic use in cancer patients.

Objectives:

(i) Evaluation of thymoquinone and metformin effects on intracellular signaling events and apoptosis and proliferation regulatory mechanisms in leukemia and lymphoma cell lines;
(ii) Characterisation of molecular targets of metformin and thymoquinone treatment in CML derived cell lines resistant and sensitive to current TKI therapy;
(iii) Determination of the efficiency of treatment with metformin in combination with PI3Kdelta and BTK inhibitors in a panel of germinal center B cell-like (GCB) and activated B cell-like (ABC) lymphoma cell lines and comparatively evaluate the effects.

Results Obtained:

Single drug treatment with metformin and TQ inhibited BrdU incorporation and proliferation of K562, LAMA84S and LAMA84R cell lines. This inhibition was shown to be concentration dependent. Sequential, non-constant ratio of a drug incubatory regimen was determined and found to be more effective when compared to simultaneous addition of drugs. Combinatorial studies using BrdU incorporation and WST-8 viability assessment have shown dominant synergistic effects at lower concentrations of drug combination. An effect of combinatorial treatment on cellular apoptosis was found to be less pronounced, as shown by Annexin V/PI assays, but have shown concentration dependent inhibitory effects in mono-therapy. Metformin inhibition of NFκB, ERK, Akt and P70S6 kinase signaling was observed in all CML derived cell lines, sensitive and resistant to TKI therapy. This inhibition resulted in reduction of anti-apoptotic Mcl-1 protein expression. Although TQ was shown to stimulate phosphorylation of Akt at Ser473 at earlier time points, synergistic effects were obvious in particular combinatorial regimens with metformin.

Importantly, in DLBCL cell lines metformin exhibited strong inhibitory potential in co-treatment experiments with ibrutinib and idelalisib, recently FDA/EMA approved drugs for therapy of CLL, NHL (FL) and DLBCL, significantly lowering their IC values and at the same time increasing the inhibition of the main pro-survival kinases. Effects of metformin on phosphorylation status of Akt have been explored by others in different cancers, such as lymphoma, glioma, fibrosarcoma, neuroblastoma, with controversial results, showing no effects, inhibition or induction of Akt activity. We have seen that in models of resistant CML and ABC type of DLBCL, metformin has shown activity against Akt, at least in part, through PTEN stimulation. Apart of Akt, inhibitory effects of metformin were seen also on NFκB and ERK signaling, which in turn resulted in decreased levels of Mcl-1 and XIAP, major apoptotic regulators, in LAMA84R and OCI-Ly10 cells, respectively.

Finally, ex-vivo analysis of primary cells isolated from AML and CLL patients have shown to be sensitive to metformin and TQ treatments to a similar extent seen in our cell line models. Importantly, western blot analysis of treated CLL B-cells identified both, Akt and NFκB as main targeting molecules, further confirming mechanisms seen in cell line models.

Results Unforeseen in the Original Project:

We aimed to explain whether TQ is able to inhibit proliferation and induce apoptosis in various leukemic cell lines and to investigate TQ’s mechanism of action on NFκB and PI3K/Akt signaling and its downstream targets. Contrary to previous reports where TQ have shown to inhibit Akt...
signaling, we observed that in DLBCL model TQ activity stimulates phosphorylation of Akt at Ser473 after 2 and 6 hours period. However, in longer incubatory settings (48h) in combination with metformin, TQ potentiated synergistic effects in all tested cell lines, as well as in primary leukemic cells, by inhibition of cellular proliferation shown by Wst-8, BrdU and 7AAD assays.
Among the non-structural proteins of DENV, NS1 is a glycoprotein of 46-55 kDa found intracellularly in the form of dimers or secreted as hexamers. NS1 is essential for viral replication, but its function is not well established. To understand the role of NS1 in DENV pathogenesis, our group identified 50 novel interactions between NS1 and human liver proteins by yeast two-hybrid system. We found vitronectin and plasminogen, which are secreted into the blood plasma and are involved in the homeostatic regulation during inflammatory processes. A common feature of the vitronectin and plasminogen proteins is the fact that they are recruited by pathogens to evade the immune system through the inhibition of specific pathways of the complement system, making them able to survive and establish an infection. Therefore, we propose that DENV NS1 could interact with these two proteins to inhibit the complement system activation.

**Objectives:**

(i) to characterise the interactions of DENV NS1 with vitronectin (VN) and plasminogen (PLG);
(ii) to understand the importance of these interactions for DENV immune evasion of the complement system;
(iii) to evaluate whether DENV NS1 affects platelet aggregation via FBG interaction, and whether NS1 is an important factor for platelet activation, cytokine release and microparticle formation.

In this project our main aims were:

(i) to produce recombinant DENV2 NS1 glycoprotein;
(ii) to assess the affinity of the NS1-VN, NS1-PLG and NS1-FBG interactions by surface plasmon resonance (SPR);
(iii) to identify the NS1-PLG complexes from dengue-infected patients’ plasma by capture ELISA;
(iv) to assess whether the NS1-VN interaction inhibits cell lysis by MAC-dependent haemolytic assay;
(v) to assess whether the NS1-PLG interaction enables PLG activation and cleavage of C3b and C5 by plasmin.

**Results Obtained:**

Our findings indicate that both recombinant and native NS1 from DENV-infected patients form a complex with VN. Most pathogenic bacteria bind VN either at its N-terminal region, its central domain containing HPX-like domains, or at the basic carboxy terminal HBD-3. Usually, bacterial-VN binding does not interfere with the VN domain involved in the inhibition of C5b-9 complex thus allowing this complement regulator to remain active in inhibiting MAC formation. Our data suggest that the NS1-VN interaction does not involve the SMB domain, HBDS, or HPX-like domains of the VN molecule. However, DENV NS1 competes with PLG for binding to VN, suggesting that these molecules have overlapping binding sites on VN. Additionally, DENV NS1 alone or in association with VN, inhibited C9 polymerisation, thus preventing MAC formation independently from its glycosylation pattern. Moreover, by binding to C9, mammalian secreted DENV, WNV, and ZIKV NS1 inhibited MAC formation on cell membranes. Taken together, these data imply a role of NS1 as a terminal pathway inhibitor of the complement system. It is more likely that NS1 acts as a complement evasion protein during the acute phase of the disease when anti-NS1 antibodies have not yet formed immune complexes, providing a less adverse extracellular environment for the released viruses. In the context of dengue secondary infections, ADE, immune complex deposition and complement activation may be critical for the development of severe cases.

Our group carried out two-hybrid assays and determined NS1 binding partners, such as the plasminogen, as described previously. Then, firstly we confirmed this result by two immunoenzimatic assays (ELISA), in which we observed that NS1 was able to interact with both purified plasminogen and plasminogen present in the serum, suggesting that this interaction could occur during DENV infection. Next, we evaluate whether NS1 was recognised as a plasmin substrate. Thus, we performed cleavage assays using both pre-activated purified plasminogen and pre-activated NHS. In the former case, using purified plasminogen, we could observe that more
NS1 bands of lower molecular weight appeared in a time-dependent manner, confirming its cleavage by plasmin. In contrast, when the plasminogen present in the NHS was used, the pattern of cleavage was not repeated, as the protein band remained intact even after 4 hours of incubation, showing that this cleavage could not happen in the presence of the other components from the serum. This result suggested that NS1 was interacting with plasminogen regulator proteins, such as uPA, in a way to prevent its degradation by plasmin. Therefore, we assessed whether NS1 could interact with uPA. By carrying out another ELISA, we showed that the two proteins interact, but more experiments need to be done in order to verify the interaction with the remaining plasminogen regulator proteins (tPA, PAI-1 and alpha-2-antiplasmin).

NS1 is known to bind the membranes of non-infected cells and promote an easier entrance of viral particles, in a context of an infection. Thus, we carried out another cleavage assay, this time assessing whether NS1 cleavage occurred at a cellular level. We showed that plasmin is able not only to cleave but also remove NS1 attached to cell membranes. This result suggested that NS1 interaction with regulators of plasminogen in order to prevent its cleavage is a mechanism of evading host innate immune response, facilitating the infection of naive cells. Our group is yet to define more roles of this interaction during an infection, characterising its regulation, chemical aspects, effects and importance.

Results Unforeseen in the Original Project:
The most striking unforeseen result that comes out of project is the capability that NS1 has to interact with several host macromolecules, subverting the cellular processes to permit viral replication. NS1 interacts with VN and PLG as well as with PLG regulator proteins (PAI-1 and alpha-2-antiplasmin). Altogether, our results reinforce the pivotal role of NS1 protein in DENV pathogenesis and replication.

Publications:
Title: Dynamic changes in replication complex

Principal Investigator: Marina Nedelcheva-Veleva, Laboratory of Genomic Stability, Structure and Function of Chromatin Department, Institute of Molecular Biology "Roumen Tsanev", Bulgarian Academy of Sciences, 21 “Acad. G. Bonchev” Str., Sofia, 1113, Bulgaria. Tel: +359-2-9793689, Fax: +359-2-8723507, E-mail: marina@bio21.bas.bg

ICGEB Contract No.: CRP/16/003

ICGEB Reference No.: CRP/BGR16-03

Abstract: In order to prevent genome instability and cancer, cells have evolved various control mechanisms to regulate the replication machinery and to ensure proper DNA synthesis even when DNA is damaged. These mechanisms induce modifications of key replisome proteins, which may lead to their degradation or dissociation from the replication fork. It is known that the MCM2-7 helicase is recruited to replication origins and, after its activation, it unwinds DNA during the elongation phase of DNA replication. The helicase is tightly regulated by various protein complexes, such as Claspin/Tim/Tipin (Mrc1/Tof1/Csm3 in S. cerevisiae). After unwinding the Polo/Primase complex synthesises RNA/DNA primers, which are, elongated by DNA Polymerases δ and ε tightly associated with PCNA. And1 (Ctf4), similar to Claspin, participates in the harmonisation of the replicative helicase and polymerases. Therefore, we are interested to find out if it is constantly present at the replication complex, or is it degraded or detached, in order to perform a specific role related to the dynamics of the replication fork. Moreover, we are interested to find out whether the dynamics of chromatin binding of And1 correlates with that of Tim/Tipin/Claspin complex, the replicative helicase and polymerases.

Objectives:
(i) To look for changes in the amount of Ctf4 (And1) throughout the cell cycle that result from the lack of functional SCF\textsuperscript{Dia2} E3 ubiquitin ligase both in yeast and in higher eukaryotes. If changes are detected - to find out the significance of Ctf4 (And1)’s degradation in the context of the dynamics of DNA replication;
(ii) To explore the dynamics of chromatin binding of And1 throughout replication and to compare it to that of Claspin, the replicative polymerases and helicase.

Results Obtained:
Our results suggest that the levels of Ctf4 and its human ortholog And1 decrease in the final stages of DNA synthesis in a proteasome-dependent manner. Furthermore, we show that Ctf4 is degraded by the SCF\textsuperscript{Dia2} E3 ubiquitin ligase. We have measured the mobility of PolA2, PolD2, And1, Claspin, MCM6 via FRAP during normal and perturbed replication. And1, similarly to PolA2 and PolD2, has high amount of mobile fraction during G1, S-phase and during checkpoint activation. In contrast, MCM6, which has a high amount of continuously bound fraction from late G1 to middle S phase and during checkpoint activation, reaches 51% immobile fraction. In addition, And1 has the fastest kinetics of recovery after photobleaching. All this results show that And1 is the most dynamic protein on the replication fork during normal and perturbed replication, which suggests regulatory rather than structural function in this process.

Results Unforeseen in the Original Project:
We measured the kinetics of recruitment and dissociation of PolA2, PolD2, PCNA and ATM at the sites of complex DNA damages, induced by UV laser. Following this, we compared the kinetics of the above mentioned proteins to the kinetics of 68 other proteins that participate in the DNA damage response. This allowed us to put these proteins in a precise time frame with respect to multiple DNA repair proteins, to generate the most comprehensive chronological map of the DNA damage response.

Publications:
**CAMEROON**

**Title:** Identification of host protective mechanisms against schistosomiasis: what role for interleukin 4 receptor?

**Principal Investigator:** Roger Moyou-Somo, Laboratory of Schistosomiasis Research and Parasitology, Medical Research Centre, Institute of Medical Research and Medicinal plants Studies, Ministry of Scientific Research and Innovation, Yaoundé, Cameroon. Tel: +237-699978625, Fax: +237-222233226, E-mail: roger_moyou@yahoo.fr

ICGEB Contract No.: CRP/15/004

ICGEB Reference No.: CRP/CMR15-05

**Abstract:** To complement the only available anti-Schistosomiasis drug praziquantel, the development of novel therapeutic measures is desperately needed. In mice, signalling via IL-4 receptor (IL-4R) was suggested a potential regulatory role during Schistosomiasis-driven pathology but this had yet to be formally determined. In this project two research Institutions collaborated (i.e., Minresi in Cameroon and ICGEB Cape Town in South Africa) and the relevance of host signalling via interleukin-4 receptor to the Schistosomiasis-induced morbidity in humans (Cameroon) and mice (South Africa) was assessed. Our study defined persistent spots of Schistosomiasis transmission in rural Cameroon and identified specific risk factors of infection and pathology. Next generation sequencing of the il-4r gene revealed an overall poor association of the gene polymorphisms with liver fibrosis amid a higher propensity to liver fibrosis in children with specific polymorphic il-4r gene variants. Moreover, making use of a newly generated murine model of inducible deletion of the il-4r gene, we demonstrated an anti-pathology effect of removing the il-4r gene during murine Schistosomiasis.

In conclusion, a role for il-4r in promoting tissue pathology during Schistosomiasis is suggested. A negative association between Schistosomiasis and measles vaccine responses in school children was also uncovered.

**Publications:**


CHILE

**Title:** Molecular-functional dissection of pericyte contribution to sprouting angiogenesis in zebrafish  
**Principal Investigator:** Julio Amigo, Hematovascular Development, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Alameda 340, Santiago, Chile. Tel. +56-2-23542850, E-mail: jamigo@bio.puc.cl  
**ICGEB Contract No.:** CRP/15/005  
**ICGEB Reference No.:** CRP/CHL15-01

**Abstract:** Genes related to development biology and cancers have a common evolutionary history, since they molecular mechanisms seen to be conserved though the species. We have been studying the evolutionary history of the Reprimo (RPRM) gene family members. These genes are implicated in p53-mediated cell cycle regulation at the G2/M checkpoint. We are using zebrafish to study RPRM requirements during neurovascular patterning and development, this will allow, for the first time, a complete organism-scale *in vivo* genetic assays for RPRM gene-function at cellular resolution on a complex vertebrate organism. We also use transgenic tools to facilitate visualisation of tissue formation and differentiation allowing simultaneous high-resolution confocal imaging of entire tissues/organs on a live organism. These technologies are being implemented for a broad spectrum of fields ranging from neurobiology, cardiovascular and regenerative biology to angiogenesis and cancer. Transgenic techniques have been extensively used to study vascular development in zebrafish embryos. The molecular mechanisms by which RPRM control tissue development are unknown, but our studies suggest that RPRM genes are involved in the central and peripheral nervous systems early embryonic specification. Recently we have demonstrated that RPRM and RPRM-Like genes appear to have differentially retained during vertebrate evolution. We have found the RPRM genes have an early common ancestor and experienced whole genome duplication through vertebrates. We characterise the spatiotemporal of RPRMs in embryonic zebrafish by whole mount in situ hybridisation (WISH). At early developmental stages RPRM genes are expressed in nervous system, mesodermal derived tissues, digestive tissues and olfactory epithelium. Currently, a large number of unanswered questions about RPRM function remain. What are the signalling pathways that mediate RPRM activity during cell proliferation and differentiation? Are these genetic interactions conserved between physiological and pathological process such a tumour/cancer growth? These essential questions will need functional and molecular analysis in animal models where to know the impact of RPRM gain- or loss-of-function in a complete organism. As mentioned, we believe that the zebrafish is an ideal model to answer these long-standing questions.

**Objectives:** Study RPRM function during developmental processes. In particular:  
(i) to characterise genetic and molecular signalling interacting with RPRM;  
(ii) to follow up cell populations during *in vivo* development by the use of transgenic tools;  
(iii) to investigate RPRM requirement during tissue specification and patterning using forward and reverse genetic screens.

**Results Obtained:**

Our results indicate that RPRM and RPRM-Like genes appear to have differentially retained during vertebrate evolution. We have found the RPRM genes have an early common ancestor and experienced whole genome duplication through vertebrates. We characterise the spatiotemporal of RPRMs in embryonic zebrafish. At early developmental stages RPRM genes are expressed in nervous system, mesodermal derived tissues, digestive tissues and olfactory epithelium. Our recent investigations suggest that RPRM is required for the proper formation and positioning of sensory organs during embryonic zebrafish development.

**Results Unforeseen in the Original Project:**

Beside its contribution to cancer progression the RPRM genes have a role during embryonic development.

**Publications:**

Title: Development of a platform to interaction of cardiac cells in an in-vitro model to reproduce the tissue pattern behavior in the endomyocardial fibrosis

Principal Investigator: John Bustamante O., Centro de Bioingeniería, Universidad Pontificia Bolivariana, Campus Universitario, Bloque 22C, Planta 2ª, Circular 1ª No. 73-76 Laureles, Medellín, Colombia. Tel: +57-4-4488388, ext. 12403, E-mail: john.bustamante@upb.edu.co
ICGEB Contract No.: CRP/17/005
ICGEB Reference No.: CRP/COL17-03

Abstract: Cardiac functions can be altered by changes in the microstructure of the heart, i.e., remodeling of the cardiac tissue, which may activate pathologies such as hypertrophy, dilation, or cardiac fibrosis. Cardiac fibrosis can develop due to an excessive deposition of extracellular matrix proteins, which are products of the activation of fibroblasts. In this context, the anatomical-histological change may interfere with the functioning of the cardiac tissue, which requires specialised cells for its operation. Of these cells, cardiomyocytes are responsible for generating the contraction of the cardiac walls, following depolarisation generated by electrical impulses. These electrical impulses are usually affected or interrupted in the presence of pathologies such as myocardial fibrosis, triggering different types of cardiac arrhythmias.

Objectives:
The aim of our project was to develop a platform emulating the biological characteristics of endomyocardial fibrosis by the cell patterning technique, to study morphological cellular changes in compact and irregular patterns of fibrosis.

Results Obtained:
It was found that cellular patterns emulating the geometrical distributions of endomyocardial fibrosis generated morphological changes after interaction of the RL-14 cardiomyocytes with the 3T3 fibroblasts. Through this study, it was possible to evaluate biological characteristics such as cell proliferation, adhesion and spatial distribution, which are directly related to the type of emulated endomyocardial fibrosis.

This research concluded that fibroblasts inhibited the proliferation of cardiomyocytes via their interaction with specific microarchitectures. This behaviour is consistent with the histopathological distribution of cardiac fibrosis; therefore, the platform developed in this research could be useful for the in vitro assessment of cellular microdomains. This would allow for the experimental determination of interactions with drugs, substrates, or biomaterials within the engineering of cardiac tissues.
Title: Deciphering the role of phosphoinositol lipids in thrombopoiesis

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ICGEB Contract No.: CRP/15/007
ICGEB Reference No.: CRP/HRV15-04_EC

Abstract: Platelets are essential for hemostasis and they also mediate diverse disease processes including inflammation, metastasis, atherosclerosis and clotting. Platelets derive from cytoplasm of megakaryocytes (MKs) in the bone marrow in a process of thrombopoiesis. MKs undergoes complex maturation process that leads towards extension of long, branched cytoplasmic structures called proplatelets that eventually release platelets. Molecular mechanisms that govern these elaborate cytoplasmic changes of MKs into proplatelets and platelets remain largely unknown. Based on our preliminary studies on primary MKs we observed that one type of phosphoinositides (PIs), phosphatidylinositol 3-monophosphate (PI3P), could have a major role in proplatelet formation. Phosphoinositides are small membrane phospholipids implicated in cellular signalling, organelle trafficking and cytoskeletal dynamics. PI3P, mostly produced by Vps34 kinase (PI3KCIII), is concentrated at the cytosolic leaflet of early endosomes and is involved in endosomal trafficking. Our hypothesis was that PI3P regulation of membrane traffic in the endocytic pathway contributes significantly to the membrane growth needed for platelet production. The proposed project attempted to reveal the exact role of PI3P in MK differentiation and/or proplatelet formation, and identify the mechanism by which PI3P is involved in platelet biogenesis.

Objectives:
In a in vitro mouse model of thrombopoiesis we aimed to: (i) define if PI3P contributes to MK maturation and/or proplatelet formation; (ii) investigate if PI3P localises to early endosomes or some other compartment in MKs; (iii) analyse PI3P effector proteins involved in this process. We visualised PI3P by using genetically engineered fluorescent probes that bind PI3P (2xFYVE, PX), a technique based on protein domains fused with fluorescent protein expressed from retroviruses, in primary mouse MKs.

Results Obtained:
We analysed localisation of PI3P, early (EE; EEA1) and late endosomes/lysosomes (LE/Lys; Rab7, LAMP1) by confocal microscopy using recombinant PI3P-binding probe (GFP-2xFYVE) in immature and mature primary MKs. We found that in immature MKs, PI3P was confined to large vesicles, mostly colocalising with EE, while in mature MKs PI3P resembled discrete vesicles localised to both EE and LE/Lys. Moreover, while in immature MKs PI3P and LE/Lys were mostly scattered or perinuclear, in mature MKs they translocated to the cell periphery. In mature MKs, PI3P (GFP-2xFYVE probe or GFP-2xFYVE and YFP-PX expressed from retroviruses) colocalised with GPIbβ, or CD61 (MK plasma membrane markers). Also, PI3P partially colocalised with both PI(4,5)P2 and LAMP1 at the same sites, and inhibition of PI3P production (PI3KCIII inhibitors) decreased LAMP1 localisation on the plasma membrane. Importantly, PI3KCIII inhibitors significantly reduced the size of MKs when applied at earlier stages. Electron microscopy analysis revealed that inhibition of VPS34 at earlier maturation stages caused defect in development of DMS that was coupled with appearance of vacuoles within the cytoplasm of cells. At later stages, overexpression of PI3P binding domains YFP-PX and GFP-2xFYVE, but not the expression of YFP/GFP, diminished proplatelets, final stages of MK development, suggesting that they act as dominant-negative inhibitors. These results were further confirmed by inhibition of PI3P production (PI3KCIII inhibitors), overexpression of MTM1, phosphatase that cleaves PI3P, or by pharmacologically inhibiting endosomal maturation from EE to LE/Lys. Since our results showed that PI3P localisation changes in immature vs. mature MK by shifting to LE, we studied well-known PI3P effector proteins, Rab5 and Rab7. Our results indicate that PI3P-positive LE/Lys contribute to the membrane growth and platelet formation from MKs.

Results Unforeseen in the Original Project:
PI3P localisation at the LE in the vicinity of plasma membrane of mature MKs was an unexpected finding. Therefore, we focused on small GTPase Rab7 at the LE and showed that Rab7 needs to be functional for proper proplatelet formation. In addition, PI3KCIII expressed equally at all stages of MK maturation, however localisation analysis revealed unexpected differences in terms of confinement to different cellular compartments warranting future investigations.

Publications:
ECUADOR

Title: Phytochemical study and anti-tumour activity of Grias neuberthii and G. peruviana two species of medicinal plants endemic in Ecuador

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ICGEB Contract No.: CRP/16/006
ICGEB Reference No.: CRP/ECU16-01_EC

Abstract: Ecuador is a nation that has the privileged to have a large number of plant species per km² as well as a broad ethno-medical knowledge base. According to ethnomedicinal information, the two species Grias neuberthii and G. peruviana are used as antipyretic, antiparasitic, anti-tumour, anti-inflammatory and antibacterial treatments by different human groups, such as the Shuar, Waorani, Kichwa, and Tsachila, which are settled in the highlands and the Ecuadorian Amazon. The uses of these species in traditional medicine are uses for which there is still high demand for new therapeutic options worldwide, including in Ecuador. The present project aims to study some of the reported activities of G. neuberthii and G. peruviana in order to validate and enhance their study.

For example cancer is one of the leading causes of death worldwide. Establishing the mechanism of cell death induced by specific extracts and isolated secondary metabolites is critical in chemotherapy because it allows us to establish new therapeutic combinations. The mechanisms involved in cell death include apoptosis, autophagy, and necroptosis and each has different mediators for death that can be activated by the compound of interest.

More than 50% of human cancers carry p53 mutations, whereas p63 and p73 mutations or epigenetic alterations are rare. However, all these genes play critical roles in controlling the expression of target genes involved in cell death and cell survival.

Under pressure from environmental chemicals, the p53 member proteins are activated by phosphorylation and proactively involved in transcriptional and post-transcriptional regulation. Serving as transcriptional factors, p53 family proteins bind and activate/inhibit the expression of multiple proteins involved in cell cycle arrest, apoptosis, and autophagy. Moreover, by interacting with other proteins, p53 members exert proactive regulatory functions on mitochondrial function, RNA splicing, epigenetic enzymes, and the production of microRNAs in tumour cells. The functions of the p53 family proteins are also controlled by multiple enzymes that catalyze phosphorylation, acetylation, and methylation. Many microRNAs negatively affect the expression of p53 members and their regulators, as well as signalling components that contribute to apoptosis, autophagy, and other types of cell death.

Objectives:
The general objective of this grant was to determine the anti-tumour activity of extracts and secondary metabolites of Grias neuberthii and Grias peruviana, both of which are endemic species of Ecuador.

Specific objectives were: (i) to isolate and characterise the secondary metabolites of G. neuberthii and G. peruviana and their cytotoxicity in cancer cell lines, and (ii) to understand the molecular mechanisms that underlie the apoptotic and autophagic effects of the extracts and secondary metabolites of G. neuberthii and G. peruviana and the modulation of p53 family members (p53, p63, and p73), which are well-known regulators of cell proliferation, cell cycle arrest, cell survival, cell death, cell metabolism, and autophagy.

Results Obtained:
Of the extracts obtained from various parts of G. neuberthii and G. peruviana, the extracts with the greatest cytotoxic potential were the extracts of the stem bark of G. neuberthii. Colon cancer represents one of the main health problems around the world and, therefore, the study of new therapies to counteract it is urgent. This is why we elected to test the most active extract in two colon carcinoma cell lines, RKO (normal p53) and SW613-B3 (mutated p53).

From what we observed, the extract has the same effective cytotoxicity as MTS since the IC₅₀ values are similar despite having different effects on p53. Likewise, in both tumour cell lines, there was no arrest of the cell cycle after exposure to the extract. However, significant differences were seen in the long-term regarding the formation of colonies, where RKO cells were more sensitive than SW613-B3: cells with mutated p53 recovered after exposure of the extract, but at a higher dose than the IC₅₀, the two tumour lines showed similar rates of cell death.

Apoptosis was not the predominant cause of cell death after exposure to the extracts, as assessed by the expression of Annexin V, among other proteins.

Both cell lines did show signs of cell death induced by autophagy after exposure to the extracts. In the autophagic pathway, Bcl-2 is associated with Beclin-1. Once Beclin-1 is released, the autophagic pathway is activated and LC3-I is converted into its active form, LC3-II, to induce phagophore formation in the nucleation phase to enclose the obsolete proteins and organelles,
tagged with p62, for degradation. Forced autophagy could generate severe damage that ends in the death of the cell. Up-regulation of Beclin-1 expression was observed in both cell lines. The dissociation between Bcl-2 and Beclin-1 is necessary for the initiation of autophagy; therefore, the increased presence of Bcl-2 observed in the RKO cell line could be explained by this dissociation. Moreover, an increase in LC-3II was detected in both the RKO and SW613-B3 cell lines. The final step in autophagy is the degradation of charges in which p62 is involved (and also degraded). In both cell lines, p62 levels were decreased, thus indicating the termination of the autophagy cell death pathway. Three chemical compounds that were probably responsible for this effect were identified: Lupeol, 3’-O-Methylellagic acid 4-O-β-D-rhamnopyranoside, and 19-α-Hydroxy-asiatic acid monoglucoside.

**Results Unforeseen in the Original Project:**
Although the compounds obtained from the active extract were in small quantities, which did not allow us to establish if they were responsible for the cytotoxic effect, previous reports show that Lupeol and 19-α-Hydroxy-asiatic acid monoglucoside have cytotoxic effects on human cancer cell lines.

**Publications:**
Title: Structural basis of Dock3-NEDD9 interactions and their role in tumour cell plasticity

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ICGEB Contract No.: CRP/15/008

ICGEB Reference No.: CRP/IND15-02

Abstract: Metastasis poses major challenges in cancer therapy and accounts for approximately 90% of the deaths in cancer. Tumour cells, migrating as individual cells, exhibit remarkable ability to modulate their morphology to counter the impediments due to the changing tissue environment. The two prominent modes of movement, a mesenchymal mode characterised by an elongated morphology and an amoeboid mode with a rounded morphology, are regulated by diverse signalling events.

Rho GTPases are the master regulators of diverse biological processes such as cell proliferation, apoptosis and cell migration. These molecules work as bio-switches, which are 'ON' (active) when bound to GTP and 'OFF' (inactive) when bound to GDP. The shuttling between the ON and OFF states is regulated by two antagonist classes of proteins known as the guanine nucleotide exchange factors (GEFs) and the GTPase-activating proteins (GAPs). GEFs activate GTPases by promoting the exchange of GDP for GTP, whereas GAPs switch off the GTPases by enhancing their intrinsic rate of hydrolysis of bound GTP to GDP. Upon activation, they interact with various downstream signalling proteins to regulate several important biological processes. Aberrations of Rho signalling particularly impact the cytoskeleton, whose organisation and reorganisation underpin the motility of cancer cells during the invasive growth and metastasis. Recently, DOCK family of non-canonical GEFs have emerged as key regulators of tumour cell migration. Studies have unequivocally shown that Dock3, a bona fide member of DOCK family GEFs, forms a complex with NEDD9 (Neural precursor expressed, developmentally down-regulated 9), a member of the p130Cas family, to activate Rac1. This particular signalling event has shown to be exploited by the malignant cells to drive mesenchymal migration by promoting actin assembly to form cellular protrusions.

Despite the proven role of Dock3-NEDD9 interaction in cancer cell migration, mechanistic details of this signalling event, such as: (i) what is the structural basis for the Dock3-NEDD9 interaction; (ii) does this interaction regulate the activity of Dock3 and finally (iii) how the interplay of Dock3 and NEDD9 regulate cell migration, remains still elusive. To address these questions, the in vitro complex of Dock3 and NEDD9 will be characterised using LC-MS/MS. The information thus obtained will be used to generate stable, homogeneous, in vitro complex of Dock3 and NEDD9 by expressing them in a heterologous Baculovirus-insect cell system.

The protein complex thus produced will be subjected to the structural studies using X-ray crystallography and/or single particle Cryo electron microscopy. The structural and biochemical information will be used to delineate Dock3-NEDD9 interactions and hence their role in Rac activation. Based on these inferences a model for Dock3 and NEDD9 induced cell migration will be developed. The model will be tested using structure based mutagenesis and cell migration assays. The study will provide insights on Dock3 and NEDD9 controlled migration of tumour cells. Since metastasis is the major clinical problem in cancer therapy, the outcome of this study will have important implications in designing novel anti-metastatic drugs. Furthermore, this study will also throw light on various DOCK regulated fundamental biological processes such as cell migration, angiogenesis and apoptotic cell debris clearance.

The Principal Investigator of the project has a strong background in structural biology and has good amount of experience in the field of DOCK signalling network, which would be extremely helpful in executing the proposed project.

Objectives:

(i) Characterisation of Dock3-NEDD9 complex and optimisation of the Dock3 and NEDD9 constructs for large-scale expression. Under this objective it was proposed to form stable in vitro complex of Dock3-NEDD9 for structural studies and also to examine the role of phosphorylation of these protein in stabilising their interaction.

(ii) To elucidate the structural basis of Dock3-NEDD9 interaction. Earlier reports have shown that interaction of NEDD9 with Dock3 is essential for the tumour cells to achieve their plasticity. But how exactly NEDD9 modulates Dock3 activity is not clear. Under this objective it was proposed to elucidate the structure of Dock3-NEDD9 structure to understand their interaction at the atomic level.

(iii) Examining the impact of Dock3-NEDD9 complexation on the GEF activity of Dock3. Some of the DOCK family GEFs are known to exist in an autoinhibited state and upon binding to scaffold proteins like NEDD9 their autoinhibition gets released. Therefore, the objective here was to test if NEDD9 has similar role with Dok3.

Results Obtained:
Dock3 and NEDD9 were cloned into pBI CMV1 and pFAST BAC vectors for co-expression in HEK293 (mammalian) and Sf9 (insect cells) expression systems. The expression level of expression of the protein complex in HEK293 was found to be extremely low and it was not sufficient for structural studies. Although the complex expressed slightly better in Sf9 expression system, but it was found to be aggregating. Modified constructs of Dock3 with N and C terminal truncations did not resolve the problem. Complexation of the proteins on stoichiometric mixture too resulted in aggregation. Negative stained micrographs of the physical mixture of the proteins showed higher order oligomerisation of the protein complex. Homo and Hetero oligomerisation of DOCK family GEFs are shown to play critical role in regulating cell migration. Perhaps, NEDD9 might control Dock3 activity by modulation its oligomeric structure. Since stable in vitro complex of Dock3-NEDD9, in sufficient quantities, for structural studies was difficult to achieve, structural studies of apo forms of Dock3 and NEDD9 were performed. In this direction NEDD9 was crystallised and single particle Cryo-EM data on Dock3 is being collected. With this the structure elucidation of Dock3 and NEDD9 is imminent. The proposed objective of understanding Dock3-NEDD9 interaction would be eventually achieved by docking the two structures. The information obtained from docking studies will be evaluated with domain deletion mutants of Dock3 and NEDD9. In this direction a library of Dock3 and NEDD9 constructs have been already prepared. Non-availability of stable Dock3-NEDD9 complex was a serious impediment in exploring the role of NEDD9 on GEF activity of Dock3. However, a mant-GTP based assay has been standardised for rapid screening of Dock3 GEF activity. On availability of Dock3 and NEDD9 constructs, role of the later on Dock3 GEFa activity will be assayed.

**Results Unforeseen in the Original Project:**
Lack of stability of in vitro Dock3-NEDD9 interaction is a completely unforeseen result. Physical mixtures of both the proteins show formation of higher oligomers of the complex. Perhaps, the physiologically the complexation might be transient or NEDD9 regulates Dock3 activity by modulating its oligomeric state.
Title: Identification of a protective sand fly salivary vaccine

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ICGEB Contract No.: CRP/17/007
ICGEB Reference No.: CRP/IRN15-02

Abstract: The vector-borne disease leishmaniasis is transmitted to humans by infected female sand flies, which transmits Leishmania parasites together with saliva during blood feeding. In Iran, cutaneous leishmaniasis (CL) is caused by Leishmania (L.) major and L. tropica, and their main vectors are Phlebotomus (Ph.) papatasi and Ph. sergenti, respectively.

In this work we tested the immune response in BALB/c mice to 14 different plasmids coding for the most abundant salivary proteins of Ph. sergenti. The plasmid coding for the salivary protein PsSP9 induced a DTH response in the presence of a significant increase of IFN-γ expression in draining lymph nodes (LN) as compared to control plasmid and no detectable PsSP9 antibody response. Animals immunised with whole Ph. sergenti SGH developed only a saliva-specific antibody response and no DTH response. Mice immunised with whole Ph. sergenti saliva and challenged intradermally with L. tropica plus Ph. sergenti SGH in their ears, exhibited no protective effect. In contrast, PsSP9-immunised mice showed protection against L. tropica infection resulting in a reduction in nodule size, disease burden and parasite burden compared to controls. Two months post-infection, protection was associated with a significant increase in the ratio of IFN-γ to IL-5 expression in the dLN compared to controls.

Objectives:
This research proposal had the objective of answering the following questions:
(i) How many salivary proteins from Ph. sergenti can induce a Th1-immune response in BALB/c mice?
(ii) What is the impact of Th1 inducing salivary proteins from Ph. sergenti on immune responses against L. tropica inoculation with Ph. sergenti SGH in BALB/c mice after booster DNA immunisation?
(iii) Which salivary protein(s) of Ph. sergenti shows the highest protection controlling parasite propagation?

Results Obtained:
This study demonstrates that while immunity to the whole Ph. sergenti saliva does not induce a protective response against cutaneous leishmaniasis in BALB/c mice, PsSP9, a member of the PpSP15 family of Ph. sergenti salivary proteins, provides protection against L. tropica infection. These results suggest that this family of proteins in Ph. sergenti, Ph. duboscqi and Ph. papatasi may have similar immunogenic and protective properties against different Leishmania species. Indeed, this anti-saliva immunity may act as an adjuvant to accelerate the cell-mediated immune response to co-administered Leishmania antigens, or even cause the activation of infected macrophages to remove parasites more efficiently. These findings highlight the idea of applying arthropod saliva components in vaccination approaches for diseases caused by vector-borne pathogens.

Publications:
Title: Bioprospecting Mauritian phytomedicines for anticancer leads
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ICGEB Contract No.: CRP/16/008
ICGEB Reference No.: CRP/MUS16-01

Objectives:
The objectives of this grant were as follows:
(i) To screen selected medicinal as well as non-medicinal endemic plants for the presence of bioactives, in order to validate their potential anti-cancer activity;
(ii) To characterise the compounds responsible for their anticancer activity;
(iii) To carry out phylogenetic analysis of chloroplast and nuclear sequences of these plants;
(iv) To generate genetic information, which will contribute in endemic biodiversity conservation;
(v) To correlate phylogeny with biological profile in order to offer a predicative approach, to enable efficient selection of plants for the discovery of lead molecules.

Results Obtained:
The results obtained at the end of the project were:
(i) One species, *Psiadia arguta*, has been identified as having significant anticancer potential;
(ii) The cytotoxic mechanism of action for *P. arguta* has been elucidated;
(iii) LC/MS data, confirming the presence of flavonols and fatty acids in *Psiadia arguta*, has been generated;
(iv) Chloroplast and nuclear sequences of *Psiadia* species have been acquired;
(v) Phylogenetic analyses of chloroplast and nuclear genes may be used for predicting the anticancer activity of *Psiadia* species.
MEXICO

**Title:** Human functional genomics of Native American variation in Mexico and Latin America  
**Principal Investigator:** Andres Moreno-Estrada, Human Evolutionary and Population Genomics Lab, Advanced Genomics Unit, LANGEBIO-CINVESTAV, Km 9.6 Libramiento Norte Carretera Irapuato-León, Irapuato, Mexico. Tel: +52-462-1663000, Fax: +52-462-6078246, E-mail: andres.moreno@cinvestav.mx

ICGEB Contract No.: CRP/15/009  
ICGEB Reference No.: CRP/MEX15-04_EC

**Abstract:** Native American populations remain underrepresented in human genomic research. This project was aimed at reducing the gap between over studied and underrepresented populations in catalogs of human genetic variation by generating genomic data from two of the major sources of Native American diversity in Latin America: Mesoamerica in Mexico and the Andean region in Peru and Chile. Targeting the coding portion of the genome allowed us to: reduce sequencing costs, reach deeper coverage, and increase discovery rate of functional variants absent in current catalogues of genetic variation. Population-specific variants were screened for signatures of adaptive evolution and a yeast model was implemented to functionally test candidate variants under selection. Whenever possible, population data was complemented with ancient DNA data from human remains of similar ancestral backgrounds to shed light on the evolutionary history of indigenous groups in Mesoamerica and the Andes. In collaboration with other ICGEB Member States, we conducted sampling and genotyping efforts in each of those regions, which fostered collaborative research across Latin America. A common challenge is the scarce availability of qualified human resources in the region, so this project also enabled specialised training of young researchers, contributing to the future generation of Latin American scientists in genomics.

**Objectives:**
(i) To sequence 75 Native American exomes from five different Mexican ethnic groups;
(ii) To generate sequencing data from Native American populations spanning the Andean region in South America;
(iii) To characterise the full site frequency spectrum (SFS) of coding and non-coding variants, and identification of functional variants with potential phenotypic effects;
(iv) To evaluate the functional impact of candidate variants with potential phenotypic effects by following *in silico* and *in vivo* approaches;
(v) To complement modern and ancient DNA data from populations for which we have access to human remains of similar or closely related ethnicity;
(vi) To foster training and genomic research in Latin America by conducting large-scale population genomic projects and promoting collaborative research among ICGEB Member States.

**Results Obtained:**
We sequenced 78 human exomes at 80x from five Mexican indigenous populations: Huichol (n=13), Maya (n=13), Nahua (n=17), Rarámuri (n=19) and Triqui (n=14) to obtain protein coding variation of the genome. We first built a demographic model for these populations using diffusion approximations with dadi, inferring a split between northern and southern ethnic groups 7.2 kya and subsequently diverging regionally 6.5 kya and 5.7 kya, respectively. We then searched for signals compatible with the action of natural selection by using the FST-based Population Branch Statistic (PBS), and we identified genes with strong differentiation between closely related populations, which could be the result of local adaptations driven by functional variants in those genes. The PBS scan revealed BCL2L13 and KBTBD8 genes as potential candidates for adaptive evolution in Rarámuris and Triquis, respectively. BCL2L13 is highly expressed in skeletal muscle and could be related to physical endurance, a well-known phenotype of the northern Mexico Rarámuri. The KBTBD8 gene has been associated with idiopathic short stature and we found it to be highly differentiated in Triqui, a southern indigenous group from Oaxaca whose height is extremely low compared to other native populations.

To explore possible shifts of genome variation between past and present gene pools, we sequenced several ancient genomes closely related to our study populations from northern and central Mexico. For example, we sequenced two ca. 900-year old pre-Columbian mummies from the Tarahumara Sierra in northern Mexico, where long-distance running is a cultural practice among the Rarámuri dating back centuries. We performed PCA to compare the ancient individuals with a reference panel of modern Native Mexican groups. Preliminary analyses show that one mummy clusters closely with present-day Rarámuris, while the second clusters with a geographically distant population. Similarly, we sequenced five bone samples yielding more than 1% of endogenous DNA from the Cañada de la Virgen archaeological site in central Mexico and found that while some ancient genomes clustered with local modern references, some showed affinities as distant as the Mayan region. These results open up questions regarding past genetic structure and migration between different indigenous groups across Mesoamerica.
As a first step to validate some of the identified variants as candidates under selection, we implemented a yeast experimental system aimed at comparing the phenotypic profiles of different polymorphisms. Before testing the variants identified in our sequenced Mexican populations, we prioritised the implementation of the experimental system using a set of candidate genes identified by ongoing selection scans in human genes with known orthologous in yeast. For example, the gene Adenylate kinase 2 (AK2) was found to be positively selected and its orthologous gene in yeast (ADK1) has 55% of sequence identity, which in turns has a paralogous gene (ADK2) with 35% of sequence identity. In order to test the functional differences between the allelic states of the AK2 human gene we constructed yeast knockouts in S. cerevisiae and conducted a series of experiments of heterologous complementation. Both yeast knockouts (Δadk1 and Δadk2) were compared in spots assays, where Δadk2 showed a similar growth rate as the wild type, while Δadk1 showed a deleterious phenotype reducing growth as a result of being an essential gene. When the transformed knockouts carrying the ancestral and modern versions of the human gene were tested in SC-Ura cultures with and without Doxycycline, the wild type phenotype was not recovered by any of the AK2 sequences.

In collaboration with our partners from the University of Chile we sequenced the complete genome of a subset of 18 samples from the ChileGenomico Project (http://chilegenomico.uchile.cl), and generated genome-wide array data from four modern indigenous populations from Central Southern Chile and Patagonia (N=61), as well as sequencing data from four ancient genomes of maritime individuals from remote Patagonia (ca. 1,000-year-old). We found that Native Patagonian Kaweskar and Yamana showed the highest genetic affinity with the ancient individuals, indicating genetic continuity in the region during the past 1,000 years. We also found that the ancient maritime individuals were genetically equidistant to a ca. 200 years old terrestrial hunter-gatherer from Tierra del Fuego, which supports a model with an initial separation of a common ancestral group to both maritime populations from a terrestrial population, with a later diversification of the maritime groups.

Publications:
Glycolysis regulation in tumour progression

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ICGEB Contract No.: CRP/15/010

ICGEB Reference No.: CRP/RUS15-01

Abstract: In this work we analysed alterations in energy metabolism in carotid body tumours (CBTs) based on the exome and transcriptome data, as well as immunohistochemistry results. We detected potential driver mutations in both known causative genes and novel genes in CBTs. The association between mutation status of several genes with changes in transcriptome profile was studied. We also compared mutation status of SDHx genes (SDHA, SDHB, SDHC, and SDHD), encoding for subunits of mitochondrial complex II, with their expression at mRNA and protein levels. It was found that mutations in KMT2D were associated with alterations in energy metabolism in CBTs. Not all mutations in SDHx genes were shown to be associated with the gene expression changes at mRNA or protein levels; other mechanisms (methylation, miRNA regulation, etc.) can be caused alterations in the gene expression. We also calculated mutational load in CBTs that was ranging on the average 0.09-0.28/megabase (Mb). Thus, we revealed several molecular genetic characteristics associated with energy metabolism, including glycolysis regulation, in CBTs.

Objectives:
(i) To estimate changes in the expression of the genes involved in energy metabolism, especially encoding for glycolytic enzymes, as well as the genes whose products may regulate this process (transcriptomic NGS analysis);
(ii) To identify associations between common driver mutations and transcriptomic profile in carotid body tumours;
(iii) To estimate changes in the expression of the proteins involved in energy metabolism; to compare the data on gene expression at the mRNA and protein levels in the same samples and to analyse correlations between changes in the gene expression and clinicopathologic characteristics of the sample;
(iv) To identify various mechanisms of changes in the expression of selected genes (transcription factors, mutations, promoter methylation, miRNA etc.);
(v) To estimate the effect of suppression for the selected key genes (if paraganglioma cell lines will be created). It should be noted that creation of CBT cell culture was challenging, because of very low viability of the tumour cells. We obtained primary CBT cell culture (immortalized with several oncogenes) enriched by supporting cells. Currently, it is going on the experiments to remove supporting cells from the CBT cell culture.

We also performed the analysis of mutation load (ML) in carotid body tumour. This is an actual and important task. It allowed evaluating number of mutations in CBT, to compare ML in CBT with others common malignant tumours, and to estimate a possibility of immunotherapy use to treatment of CBT.

Results Obtained:

In this work we analysed alterations in energy metabolism in CBTs basing on the exome and transcriptome data, as well as immunohistochemistry results. We detected potential driver mutations (PDMs) in several genes (ATRX, BAP1, BRAF, IDH1, IDH2, KMT2D, RET, SDHB, SDHC, and SDHD) reported previously as involved in the development of paragangliomas/pheochromocytomas. We studied association between mutation status of these genes with changes in transcriptome profile. It was found that mutations in KMT2D gene were associated with alterations in energy metabolism in CBTs, including down-regulation of genes involved in the oxidative phosphorylation, overexpression of genes responsible for insulin-dependent glucose import, etc. Mutations in SDHx genes (SDHB, SDHC, and SDHD) were associated with changes in the mechanisms of cell migration regulation, extracellular matrix organisation, angiogenesis, reparation, deregulation in the expression of known cancer-associated genes, etc.

We compared mutation status of SDHx genes (SDHA, SDHB, SDHC, and SDHD) with their expression at mRNA and protein levels. We found, that not all mutations in SDHx genes were associated with the gene expression changes at mRNA or protein levels. In most samples, the expression of SDHx genes at mRNA level was not correlated with their protein expression levels. The most interesting result was down-regulation of SDHB gene in all CBT samples, which had mutations in either SDHB or other SDHx genes (SDHA, SDHC, or SDHD). Analysis of associations between mRNA and protein expression levels, as well as mutation status of SDHA, SDHC, and SDHD genes revealed that in many cases their inactivation could be mediated by the other mechanisms (methylation, miRNA regulation, etc.) than mutations.
We obtained primary CBT cell culture (immortalised with several oncogenes) enriched by supporting cells. Currently, it is going on the experiments to remove supporting cells from the CBT cell culture.

**Results Unforeseen in the Original Project:**
We calculated mutational load (ML) in CBTs. Our results showed ML ranging on the average 0.09-0.28/megabase (Mb) in CBTs. Thus, ML in CBTs is lower than in other common tumours. Additionally, several pathogenic/likely pathogenic somatic and germline variants in both known causative genes and novel genes were identified in CBTs.

**Publications:**


Survey for antimicrobials effective against carbapenem-resistant Gram-negative bacteria

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Abstract: The 16S rRNA gene-based metagenomics revealed impact of human population to diversity of bacterial communities. Shotgun metagenomics revealed that Plav Lake metagenome was the richest concerning potential antimicrobial biosynthetic gene clusters (BGC). AntiSMASH analysis identified 49, 33 and 39 secondary metabolite BGCs in metagenomes. Detected antibiotic resistance genes could be classified into several groups: genes coding efflux pumps of RND superfamily and blaTEM, tetc, aacA, aadA, catA1 and ermB gene. RT-qPCR confirmed and quantified seven of these genes. Functional metagenome libraries were constructed successfully. The presence of antimicrobial compounds active against multidrug resistant Gram-negative bacteria was screened against carbapenem-resistant clinical isolates from laboratory collection. We selected four fosmid clones, which were active against Klebsiella pneumoniae and/or E. coli. Two of those were clones from Plav Lake metagenome, one from Black Lake metagenome, and one from Black Lake metagenome. However, the production of antimicrobial components was unstable. Five hundred colonies of cultivable bacteria from sediments were selected basing on morphological differences. Eight isolates showed inhibitory activity against tested pathogenic bacteria. Isolates were identified as Paenibacillus terrae, P. peoriae, Bacillus pumilus and P. polymyxa.

Objectives:

Traditional sources of novel antibiotics are overexploited and we are now aware that novel ecological niches must be exploited and screening should be expanded towards other bacterial species as potential antibiotic producers. Sediments of glacial lakes from Western Balkans are unexplored natural treasure and represent a challenge regarding their microbial genomic and metabolic potential. Therefore, we thought that exploiting their microbial potential is of crucial importance. During the project we aimed to analyse sediments of three glacial lakes selected due to possible anthropogenic influences, ranging from lakes with minimal anthropogenic impact to that with significant anthropogenic impact. In order to get the full insight into these microbial communities and their genetic potential, both microbiological and metagenomics approaches were planned to be used for analyses. The main objectives were: (i) isolation of cultivable bacteria that produce antimicrobial compounds; (ii) 16S rRNA gene-based metagenomics of sediments in order to get insight into bacterial communities and their diversity, as well as to assess anthropogenic impact to changes in bacterial diversity; (iii) shotgun metagenomics to get insight into genomic potential of analysed metagenomes considering both antimicrobial molecules production and antibiotic resistance genes; (iii) functional metagenomics analyses in order to detect novel antimicrobials with unknown genetic determinants of production; (iv) to asses potential of laboratory collection of lactic acid bacteria (LAB) against carbapenem-resistant Gram-negative pathogens and, finally (v) to isolate and characterise novel antimicrobial compound from any of previously mentioned approaches active against carbapenem-resistant Gram-negative bacteria.

Results Obtained:

Sediment samples were collected from three glacial lakes: Plav Lake (42.595833° N 19.925000° E), Black Lake (43.143333° N 19.087500° E) and Donje Bare Lake (43.318333° N 18.630833° E). In general, sediment from the Black Lake was carbonate and alkaline, with low levels of TOC and TN and with low amounts of nitrogen, phosphorous and potassium. Sediment from the Plav Lake was also carbonate but neutral, with medium content of TOC and TN with low levels of nitrogen, potassium and especially phosphorous. Sediment from the Donje Bare Lake was non-carbonate, slightly acidic, with medium content of TC and low TN content. The pH values ranged from 6.52 to 8.67 and 6.32 to 8.43 (soil to water or KCl, respectively). Average concentration of TN in analysed samples ranged from 0.042 to 0.301%. The sediment from the Plav Lake had almost four times higher NH4-N concentration comparing to other samples (values ranged between 7.0 and 25.9 mg/kg). Total organic carbon varied from 0.452 to 2.813%, with sample from the Black lake that had significantly lowest value comparing to other samples. Phosphorous was the most abundant in the sediment from Black Lake (3.1 mg/100g). Calcium carbonate was not detected in sediment from the Donje Bare Lake, and content in other samples varied from 31.1 to 51.6%.

To determine the bacterial communities composition from in sediments selected glacial lakes 454-pyrosequencing technology was used. The Shannon index of the Plav Lake sediment was the lowest compared with the other samples. The Simpson index was also lowest in this sample. The highest number of unique OTUs was present in sediment of the Black Lake (388), while sediment from the Plav Lake was the poorest in unique OTUs (169).
20 different bacterial phyla were identified across the three sediment samples (Black Lake 14, Plav Lake 15 and Donje Bare Lake 19). The raw data of all libraries generated during this study is publicly available at the Sequence Read Archive (SRA) portal of NCBI under accession number SRP099879. For Plav Lake sediment: sample SRS1961325, experiment SRRX2542175, run SRR5234824. For Black Lake: sample SRS1961324, experiment SRRX2542174, run SRR5234823. For Donje Bare Lake sediment: sample SRS1961323, experiment SRRX2542173, run SRR5234822. Shotgun sequencing of sediment metagenomes was performed on HiSeq2000 2x100bp sequencing platform. Identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in sequenced metagenomes were performed using the antibiotics and secondary metabolite analysis shell – antiSMASH. AntiSMASH analysis of sequenced metagenomes identified 49, 33 and 39 secondary metabolite BGCs in metagenomes from Plav Lake, Black Lake and Donje Bare Lake respectively for a broad range of different molecule classes including non-ribosomal peptides (NRPS), polyketides (PKS), bacteriocins and others. Shotgun metagenomes sequences data derived from bacterial communities from sediments of Western Balkan glacial lakes were submitted to GenBank database at NCBI. The sediment metagenome whole genome shotgun (WGS) project from Plav Lake has the project accession PDVJ0000000. The first version of the project (01) has the accession number PDVJ01000000, and consists of sequences PDVJ01000001 - PDVJ01092463 (https://www.ncbi.nlm.nih.gov/Traces/wgs/?val=PDVJ01#contigs).

Sediment metagenome from Black Lake is available under accession number PDVI01000000, and consists of sequences PDVI01000001-PDVI01028961 (https://www.ncbi.nlm.nih.gov/Traces/wgs/?val=PDVI01#contigs), while metagenome sequences from Donje Bare Lake sediments can be reached under accession number PDHV01000000, and consists of sequences PDHV01000001-PDHV01074614 (https://www.ncbi.nlm.nih.gov/Traces/wgs/?val=PDHV01#contigs). Identified genetic determinants encoding for antimicrobial compounds enabled us selection, PCR amplification using total metagenome DNA and cloning of those using different expression systems (please see below). Additionally, metagenomes were screened for the presence of the genetic determinants of antibiotic resistance using The Comprehensive Antibiotic Resistance Database (CARD, https://card.mcmaster.ca/), Antibiotic Resistance Genes Database (ARDB, https://ardb.cbcb.umd.edu/); integrons and CALLIN elements using Integron Finder and insertion elements using IS Finder (https://www.is.biotoul.fr/). Detected resistance genes could be classified into several groups: genes coding efflux pumps of Resistance-Nodulation-Division (RND) superfamily: mexF, mexY, mexB, mexD, mexI, smeB, smeE, amrB, cebB, acrD, mdtB, mdtF and blaTEM, tetC, aacA, aadA, catA1 and ermB gene. Complete integrin was detected only in metagenome from the Plav Lake. The total number of detected CALIN elements with two or more attC sites was 38: 20, 4 and 14 from Plav Lake, Black Lake and Donje Bare Lake respectively. RT-qPCR method was used for detection and quantification of antibiotic resistance and integrate genes in three sampled sediments. For all three lakes was confirmed presence of seven genes (blaTEM, catA1, ermB, mexA, mexB, intI1 and intI3). In order to screen the metagenomes for antimicrobial compounds that could not be detected by bioinformatics metagenome mining, we constructed functional metagenome libraries. We selected a total of 15 360 fosmid clones, 5 760 for Plav Lake metagenome, 5 376 for Black Lake metagenome and 4 224 for Donje Bare Lake metagenome. The presence of antimicrobial compounds active against multidrug resistant Gram-negative bacteria was screened in Luria agar plates with clinical isolates from laboratory collection: Pseudomonas aeruginosa MMM83, Klebsiella pneumoniae IT977, Acinetobacter baumannii 6077/12, Achromobacter xylosoxidans FB2 and Escherichia coli IMD989. We selected four fosmid clones, which were active against Klebsiella pneumoniae and/or E. coli. Two of those were clones from Plav Lake metagenome, one from Black Lake metagenome, and one from Black Lake metagenome. We experienced troubleshooting with the stability of the antimicrobial phenotype thus we were unable to further analyse these clones. In order to isolate and characterise cultivable bacteria from glacial lake sediments moderately low-nutrient media DR2A with cycloheximide was used. Five hundred colonies were selected basing on morphological differences. All isolates were tested for antimicrobial activity against multiresistant pathogenic clinical isolates Pseudomonas aeruginosa MMM83, Klebsiella pneumoniae IT977, Acinetobacter baumannii 6077/12, Achromobacter xylosoxidans FB2, Escherichia coli. For detection of antimicrobial activity, an agar well diffusion assay was used. Eight isolates showed inhibitory activity against tested pathogenic bacteria. Among them, seven were isolated from Plav Lake (PL9, PL57, PL61, PL75, PL79, PL101, PL120) and one was isolated from Donje Bare Lake (DBL42). According to the results, isolates PL9 and DBL42 were identified as Paenibacillus terrae, PL57 as a P. peoriae, PL61 and PL75 as a Bacillus pumilus, PL101 and PL120 as a P. polymyxa.

Analysis of the laboratory collection of Lactic Acid Bacteria (LAB) for the producers of antimicrobial peptides active against multidrug-resistant Gram-negative bacteria did not result in any active strain. 115 strains were selected on the base of their origin and characteristics, of which 85 were Enterococcus spp. strains and 30 were Lactococcus lactis strains. None of the analysed strains showed activity against carbapenem-resistant clinical isolates.

Publications:
Title: Application of nanobody technology in the diagnosis of Leishmaniasis in Syria

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Abstract: Cutaneous leishmaniasis (CL), caused by unicellular leishmania parasites, is a very old endemic disease and one of the most important public health problems aggravated by the Syrian crises. Antibodies have long been implicated in the identification and isolation of leishmania antigens beside their role in the detection and treatment of the pathogens. Recombinant single fragments of antibodies, such as nanobodies extrapolated from the camel heavy chain antibodies (HCAbs), have even greater potentials in biomedicine owing to their outstanding physicochemical properties.

The aim of this project was to prepare specific nanobodies against *L. tropica*, which is a predominant species known to cause CL in the region. Therefore, a standard camel immunisation procedure was carried out using *L. tropica* cells, followed by assessing the solicited immune response and the active participation of HCAbs in this response by ELISA. A relatively large nanobody “immune” library of 5 × 108 individual transformants, with 96.5% positivity, was prepared from the blood of the immunised camel. Phage display biopanning on this nanobody library resulted in the isolation of five anti *L. tropica* specific nanobodies (NbLt05, 06, 14, 24, 36). These nanobodies were more specific to *L. tropica* than to *L. major*, but could recognise equally the lysate and the outer surface of the intact cells as well as the two main stages of the parasite, promastigotes and amastigotes. In addition, nanobodies, either when they are in the free or phage–displayed forms, recognised several leishmanias proteins (majorly between 75 and 63 kDa), speculating their target to be the metalloprotease gp63 immonodominant surface antigen of leishmania.

Interestingly, and because of the important role of gp63 in leishmania life cycle, all these nanobodies have a negative effect on the infectivity of *L. tropica*, as they decreased the number of infected macrophages as well as of amastigotes inside those macrophages. The outstanding characteristics of anti-leishmania nanobodies could be of a great use in the diagnosis of this pathogen in the blood and patient’s tissues, along with the potential applications in the development of new treatment against leishmaniasis.

Objectives:

During the project, we tried to achieve several objectives:

(i) Isolation of PCR confirmed leishmania local strains;
(ii) Preparation of leishmania vaccines for camel immunisation;
(iii) Evaluation of the immune response against Leishmania;
(iv) Purification of different camel IgG subclasses and assessing their participation in the raised immune response;
(v) Construction of nanobody “immune” cDNA library using pMES4 phagemid in *E. coli*;
(vi) Retrieving specific anti-*L. tropica* nanobodies from the library using phage display technology;
(vii) Testing phage–displayed nanobodies (phage-Nbs) for interaction with Leishmania antigens;
(viii) Sequence determination of the new anti-*L. tropica* nanobodies;
(ix) Protein expression of nanobodies in *E. coli* strain WK6;
(x) Protein purification of nanobodies using affinity chromatography;
(xi) Characterisation of new nanobodies for interaction with *L. tropica* antigens using immunoblotting and ELISA;
(xii) Testing nanobodies against several local strains of *L. tropica*.

Results Obtained:

(i) Assessing camel immunisation with leishmania

Leishmania local strains were collected directly from skin scars of the patients and tested by microscopy on slides and confirmed by PCR. An adult Arabian one-humped female camel was used for immunisation with five doses of leishmania vaccine given by biweekly subcutaneous injections. A solid phase ELISA, of the blood samples collected at several time points from the start of immunisation over a period of 70 days samples, revealed a raise of an important and specific immune response toward both species of leishmania, *L. tropica* and *L. major*.

IgG subclasses from immunised camel serum were fractionated by differential purification in order to determine the importance of each IgG fraction in the developed immune response against *L. tropica*.

(ii) Construction of anti-*L. tropica* nanobody library
Starting with 50 ml of blood sample collected from immunised camel at day 42, VHH genes were amplified by PCR, digested with PstI/BstEII and then ligated in pMES4 phagemid. The freshly ligated nanobody-vector constructs were electro-transformed in E. coli TG1 cells to obtain a large library of $3 \times 10^8$ individual colonies, with 95.6 % of positivity and > 90 % of diversity.

(iii) Phage display panning of nanobody library
A standard phage display biopanning procedure was followed to isolate specific nanobodies against the intact parasites of *L. tropica*. Enrichment with specific anti-*L. tropica* phage-Nbs was determined by polyclonal phage-ELISA test using the rescued phage particles from the primary library and after each round of panning. Eluted phage-Nbs were used to re-infect exponentially growing TG1 cells. Five positive colonies of TG1 cells from the previous phage-ELISA experiment were selected, named NbLt05, 06, 24 and 36, resulted in a distinct RFLP profile when their genes were amplified by PCR from pMES4 plasmid in their colonies then digested with HinfI.

(iv) Characteristics of anti-*L. tropica* specific nanobodies
Plasmid constructs of pMES4 containing the different five nanobodies were prepared from the positive colonies of *E. coli* TG1 cells and sent for sequencing. Aligning the predicted amino acid (AA) sequences confirmed the difference between these nanobodies especially within the three CDRs of the structure. Protein characteristics of these nanobodies were determined after expression as soluble recombinant proteins using different immunological methods.

(v) Testing the reactivity of anti-*L. tropica* nanobodies by immunoassays
Leishmania electrophoresed proteins were blotted on nitrocellulose membrane then detected with each of the nanobodies or the rabbit anti-*L. tropica* antibody as positive control. All five nanobodies detect immunogenic proteins (63, 57 kDa). The reactivity of soluble nanobodies with *L. tropica* (cells, lysate, and two main stages; promastigote and amastigote) was also confirmed by ELISA and compared with anti *L. major* nanobody (NbLm01) as a negative control.

(vi) The effect of nanobodies on leishmania infection
Treatment of leishmania with the different nanobodies decreased the number of infected macrophages, between (30 – 40 %), and the number of amastigotes per macrophage, as found by microscopic examination. These results were confirmed through detection of parasitic DNA inside the infected cells.

In conclusion, the widespread of leishmania in Syria and the absence of effective methods for treatment, together with the great potentials of camel nanobodies in different research and medical fields, were all the motivation of this project. The specific anti-leishmania nanobodies isolated in this work showed reactivity towards two different species of leishmania (*L. tropica* and *L. major*) using several immunological approaches, making them useful as universal leishmania molecular markers. One immunodominant antigen (gp63) could be among their recognised target antigens, which could explain their failure in distinguishing between the two major stages of the parasite (promastigotes and amastigotes). In addition, these nanobodies showed undeniable capacity to affect leishmania infectivity into macrophages, inspiring a possible application in the treatment of leishmaniasis.
TUNISIA

Title: Characterisation of mycorrhizal symbiotic interface in prickly pear for enhancing growth and stress tolerance of economically important-Mediterranean crops

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Abstract: Soil bacteria and fungi are largely responsible for key ecosystem services, including soil fertility and climate regulation. The specie Opuntia ficus indica is considered one of the most known natural tolerant plants widely planted in dry lands. However, the associations Opuntia cacti establish with microorganisms and the rules governing microbial community assembly, remain poorly understood. Microbial diversity was investigated using cultivation-independent and high-throughput sequencing approaches. Soil and roots samples were collected from cactus following an increased aridity degree. Bacterial and fungal sequencing analysis showed that the microbial profiles were highly influenced by the bioclimatic stages and soil physicochemical proprieties. The effectiveness of the combined spiny-spineless cacti inoculum was evaluated by inoculating grapevine rootstocks Ruggeri 140 treated with salt, drought and phosphorus deficiency. And thus it allowed the identification of the involved specific-stress microbial communities. Microbial correlation networks analysis showed that specific-stress cacti microbes are highly correlated to soil parameters, suggesting their beneficial synergic role for enhancing stress tolerance. These findings underlined that natural tolerant plants’ local microbes could be a promising bio-fertiliser to develop a sustainable agriculture.

Objectives:
The main objective was the characterisation of the cactus/specific stress-microbes association mechanism under adverse conditions and its microbial symbionts effects for enhancing the tolerance of Mediterranean economic plants to abiotic stress.

Results Obtained:
(i) Identification of bacterial profile associated to the rhizosphere (soil) and roots of spineless Opuntia ficus indica collected following increased aridity degree in Tunisia;
(ii) Identification of fungal profile associated to the rhizosphere (soil) and roots of spineless Opuntia ficus indica collected following increased aridity degree in Tunisia;
(iii) Microbial characterisation difference between the two types: spiny and spineless cacti in the bioclimatic upper-arid stage in Tunisia;
(iv) Microbial correlation networks of Opuntia ficus indica associating bacterial or fungal OTUs and soil physico-chemical parameters under increased aridity degree;
(v) Effectiveness of the Opuntia ficus indica microbial consortia for enhancing the tolerance of grapevine rootstocks 140RU to abiotic and nutritive stresses;
(vi) Identification of prokaryotic and eukaryotic microbial profiles specifically involved in salinity, drought and phosphorus deficiency stresses and named in this project as specific-stress communities;
(vii) Effectiveness of two rhizospheric desert shrubs (Ziziphus lotus and Ephedra altissima) associated to Opuntia ficus indica plantations for enhancing the drought tolerance of tomato plants.

Results Unforeseen in the Original Project:
The significant difference of microbial profile structures between the spiny and the spineless cacti types, unless they are naturally growing in the same field spots/same bioclimatic stage and occurring the same soil physico-chemical proprieties. Bacteria community is host-dependent in each compartment (soil or root). However, fungi community is compartment-dependent.
Abstract: The primarily therapeutic modality for many hematopoietic disorders is bone marrow transplantation, which relies on the ability of a small number of hematopoietic stem cells (HSCs) to repopulate all blood lineages. We have recently demonstrated that HSCs pool is expanded in vivo following stem cell specific knockout of hematopoietic factors such as Meis1 and Hif-1α. We have shown that deletion of such HSC quiescence regulators in HSC compartment not only leads to cell cycle entry but also HSC expansion. Thus, targeting of HSC quiescence regulators by RNA interference may provide valuable tools for *ex vivo* and *in vivo* expansion of HSCs.

Objectives:
We aimed to develop hematopoietic stem cell expansion technologies based on HematoMiRs, namely miRNA-like targeting approaches, to modulate hematopoietic factors involved in hematopoietic stem cell expansion and self-renewal. To this end, we analysed about 200 genes that are specifically knockout in HSCs *in vivo* and determined the loss-of-function phenotypes of genes. 57 of these genes following loss-of-function showed at least 1.5 fold expanded stem cell pool or cell cycle activity (loss of quiescence). In addition, using prediction algorithms, we have identified 8 seed sequences (namely HematoMiRs) targeting 3′ UTR of these hematopoietic factors.

Results Obtained:
We determined the effect of these 8 different proHematoMiRs targeting over 57 HSC quiescence regulators. We have found intriguing results in terms of upregulation of phenotypical HSC surface antigens post HematoMiR treatments. HematoMiR #2 and #5 treatments particularly led to the robust increase in LSKCD34low HSC population. In addition, we have found that HematoMiR #2 and #5 treatments had broadest downregulation of their predicted target genes in HSC quiescence regulators. Furthermore, we have seen that HematoMiR #2 and #5 had robust downregulation of CDKIs during course of 5 day HSC expansion. In addition, we have addressed the possibility of determining a robust HSC expansion procedure by mixing HematoMiRs in various combinations. We have tested All HematoMiRs together and by excluding one-by-one. We have found that removal of HematoMiR-2, HematoMiR-5, HematoMiR-3, HematoMiR-1, and HematoMiR-8 leads to lower HSPCs content analysed by flow cytometer. This result indicates that HematoMiR-2, HematoMiR-3, HematoMiR-5, HematoMiR-1 and HematoMiR-8 are required in the mixture for optimum HSC expansion. On the other hand, removal of HematoMiR-6 and HematoMiR-7 leads to higher HSC content, which indicates that these two HematoMiRs are dispensable in the final mixture of HematoMiRs. Besides, we determined the effect of various mixture combinations of selected HematoMiRs comparing to all 8 of them together and each of them separately. Triple and different binary combination of selected HematoMiR-2, 5.1 treatment showed the increased HSPCs population. HematoMiR mixture (HM-MIX) including HematoMiR-2 and HematoMiR-5 treatment robustly increased the LSK cell population. We have studied HematoMiR-5 and HM-MIX-2.5 in HSC cell cycle, apoptosis, CFU assays post HematoMiR-5 and HM-MIX-2.5 treatment, human HSC expansion, engraftment and repopulation of HematoMiR-5 and HM-MIX-2.5 treated HSCs *in vivo*. HematoMiR-5 and HM-MIX-2.5 treatment led to increased progression into G0 and S phase of cell cycle, no change in apoptosis rates of HSCs, increased human HSC expansion post HematoMiR-5, and better engraftment capacity of transplanted HSCs into immunocompromised animals.

Results Unforeseen in the Original Project:
We have developed new HSC expansion strategies based on HematoMiRs by targeting over 50 different hematopoietic factors. Overall, these studies suggest that HematoMiR-5 and the combination of HematoMiR-2 and HematoMiR-5 (HM-MIX-2.5) treatment could allow robust HSC expansion *ex vivo*. Targeting quiescence factors with these HematoMiRs treatment is able to induce HSCs proliferation and their cycling ability without leading to loss of self-renewal and apoptotic issue. We have showed that developed HematoMiRs are effective in human HSC expansion as well.

Publications: