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ICGEB Research Grants Programme

RESEARCH GRANTS COMPLETED IN 2020

CRP - ICGEB RESEARCH GRANTS COMPLETED IN 2020

COUNTRY	PRINCIPAL INVESTIGATOR	PROJECT TITLE
ARGENTINA	José M. ESTEVEZ	Masters molecular regulators of polarized growth in plant cells
BRAZIL	Renata DE MEIRELLES SANTOS PEREIRA	The role of PRC2 in controlling T exhaustion during HIV infection (*Early Career Return Grant*)
CHILE	Eugenia MORSELLI	Autophagy, primary cilium and inflammation in neurons and astrocytes
CHINA	XiangBo WAN	Identification of novel hypoxia induced radioresistance signaling for endemic nasopharyngeal carcinoma (*Early Career Return Grant*)
COSTA RICA	Elías BARQUERO-CALVO	Regulation of adaptive immunity during a Brucellosis infection
CROATIA	Vjekoslav TOMAIC	Biological factors determining Human Papillomavirus (HPV) driven carcinogenesis (*Early Career Return Grant*)
CROATIA	Vlatka ZOLDOŠ	Comprehensive toolbox for epigenetic modulation of gene expression
HUNGARY	Zoltán WIENER	Exosomes in the inflammatory intestinal stem cell niche (*Early Career Return Grant*)
MALAYSIA	Woei Yenn TONG	Mode of action of a new antibiotic Phomopsidione on *Pseudomonas aeruginosa*
MEXICO	Lorena AGUILAR ARNAL	Metabolic regulation of chromatin dynamics in obesity (*Early Career Return Grant*)
MEXICO	Juan Ernesto LUDERT	Antibody facilitated Zika virus infection of Hofbauer cells and study of the mechanisms used by the virus to cross the transplacental barrier
NIGERIA	Marycelin Mandu BABA	Establishment of a laboratory facility for the diagnosis and surveillance of Arbovirus infections in Nigeria
PERU	Juan LOPEZ	Molecular mechanism of *T. cruzi* virulence factors (*Early Career Return Grant*)
POLAND	Joanna JAZOWIECKA-RAKUS	Mesenchymal stem cells and myxoma virus in oncolytic melanoma therapy (*Early Career Return Grant*)
ROMANIA	Violeta RISTOIU	Iba-1 (+) macrophages contribution to peripheral neuropathic pain development
SERBIA	Lidija SENEROVIC	New synergistic strategy to treat chronic wound infections
SLOVENIA	Boris TURK	Lysosomal proteases in semaphorin signaling and cell polarity

ARGENTINA

Title: Masters molecular regulators of polarised growth in plant cells
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Abstract: Both pollen tubes and root hairs are single-plant cells that growth in a polar manner and both play crucial roles in the survival of plants either in fertilisation and seed formation or in acquisition of nutrients and water. This project proposes to study the polar-growth of both cell types in the model plant *Arabidopsis thaliana*. Polar-cell growth is maintained by coordinated regulators that control calcium ions (Ca^{2+}) flow, reactive oxygen species (ROS) and pH oscillations. Our main goal is to identify Ca^{2+} transporters-channels localised in plasma membrane (CaC) and intracellular organelles involved in the development of growing root hairs and pollen tubes. To this end, we plan to use pharmacological inhibitors, genetic tools as well as fluorescent biosensors coupled to advance Fluorescent Microscopy highly compatible to root hair and pollen tube single-cell imaging. The knowledge acquired would have a great impact in the inorganic Phosphorus (Pi) uptake in poor soils, under salt- and water-stress conditions. It is predicted that world resources of inexpensive Pi may be depleted by ~2050. Consequently, improving plant's ability to efficiently use Pi is vital to developing more sustainable agriculture. ICGEB award will help to fully develop and expand my research perspective. Giving the resources and the freedom necessary will allow me to take all the risk required to make truly-innovative research.
Objectives:
(i) Identifying Ca^{2+} transporters involved in the generation of gradient producing Ca^{2+} and ROS that regulate the polarised growth of plant cells, in root hairs and pollen tubes; (ii) Characterise absolute levels of cytoplasmic Ca^{2+}, ROS production, and pH using fluorescent reporter probes such as Cameleon YC3.6, DCF, roGFP, pHFluorin and PRpHluorin in root hairs and pollen tubes; (iii) Characterise the surface cell wall integrity sensors of LRX and PERK types in pollen tubes. In this project we plan to discover the molecular mechanisms that maintain polarised growth with emphasis in the gaps of our understanding of how Ca^{2+}-ROS-pH complex network is coordinated for its correct function.
Results Obtained:
<u>(i) Root hair model</u>
(ia) How Auxin and ROS are connected at the molecular level. Root hair development varies by plant species; it either occurs randomly, starting with an asymmetrical cell division, or via a position-dependent mechanism. The latter mechanism is better studied and occurs in the model plant Arabidopsis thaliana, where root hair cells or trichoblasts and non-hair cells or atrichoblasts differentiate from the epidermal cell layer. A well-defined developmental program and multiple environmental signals coupled to several hormones are integrated to define the final size of root hairs. Root hair size has vital physiological implications for the plant, determining the surface area/volume ratio of the whole roots exposed to the nutrient pools, thereby likely impacting nutrient uptake rates. We have obtained results that show that ROS production is controlled by the transcription factors RSL4, which in turn is transcriptionally regulated by auxin through several Auxin Responsive Factors (ARFs). In this manner, auxin controls ROS-mediated polar growth by activating RSL4, which then upregulates the expression of genes encoding NADPH oxidases (also known as RBOHs, RESPIRATORY BURST OXIDASE HOMOLOG proteins) and Class-III Peroxidases (PER), which catalyse ROS production. Chemical or genetic interference with the ROS balance or peroxidase activity affect root hair final cell size. Overall, our findings establish a molecular link between auxin regulated ARFs-RSL4 and ROS-mediated polar root hair growth. These results were recently published in Mangano et al. (2017).
<u>(i) Root hair model</u>
(ib) How conflicting signals such as Auxin and low-phosphate affect cell growth. Root hair size determines the surface area/volume ratio of the whole roots exposed to the nutrient and water pools, thereby likely impacting nutrient and water uptake rates. The speed at which they grow is determined both by cell-intrinsic factors like hormones (e.g., auxin) and external environmental signals like nutrient availability in the soil (e.g., phosphate). Overall root hair growth is controlled by the transcription factors RSL4 and RSL2. While high levels of auxin promote root hair growth, high levels of inorganic phosphate (Pi) in the media are able to strongly repress RSL4 and RSL2 expression linked to a decreased polar growth. In this work, we inquired the mechanism used by root hairs to integrate conflicting growth signals like the repressive signal of high Pi levels and a concomitant high auxin exposure that promotes growth and questioned whether these complex signals might activate known molecular players in root hair polar growth. Under these conditions, RSL2 expression (but not RSL4) is activated linked to ROS production and root hair growth. On the

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other hand, by blocking ROS production derived from the NADPH Oxidase C (or RBOHC for RESPIRATORY BURST OXIDASE HOMOLOG C) and ROS production from Secreted type-III Peroxidases (PERs), it was possible to repress the auxin growth-promoting effect. This study (Mangano et al. 2018) identifies a new layer of complexity between auxin, Pi nutrient availability and RSL2/RSL4 transcription factors all acting on ROS homeostasis and growth at the root hair level.

(i) Root hair model

(ic) How pH might control oscillating polar growth. Polar growth in root hairs and pollen tubes is an excellent model for investigating plant cell size regulation. While linear plant growth is historically explained by the acid growth theory, which considers that auxin triggers apoplastic acidification by activating plasma membrane P-type H+-ATPases (AHAs) along with cell wall relaxation over long periods, the apoplastic pH (apopH) regulatory mechanisms are unknown for polar growth. Polar growth is a fast process mediated by rapid oscillations that repeat every 20–40 seconds. In a revision article (Martinez Pacheco et al. 2018), we explore a reactive oxygen species (ROS)-dependent mechanism that could generate oscillating apopH gradients in a coordinated manner with growth and Ca^{2+} oscillations. We propose possible mechanisms by which apopH oscillations are coordinated with polar growth together with ROS and Ca^{2+} waves.

(ii) Pollen tube model

(iia) Ca^{2+} dynamics, pharmacological inhibition of Ca^{2+} channels and polar growth. Pollen tube growth requires coordination between the tip-focused cytoplasmic calcium concentration ([Ca2+]cyt) gradient and the actin cytoskeleton. This $[Ca^{2+}]$cyt gradient is necessary for exocytosis of small vesicles, which contributes to the delivery of new membrane and cell wall at the pollen tube tip. The mechanisms that generate and maintain this $[Ca^{2+}]$cyt gradient are not completely understood. Here, we have studied calcium dynamics in tomato (Solanum lycopersicum) pollen tubes using transgenic tomato plants expressing the Yellow Cameleon 3.6 gene under the pollen-specific promoter LAT52. We use tomato as an experimental model because tomato is a Solanaceous plant that is easy to transform, has an excellent genomic database and genetic stock center and unlike Arabidopsis, tomato pollen is a good system to do biochemistry. We found that tomato pollen tubes showed an oscillating tip-focused $[Ca^{2+}]$cyt gradient with the same period as growth. Then, we used a pharmacological approach to disturb the intracellular Ca^{2+} homeostasis, evaluating how the $[Ca^{2+}]$cyt gradient, pollen germination and in vitro pollen tube growth were affected. We found that cyclopiazonic acid (CPA), a drug that inhibits plant PIIA-type Ca^{2+}-ATPases, increased $[Ca^{2+}]$cyt in the subapical zone, leading to the disappearance of the Ca^{2+} oscillations and inhibition of pollen tube growth. In contrast, 2-aminoethoxydiphenyl borate (2-APB), an inhibitor of Ca^{2+} release from the endoplasmic reticulum to the cytoplasm in animals cells, completely reduced $[Ca^{2+}]$cyt at the tip of the tube, blocked the gradient and arrested pollen tube growth. Although both drugs have antagonistic effects on $[Ca^{2+}]$cyt, both inhibited pollen tube growth triggering the disappearance of the $[Ca^{2+}]$cyt gradient. When CPA and 2-APB were combined, their individual inhibitory effects on pollen tube growth were partially compensated. Finally, we found that GsMTx-4, a peptide from spider venom that blocks stretch-activated Ca2+ channels, inhibited tomato pollen germination and had a heterogeneous effect on pollen tube growth suggesting that these channels are also involved in the maintenance of the $[Ca^{2+}]$cyt gradient. All these results indicate that tomato pollen tube is an excellent model to study calcium dynamics. These results were recently published in Barberini et al. (2017).

Publications:

Mangano, S., Denita-Juarez, S.P., Choi, H.S., Marzol, E., Hwang, Y., Ranocha, P., Velasquez, S.M., Borassi, C., Barberini, M.L., Aptekmann, A.A., Muschietti, J.P., Nadra, A.D., Dunand, C., Cho, H.T., Estevez, J.M. Molecular link between Auxin and ROS-mediated polar growth. 2017. Proc. Natl. Acad. Sci. USA. **114(20)**, 5289-5294

Marzol, E., Borassi, C., Juárez, S.P.D., Mangano, S., Estevez, J.M. RSL4 takes control: Multiple signals, one transcription factor. 2017. Trends Plant. Sci. **22(7)**, 553-555

Møller, S.R., Yi, X., Velásquez, S.M., Gille, S., Hansen, P.L.M., Poulsen, C.P., Olsen, C.E., Rejzek, M., Parsons, H., Yang, Z., Wandall, H.H., Clausen, H., Field, R.A., Pauly, M., Estevez, J.M., Harholt, J., Ulvskov, P., Petersen, B.L. Identification and evolution of a plant cell wall specific glycoprotein glycosyl transferase, ExAD. 2017. Sci. Rep. **7**, 45341

Xue, H., Veit, C., Abas, L., Tryfona, T., Maresch, D., Ricardi, M.M., Estevez, J.M., Strasser, R., Seifert, G.J. *Arabidopsis thaliana* FLA4 functions as a glycan-stabilised soluble factor via its carboxy-proximal Fasciclin 1 domain. 2017. Plant J. **91(4)**, 613-630

Barberini, M.L., Sigaut, L., Huang, W., Mangano, S., Juarez, S.P.D., Marzol, E., Estevez, J.M., Obertello, M., Pietrasanta, L., Tang, W., Muschietti, J. 2017. Calcium cytoplasmic reservoirs contribute to maintain the calcium gradient necessary for *in vitro* tomato pollen tube growth. 2018. Plant Reprod. **31(2)**, 159-169

Juan Ignacio Cagnola, J.I., Dumont de Chassart, G.J., Ibarra, S.E., Chimenti, C., Ricardi, M.M., Delzer, B., Ghiglione, H., Zhu, T., Otegui, M.E., Estevez, J.M., Casal, J.J. Reduced ARABINOGALACTAN PROTEIN abundance anticipates stress-induced kernel abortion in field crops of maize. 2018. Plant Cell. Environ. **41**, 661-674

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Mangano, S., Denita-Juarez, S.P., Marzol, E., Borassi, C., Estevez, J.M. High auxin and high Pi impact on RSL2 and ROS-linked root hair growth in Arabidopsis. 2018. Front. Plant Sci. 9, 1164
Mangano, S., Martínez Pacheco, J., Marino-Buslje, C., Estevez, J.M. How does pH fit in oscillating polar growth? 2018. Trends Plant Sci. **23(6)**, 479-489
Marzol, E., Borassi, C., Bringas, M., Sede, A., Rodríguez Garcia, D.R., Capece, L., Estevez, J.M. Filling the gaps to solve the Extensin puzzle. 2018. Mol. Plant. **11(5)**, 645-658
Sede, A.R., Borassi, C., Weinger, D., Mecchia, M.A., Estevez, J.M., Muschietti, J.P. Multiple Arabidopsis pollen extensin-like (PEXs) proteins are needed for cell wall integrity during pollen tube growth. 2018. FEBS Letters **592(2)**, 233-243
Silva-Sanzana, C., Estevez, J.M, Blanco-Herrera, F. Influence of cell wall polymers and their modifying enzymes during plant-aphid interactions. 2019. J. Exp. Bot. erz550
Borassi, C., Gloazzo Dorosz, J., Ricardi, M.M., Fachin, L.P., Carignani, M., Marzol, E., Mangano, S., Rodríguez Garcia, D.R., Martínez Pacheco, J., Rondón Guerrero, J.C., Velasquez, S.M., Villavicencio, B., Ciancia, M., Seifert, G., Verli, H., Estevez, J.M. A cell surface O-glycosylated peptide modulates root hair cell fate. 2020. New Phytologist, doi: 10.1111/nph.16487
García Bossi, J., Kumar, K., Barberini, M.L., Domínguez, G.D., Rondón Guerrero, Y.D.C., Marino-Buslje, C., Obertello, M., Muschietti, J.P., Estevez, J.M. Functions of Plant P-type IIA ECA and P-type IIB ACA Ca2+-ATPases in growth and development. 2020. J. Exp. Bot. **71(4)**, 1239-1248
Li, L., Li, B., Xie, C., Zhang, T., Borassi, C., Estevez, J.M., Li, X., Liu, X. Arabidopsis RAD23B regulates pollen development by mediating KRP1 degradation. 2020. J. Exp. Bot. eraa167 (in press)
Sede, A., et. al., Estevez, J.M., Muschietti, J.P. Imaging and analysis of the content of callose, pectin and cellulose in the cell wall of Arabidopsis pollen tubes grown *in vitro*. 2020. Methods in Molecular Biology (in press)
Zhu, S., Estévez, J.M., Liao, H., Zhu, Y., Yang, T., Li, C., Wang, Y., Li, L., Liu, X., Pacheco, J.M., Guo, H., Yu, F. The RALF1–FERONIA complex phosphorylates eIF4E1 to promote localised protein synthesis and Polar Root Hair Growth. 2020. Mol. Plant. **13(5)**, 698-716
Zhu, S., Martínez Pacheco, J., Estevez, J.M., Yu, F. Autocrine regulation of root hair size by RALF-FERONIA-RSL4 signaling pathway. 2020. New Phytologist, doi: 10.1111/nph.16497

Developing knowledge

Brazil

Title: The role of PRC2 in controlling T cell exhaustion during HIV infection
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Abstract: The immune control of infections or cancers is compromised by the fact that T lymphocytes, during chronic antigen stimulation, display a state of hypo-responsiveness, known as exhaustion. Understanding the molecular mechanisms that govern the commitment towards effector and memory T cells or the state of T cell exhaustion is critical for improving immunotherapy strategies. We previously observed that the expression of Ezh2 (Enhancer of zeste homolog 2) is increased in effector CD8 T cells, whereas its levels are decreased in exhausted cells. Ezh2 is the catalytic subunit of PRC (Polycomb Repressive Complex) 2, responsible for the deposition of the repressive histone modification H3K27me3. PRC2 collaborates with other Polycomb complex, PRC1 (that adds ubiquitin to H2AK119), in chromatin modification and gene regulation. We hypothesised that PRC2 is important for CD8 T cells effector function and counterbalances exhaustion. During the first year of this work, two very important papers confirmed the role of Ezh2 in the differentiation of effector CD8 T cells. The aims of the present project were slightly adjusted to include addressing the role of PRC1 on CD8 T cell differentiation.
Objectives:
(i) To investigate the role of PRC1 proteins in CD8 T cells activation and function, addressing the collaboration between PRC1 and PRC2;
(ii) To understand if PcG proteins modulate effector/memory CD8 T cells differentiation and control early events that determine the exhausted phenotype;
(iii) To investigate the impact of PcG proteins levels in the exhaustion phenotype of CD8 T cells from HIV patients.
Results Obtained:
Initially we showed that Ezh2 deletion not only compromises effector CD8 T cell differentiation during LCMV (Lymphocytic Choriomeningitis Virus) infection, as previously demonstrated, but during protozoa parasite infection (*Trypanosoma cruzi*) in mouse. We extended our analyses for the investigation of PRC1 proteins during infection and cancer. Through *in vivo* experiments with LCMV infection and *in vitro* experiments with CD8 T cell culture we observed that the deletion of a PRC1-component Cbx4, that works in conjunction with Ezh2, induced an increase in memory cell generation, which resembles the phenotype observed for Ezh2 deletion, suggesting that PRC1 and PRC2 complexes can act in collaboration. Additionally, our RNA-seq experiments confirmed the differential expression and enrichment of genes associated with memory phenotype upon deletion of Cbx4. The generation of cells with memory phenotype upon Cbx4 deletion suggested that these cells could be more efficient in immunotherapy protocols against tumour, but CD8 T cells expressing shCbx4 display reduced cytotoxicity capacity and do not control tumour efficiently compared to control cells. Once we determined that PRC1 and PRC2 are critical for effector CD8 T cells generation, we investigated the impact of Ezh2 deletion during chronic antigen stimulation. First, we observed in LCMV model that the exhaustion profile of exhausted cells develops very early during CD8 T cell activation/differentiation, what opposes the common-sense idea that antigen persistence is critical for the acquisition of exhausted phenotype. In that sense, epigenetic modulation could be critical for the early determination of exhaustion and our data showing distinct Ezh2 expression in different CD8 T cell subpopulations reinforces that hypothesis. We observed that Ezh2 deletion is associated with the development of a hypo-responsive phenotype in T cells, even *in vitro*. We then determined the levels of expression of Polycomb genes in HIV patients during different stages of infection from published transcriptomic datasets and observed that reduced levels of Ezh2 (and other PcG genes) are associated to CD8 T cell hypo-responsive phenotype. Our results confirmed that PRC2 (Ezh2) and PRC1 (Cbx4) members are required for effector CD8 T cell generation. Moreover, we show that low levels of Ezh2 are associated to T cell hypo-responsiveness.
Results Unforeseen in the Original Project:
Even tough the deletion of Cbx4 favours the generation of memory cells we did not observe the same effect for other PRC1 members, including the catalytic subunits. These results suggest that distinct PRC1 complexes with different composition may regulate expression of specific and distinct genes upon binding to their loci. Future experiments will be conducted to address this complexity.
Publications:
Pereira, R.M., Rao, A., Hogan, P.G., Martinez, G.J. Transcriptional and epigenetic regulation of T cell hyporesponsiveness. 2017. J. Leukoc. Biol. **102**, 601-615

Developing knowledge

CHILE

Title: Autophagy, primary cilium and inflammation in neurons and astrocytes
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Abstract: Consumption of high fat diets (HFD) rich in saturated fatty acids (SatFAs) and poor in ω3 poly-unsaturated fatty acids (PUFAs) is growing worldwide and is associated with the exponential increase in the rates of obesity and obesity-associated dysfunctions. Growing evidence indicates excessive hypothalamic accumulation of SatFAs, accompanied by the decrease in PUFAs drives to insulin resistance, the central feature of obesity-related metabolic dysfunctions.
Chronic exposure to HFD dysregulates hypothalamic autophagy, a cellular process required to maintain cellular and tissue homeostasis, promoting obesity, and insulin resistance. Autophagy regulates primary cilia formation, or ciliogenesis. The primary cilium (cilium) is a microtubule-based antenna-like structure that emanates from the surface of different cell types that receives signals from the environment regulating intracellular signaling pathways. Within the hypothalamus astrocytes and neurons are ciliated. Humans carrying ciliary dysfunctions show features of metabolic syndrome, and are obese and shortened or elongated cilia are associated with pathological conditions. Interestingly, the molecular machinery involved in autophagy participates in ciliogenesis and regulates cilia length suggesting that alterations in autophagy might affect cilium-related functions.
Objectives:
(i) To evaluate if palmitic acid inhibits autophagy *in vitro*, in hypothalamic neurons and astrocytes, causing inflammation via ciliary TGFβ receptors;
(ii) To assess whether α-linolenic acid promotes autophagy *in vitro*, in hypothalamic neurons and astrocytes, suppressing palmitic acid-mediated inflammation, via ciliary TGFβ receptors;
(iii) To determine the role of α-linolenic acid in hypothalamic autophagy, ciliogenensis, inflammation and insulin sensitivity *in vivo*, in control and chronic-HFD fed mice.
Results Obtained:
Our results indicate autophagy is inhibited by exposure to saturated fatty acids (SatFAs)(Palmitic and Stearic acid) in hypothalamic neurons and in astrocytes. Additionally, exposure to SatFAs reduces the number and the size of primary cilia. Interestingly, this effect on the primary cilium occurs only in neurons, as the size and number of cilia is not affected in hypothalamic astrocytes. Loss of cilia in neurons is linked to autophagy inhibition, as chemical and genetic autophagy blockade reduces ciliogenesis. Notably, treatment with α-linolenic acid restores both autophagy and ciliogenesis in neurons, by a mechanism that we are still evaluating. Functionally, autophagy blockade in hypothalamic neurons (by palmitic acid exposure, by genetic and chemical inhibition) reduces insulin signaling, an effect that has been observed also following primary cilium depletion. *In vivo*, chronic HFD consumption modifies the shape of hypothalamic primary cilia, further studies need to determine if this affects their function.
Results Unforeseen in the Original Project:
In the original project we focused on inflammation as effect induced by primary cilium loss, however we could not obtain consistent results, so we decided to observe the effect of primary cilium loss on insulin response. Interestingly, our results show lack of primary cilium in hypothalamic neurons inhibits insulin signaling.
Publications:
Budini, M., Buratti, E., Morselli, E., Criollo, A. Autophagy and its impact on neurodegenerative diseases: New roles for TDP-43 and C9orf72. 2017. Front. Mol. Neurosci. **10**, 170
Ávalos, Y., Peña-Oyarzun, D., Budini, M., Morselli, E., Criollo A. New roles of the primary cilium in autophagy. 2017. Biomed. Res. Int. **2017**, 4367019
Peña-Oyarzun, D., Troncoso, R., Kretschmar, C., Hernando, C., Budini, M., Morselli, E., Lavandero, S., Criollo, A. Hyperosmotic stress stimulates autophagy via polycystin-2. 2017. Oncotarget **8(34)**, 55984-55997
Morselli, E., Santos, R.S., Gao, S., Ávalos, Y., Criollo, A., Palmer, B.F., Clegg, D.J. Impact of estrogens and estrogen receptor-α in brain lipid metabolism. 2018. Am. J. Physiol. Endocrinol. Metab. **315(1)**, E7-E14
Peña-Oyarzun, D., Bravo-Sagua, R., Diaz-Vega, A., Aleman, L., Chiong, M., Garcia, L., Bambs, C., Troncoso, R., Cifuentes, M., Morselli, E., Ferreccio, C., Quest, A.F.G., Criollo, A., Lavandero, S. Autophagy and oxidative stress in non-communicable diseases: A matter of the inflammatory state? 2018. Free Radic. Biol. Med. **124**, 61-78

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Hernández-Cáceres, M.P., Toledo-Valenzuela, L., Díaz-Castro, F., Ávalos, Y., Burgos, P., Narro, C., Peña-Oyarzun, D., Espinoza-Caicedo, J., Cifuentes-Araneda, F., Navarro-Aguad, F., Riquelme, C., Troncoso, R., Criollo, A., Morselli, E. Palmitic acid reduces the autophagic flux and insulin sensitivity through the activation of the free Fatty Acid Receptor 1 (FFAR1) in the hypothalamic neuronal cell line N43/5. 2019. Front. Endocrinol. (Lausanne) **10**, 176

Kretschmar, C., Peña-Oyarzun, D., Hernando, C., Hernández-Moya, N., Molina-Berríos, A., Hernández-Cáceres, M.P., Lavandero, S., Budini, M., Morselli, E., Parra, V., Troncoso, R., Criollo, A. Polycystin-2 is required for starvation- and rapamycin-induced atrophy in myotubes. 2019. Front. Endocrinol. (Lausanne) **10**, 280

Hernández-Cáceres, M.P., Cereceda, K., Hernández, S., Li, Y., Narro, C., Rivera, P., Silva, P., Ávalos, Y., Jara, C., Burgos, P., Toledo-Valenzuela, L., Lagos, P., Cifuentes Araneda, F., Perez-Leighton, C., Bertocchi, C., Clegg, D.J., Criollo, A., Tapia-Rojas, C., Burgos, P.V., Morselli, E. Palmitic acid reduces the autophagic flux in hypothalamic neurons by impairing autophagosome-lysosome fusion and endolysosomal dynamics. 2020. Mol. Cell. Oncol. **7(5)**, 1789418

Peña-Oyarzun, D., Batista-Gonzalez, A., Kretschmar, C., Burgos, P., Lavandero, S., Morselli, E., Criollo, A. New emerging roles of Polycystin-2 in the regulation of autophagy. 2020. Int. Rev. Cell. Mol. Biol. **354**, 165-186

Peña-Oyarzun, D., Rodriguez-Peña, M., Burgos-Bravo, F., Vergara, A., Kretschmar, C., Sotomayor-Flores, C., Ramirez-Sarmiento, C.A., De Smedt, H., Reyes, M., Perez, W., Torres, V.A., Morselli, E., Altamirano, F., Wilson, C.A.M., Hill, J.A., Lavandero, S., Criollo, A. PKD2/polycystin-2 induces autophagy by forming a complex with BECN1. 2020. Autophagy **30**, 1-15

CHINA

Title: Identification of novel hypoxia induced radioresistance signalling for endemic nasopharyngeal carcinoma
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Abstract: Enhanced DNA repair capacity is an essential reason of tumour radioresistance, which leads to the failure of cancer treatment. Through Crispr/cas9 knockout library screening, we identified that NONO, a DNA and RNA binding protein, played important roles in tumour radioresistance. Knockout of NONO remarkably sensitised tumour cells to irradiation (IR), and suppressed the repair of IR-induced double strand DNA damage. With bioinformatics and experimental analysis, we found that after radiation or epidermal growth factor (EGF) stimulation, epidermal growth factor receptor (EGFR) internalised and bound to NONO in nucleus, but NONO was not the substrate of EGFR kinase. Further investigation showed that NONO enhanced the association of nuclear EGFR and DNAPK, and increased the phosphorylation of DNAPK. Besides, a ribosomal protein RPLP0 was also characterised as an IR-induced NONO-interacting protein. RPLP0/NONO complex are required for the DNA damage-induced phosphorylation of DNAPK, with plays essential roles in dsDNA repair. These results provide a new insight into the mechanism of dsDNA break repair and tumour radioresistance, which may be exploited to radiotherapy sensitisation.
Objectives:
(i) Identification of NONO might be the key player racing HIF-1α induced NPC radioresistance; (ii) The molecular mechanism of EGFR--NONO--DNA repair signalling in regulation of NPC radiosensitivity; (iii) The molecular mechanism of HIF-1α--nuclear EGFR--NONO--DNA repair signalling in NPC radioresistance; (iv) The crosstalk among HIF-1α--nuclear EGFR--NONO--DNA repair signalling, HIF-1α--Beclin 1--autophagy and HIF-1α--Aurora-A--apoptosis pathways, and the potential to sensitise the radioresistant NPC cells by targeting the key molecules in these pathways; (v) Mechanism validation in NPC patients and animal models.
Results Obtained:
(i) Nuclear EGFR-NONO-p-DNAPKs axis enhances DNA damage repair and tumour radioresistance.
Colony formation assay showed that NONO-knockout remarkably sensitised HN5 and UMSCC-10A cells to radiation. We hypothesised that NONO may interacted with EGFR. CoIP assay showed that EGFR and NONO was associated in HN5 cells. Furthermore, NONO was interacted with the extra-cellular domain of EGFR, and EGFR bound to RRM1 domain of NONO. NONO interacted with EGFR in nucleus but not cytoplasm. Immunofluorescent assay showed that EGF stimulation induced EGFR translocating to nucleus and co-localised with NONO. Besides, both EGF and irradiation treatment, which induced EGFR internalisation, enhanced the interaction of NONO and EGFR. Meanwhile, AG1478, the inhibitor of EGFR, abolished the irradiation-induced association of NONO and EGFR. As reported previously, irradiation induced the association of EGFR and DNAPK in nucleus, and NONO knockout inhibited their interaction. Accordingly, NONO knockout abolished the radiation-induced phosphorylation of DNAPK.
(ii) NONO/RPLP0 complex promotes DNA repair and radioresistance by enhancing pT2609-DNA-PK.
Ribosomal protein RPLP0 was identified as a novel NONO binding protein, and their association was remarkably enhanced after irradiation. The interaction of NONO and RPLP0 was further confirmed by CoIP assay. Most importantly, irradiation enhanced the association of NONO and RPLP0 in U2OS cells. Furthermore, the results of live cell imaging and proximal ligation assay showed that RPLP0 and NONO were associated in nucleus. The examination of γ-H2A.X by western blot and immunofluorescence showed that silencing RPLP0 suppressed DNA repair. Furthermore, in vitro NHEJ assay showed siRPLP0 inhibited NHEJ. DNAPK is an essential kinase in NHEJ. We found that both NONO knockout and RPLP0 silencing reduced the phosphorylation of DNAPK. Interestingly, compared with tissues from TRG0 patients, NONO was higher expressed in CRC tissues from TRG3 patients, indicating an important role of NONO in tumour radioresistance.
(iii) Impact of chemotherapy regimens on normal tissue complication probability models of acute hematologic toxicity in rectal cancer patients receiving intensity modulated radiation therapy with concurrent chemotherapy from a prospective phase III clinical trial.
To determine whether there are differences in bone marrow tolerance to chemo-radiotherapy (CRT) between two chemotherapy regimens according to FOWARC protocol and how chemotherapy regimens affect radiation dose parameters and normal tissue complication probability (NTCP) modelling that correlate with acute hematologic toxicity (HT) in rectal cancer patients treated with intensity modulated radiation therapy (IMRT) and concurrent chemotherapy. Patients receiving

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FOLFOX have lower BM tolerance to CRT than those receiving 5FU. Low-dose radiation to the PBM is predictive for HT2+ in patients who received 5FU. NTCP modelling in FOLFOX group predicts much higher risk of HT3+ than 5FU group.

Results Unforeseen in the Original Project:

As described in the original project, we characterised the irradiation or EGF-induced interaction of NONO and EGFR in nucleus, and investigated their underlying mechanisms in DNA repair and tumour radioresistance. Moreover, beyond the objectives in proposal, we further identified a novel NONO-binding protein, RPLP0, which binds to NONO upon irradiation and enhances DNA repair and tumour radioresistance.

Publications:

Cai, H., Pang, X., Dong, D., Ma, Y., Huang, Y., Fan, X., Wu, P., Chen, H., He, F., Cheng, Y., Liu, S., Yu, Y., Hong, M., Xiao, J., Wan, X., Lv, Y., Zheng, J. Molecular decision tree algorithms predict individual recurrence pattern for locally advanced nasopharyngeal carcinoma. 2019. J. Cancer **10**, 3323-3332

Cheng, Y., Ma, Y., Zheng, J., Deng, H., Wang, X., Li, Y., Pang, X., Chen, H., He, F., Wang, L., Wang, J., Wan, X. Impact of chemotherapy regimens on normal tissue complication probability models of acute hematologic toxicity in rectal cancer patients receiving intensity modulated radiation therapy with concurrent chemotherapy from a prospective phase III clinical trial. 2019. Front. Oncol. **9**, 244

Fan, X., Xie, Y., Chen, H., Guo, X., Ma, Y., Pang, X., Huang, Y., He, F., Liu, S., Yu, Y., Hong, M., Xiao, J., Wan, X., Li, M., Zheng, J. Distant metastasis risk definition by tumour biomarkers integrated nomogram approach for locally advanced nasopharyngeal carcinoma. 2019. Cancer Control. **26(1)**, 1073274819883895

Fan, X.J., Huang, Y., Wu, P.H., Yin, X.K., Yu, X.H., Fu, X.H., Feng, L.L., Wang, Y.L., Yi, H.J., Chen, Z.T., Yin, J.X., Zhang, D.L., Feng, W.X., Bai, S.M., Kim, T., Mills, G.B., Lu, Y.L., Wan, X.B., Wang, L. Impact of cold ischemic time and freeze-thaw cycles on RNA, DNA and protein quality in colorectal cancer tissues biobanking. 2019. J. Cancer **10(20)**, 4978-4988

COSTA RICA

Title: Regulation of adaptive immunity during a brucellosis infection
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ICGEB Contract No.: CRP/16/005
ICGEB Reference No.: CRP/CRI16-02
Abstract: Neutrophils (PMNs) and complement proteins (C´) are in the first line of defence of innate immunity. The detection of various microbial structures by these elements influences the downstream activation events of the immune response. In spite of the significant role of complement and PMNs against most acute infections, the role of these elements of the innate immunity in bacterial chronic infections has remained unexplored for many years. In order to understand this, we propose to use the intracellular pathogen *Brucella abortus* as a model. Our studies have shown that *Brucella* resists the killing action of PMNs and C´, albeit these elements participate in the regulation of the adaptive immune response during brucellosis.
Objectives:
Our key objective is to unveil the events associated with the interaction of *Brucella abortus* with PMNs and C´, and determine the role that these elements play during in the regulation of the immune response and in the dispersion of the bacterium during experimental brucellosis.
To achieve this, we will focus on three specific aims:
(i) To determine the mechanisms by which *Brucella* interacts and resists the bactericidal action of PMNs and C´;
(ii) To determine the role of C´ and PMNs in the modulation of adaptive immunity during experimental brucellosis;
(iii) To develop a model for exploring the role of PMNs as "Trojan horse" vehicles for the dispersion of *Brucella* to different organs.
Results Obtained:
Our main results show that C´ and PMN resistance may relate to host protein modulation by the bacteria; using a proteomic based approach we identified that most of the proteins interacting with the bacteria were related to the complement and coagulation systems. Among these, complement system regulatory proteins were identified in *Brucella* surface. Based on our results we propose that interaction with serum factors is a key event in the modulation of innate immunity in brucellosis. Specifically, the sequestration of complement regulatory proteins and the attachment of conglutinin (bovine host), that would favour entry to host cells (unpublished results). Interestingly, and in accordance with PMN depletion, complement-depleted mice readily eliminated *B. abortus* from the spleen and did so more efficiently than the infected controls after 7 days of infection. The levels of the immune activator cytokine IFN-gamma and the regulatory cytokine IL-10 were significantly increased. No significant histopathological changes in the liver and spleen were observed between the complement-depleted *B. abortus*-infected mice and the corresponding controls. The action exerted by *Brucella* on the immune system in the absence of complement may correspond to a broader phenomenon that involves several components of innate immunity (González-Espinoza, et al., 2018). We also determined the role of PMN in adaptative immunity. Removal of polymorphonuclear neutrophils (PMNs) at the onset of adaptive immunity against *Brucella abortus* favoured bacterial elimination in mice. This was associated with higher levels of interferon gamma and a higher proportion of cells expressing interleukin 6 and inducible nitric oxide synthase, compatible with M1 macrophages, in PMN-depleted *B. abortus*-infected mice. At later times in the acute infection phase, the amounts of IFN gamma fell while IL-6, IL-10, and IL-12 became the predominant cytokines in PMN depleted mice. IL-4, IL-1, and TNF-alpha remained at background levels at all times of the infection. The depletion of PMNs at the acute stages of infection promoted the premature resolution of spleen inflammation. The efficient removal of bacteria in the PMN depleted mice was not due to an increase of antibodies, since the immunoglobulin isotype responses to *Brucella* antigens were dampened. Anti-*Brucella* antibodies abrogated the production of IL-6, IL-10, and IL-12 but did not affect the levels of IFN gamma at later stages of infection. These results demonstrate that PMNs have an active role in modulating the course of *B. abortus* infection after the adaptive immune response has already developed (Mora-Cartín, et al., 2019).
Finally, we developed a model to explore the "Trojan horse hypothesis" in brucellosis. Within this context, we concluded that loads of *B. abortus* in the bone marrow (BM) remain constant and are long lasting. In addition, we hypothesise that: (i) BM PMNs may serve as vehicles for dispersion of *Brucella*, following the Trojan horse hypothesis; (ii) that *B. abortus*-infected myeloid oligopotent progenitor cells may differentiate into mature infected cells; and (iii) that monocytes are the most likely *Brucella* reservoirs in the BM and that these cells may be the source of the frequent relapses

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observed in antibiotic-treated individuals (Gutiérrez-Jiménez et al, 2018). We also observed that (iv) *Brucella abortus* infected up to 96% of BM-PMNs, inducing a premature death of these cells; (v) the *Brucella*-infected PMNs displayed PS as "eat me" signal, promoting the association with Mφs and favoring the bacterial replication within these mononuclear phagocytes; (vi) the low production of proinflammatory cytokines and the high production of the anti-inflammatory IL-10 at the initial stages of infection, correlated with the non-phlogistic Mφ *Brucella*-PMN uptake and subsequent bacterial replication (Gutiérrez-Jiménez, et al., 2019).

Publications:

González-Espinoza, G., Barquero-Calvo, E., Lizano-González, E., Alfaro-Alarcón, A., Arias-Gómez, B., Chaves-Olarte, E., Lomonte, B., Moreno, E., Chacón-Díaz, C. Depletion of complement enhances the clearance of *Brucella abortus* in mice. 2018. Infection and Immunity **86**, e00567-18

Gutiérrez-Jiménez, C., Hysenaj, L., Alfaro-Alarcón, A., Mora-Cartín, R., Arce-Gorvel, V., Moreno, E., Gorvel, J.P., Barquero-Calvo, E. Persistence of *Brucella abortus* in the bone marrow of infected mice. J Immunol Res. 2018. **69:1-8**. doi: 10.1155/2018/5370414

Gutiérrez-Jiménez, C., Mora-Cartín, R., Altamirano-Silva, P., Chacón-Díaz, C., Chaves-Olarte, E., Moreno, E., Barquero-Calvo, E. Neutrophils as Trojan horse vehicles for *Brucella abortus* macrophage infection. 2019. Front. Immunol. **7(10)**, 1012

Mora-Cartín, R., Gutiérrez-Jiménez, C., Alfaro-Alarcón, A., Chaves-Olarte, E., Chacón-Díaz, C., Barquero-Calvo, E., Moreno, E. Neutrophils dampen adaptive immunity in brucellosis. 2019. Infection and Immunity. IAI **00118-19**.

CROATIA

Title: Biological factors determining Human Papillomavirus (HPV) driven carcinogenesis
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ICGEB Reference No.: CRP/HRV16-05_EC
Abstract: Human Papillomaviruses (HPVs) are small DNA tumour viruses, which cause approximately 600,000 cancers annually and they include cervical cancers, other ano-genital cancers, and a number of head and neck cancers. HPV E6 and E7 oncoproteins are critical for development and maintenance of the malignant phenotype in HPV-induced cancers. These two viral oncoproteins interfere with a plethora of cellular pathways, such as cell cycle regulation and the control of apoptosis, which are critical in maintaining normal cellular functions. Furthermore, their continuous expression is required for maintenance of the transformed phenotype, while loss of their protein expression will result in cellular growth arrest and/or apoptosis. Therefore, these two viral oncoproteins are considered to be excellent targets for development of novel therapies against HPV-induced malignancies, and a better understanding of the interplay between these oncoproteins and their cellular substrates is crucial for this process. In addition, understanding the molecular mechanisms underlying the respective functions of E6 and E7 can also help in determining novel and more reliable biomarkers that could help in predicting the disease development at early stages in atypical anatomic sites, such as the head-and-neck region, where the virus replicates.
Objectives:
(i) To establish immortalised cell cultures of human keratinocytes from cells isolated from different anatomical sites harbouring HPV-16 genomes. This will allow us to follow the progression of malignant potential over time in these tissues and to determine if there are differences or similarities in the way in which HPV-16 initiates tumour progression. This will also allow us to investigate potential novel prognostic markers associated with the development of HPV-mediated cancers at different anatomical sites.
(ii) To perform proteomic analyses to identify E6- and E7-interacting partners and use them to determine whether the target proteins of the virus oncoproteins are the same in cells from diverse tissue types. In this way, we could potentially identify novel therapeutic targets of E6/E7.
Results Obtained:
We created HPV-16 genome-containing cell lines from different anatomical sites (HFKs - human foreskin keratinocytes and TONs - tonsillar keratinocytes). Using various biochemical and mechanistic analyses we monitored the potential similarities and differences in HPV-16-driven malignant progression between these cell lines. After extensive analyses, we did not observe any significant aspects which could indicate that there are major differences in the process of HPV-16-mediated malignant progression between cells isolated from different anatomical sites. Currently, we are in the process of generating HPV-18 genome-expressing cell lines from the same tissue origins. We will then perform comparative analyses with the equivalent cell lines expressing HPV-16, investigating the interactions of the viral oncoproteins with some of their known target substrates. In this way, we will be able to assess whether the profile of the interacting partners and consequences of the interactions with HPV-16 and -18 E6/E7 oncoproteins can affect the process of HPV-mediated malignancy at different anatomical sites.
We have identified Na+/H+ exchange regulatory factor 2 (NHERF-2) as a novel interacting partner of HPV-16 and HPV-18 E6 oncoproteins. We demonstrated that both E6 oncoproteins interact with NHERF-2, a PDZ domain-containing protein, which amongst other cellular functions, also behaves as a tumour suppressor regulating endothelial proliferation. The interaction between the E6 oncoproteins and NHERF-2 is dependent on E6's PDZ-binding motif (PBM) and results in proteasome-mediated degradation of NHERF-2. We further confirmed this effect in cells derived from HPV-16- and HPV-18-positive cervical tumours, where we show that NHERF-2 protein turnover is increased in the presence of E6. Finally, our data indicate that E6-mediated NHERF-2 degradation results in p27 downregulation and cyclin D1 upregulation, leading to accelerated cellular proliferation. The findings of this study are the first report demonstrating that E6 oncoproteins can stimulate cell proliferation by indirectly regulating p27 through targeting a PDZ domain-containing protein. Our current aim is to investigate whether the E6-NHERF-2 association can be used as a basis for a potential therapeutic approach against HPV-positive cancers.
Results Unforeseen in the Original Project:
The ICGEB funding has contributed significantly in the scientific training of 2 PhD students, 2 postdoctoral fellows and 1 MSc student, who completed his Master thesis under this project. We were also invited to submit a review article for Pathogens special issue on “Viral-host interactions and determinants of human papillomavirus (HPV) life cycle and pathogenesis”.
Publications:

Developing knowledge

Saidu, N.E.B., Filić, V., Thomas, M., Sarabia-Vega, V., Đukić, A., Miljković, F., Banks, L., Tomaić, V. PDZ domain-containing protein NHERF-2 is a novel target of Human Papillomavirus 16 (HPV-16) and HPV-18. 2019. J Virol. **94(1)**
Đukić, A., Lulić, L., Thomas, M., Skelin, J., Saidu, N.E.B., Grce, M., Banks, L., Tomaić, V. HPV Oncoproteins and the Ubiquitin Proteasome System: a Signature of Malignancy? 2020. Pathogens **9(2)**, 133

CROATIA

Title: Comprehensive toolbox for epigenetic modulation of gene expression
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Abstract: The CRISPR/dCas9-based platform for easy and convenient assembly was designed, constructed and validated. It consists of different promoters, Cas9 orthologs, effector domains and selection markers, along with specific sgRNAs to target multiple candidate loci within the same cell. Basic backbone is provided for delivery by transfection, and the alternative backbones are also available for lentiviral delivery and for co-expression of up to 6 sgRNAs for both orthologous Cas9 proteins from *S. pyogenes* and *S. aureus* (SpCas9 and SaCas9). Four effector domains were fused to dCas9: DNMT3A (for targeted methylation); TET1 (for targeted demethylation); KRAB and VPR (for direct gene repression and activation, respectively). The fusion constructs were well characterised for both SaCas9 and SpCas9 orthologs and N- and C- terminal fusions as well as validated by transfections of different cell lines. Time course experiments for all dCas9 fusions were done in order to determine the exact day of the maximal activity of the catalytic domains within fusions following transfection, as well as the time frame in which an achieved effect persist. As a proof of principle we used CRIPSR/dCas9 toolbox for epigenetic manipulations and direct activation/silencing of the candidate genes. We demonstrated successful manipulation of the gene pairs within the same cells using dCas9-fusions with catalytic domains of synergistic and antagonistic activities.
Objectives:
(i) Design and construction of the modular system for manipulation of epigenetic modifications and direct manipulation of gene expression level: (a) basic backbone/assembly for delivery by transfection; (b) backbone/assembly for by lentiviral delivery; (c) development of dual marker system; (d) design and construction of the multi-guide system;
(ii) Validation and characterisation of C- and N-terminal fusions of SpCas9 and SaCas9 with various effector domains;
(iii) Use of the SpCas9 and SaCas9-fusions with effector domains of antagonistic (DNMT3A and TET1, or VPR and KRAB) or synergistic (DNMT3A and KRAB or TET1 and VPR) activities within the same cell;
(iv) Functional analysis of *HNF1A* gene regulation by CpG methylation using dCas9 fusions;
(v) Analysis of stability and propagation of introduced epigenetic marks following epigenetic manipulation using CRISPR/dCas9 molecular tools.
Results Obtained:
A modular and extensible CRISPR/dCas9-based toolbox for epigenetic editing and direct gene regulation was designed and validated on the candidate loci in several different cell lines. It features a system for expression of orthogonal dCas9 proteins fused to various effector domains and includes a multi-gRNA system for simultaneous targeting dCas9 orthologs to up to six loci. The C- and N-terminal dCas9 fusions with DNMT3A and TET1 catalytic domains were thoroughly characterised. We demonstrated simultaneous use of the dCas9 fusions with effector domains with antagonistic and synergistic activities in the same cells. We targeted two gene pairs (*BACH2-IL6ST* and *HNF1A-MGAT3*) with DNMT3A-dSpCas9 and TET1-dSaCas9 fusions at the same time in same cells. We showed successful epigenetic manipulation of their promoters: imposed cytosine hyper- and hypo-methylation altered level of gene transcription if targeted CpG sites were functionally relevant. We were also able to demonstrate that the dual epigenetic manipulation of the *HNF1A* and *MGAT3* genes, involved in protein N-glycosylation, resulted in change of the glycan phenotype in BG1 cells. Furthermore, simultaneous targeting of the TET1-dSaCas9 and VPR-dSpCas9 fusions to the HNF1A regulatory region revealed strong and persistent synergistic effect on gene transcriptional activation, up to 30 days following cell transfection, suggesting involvement of epigenetic mechanisms in maintenance of the reactivated state. In addition, if we targeted narrow region of 4 CpG sites in promoter of *HNF1A,* the effect of forced methylation spread down- and upstream of the targeted region.
Results unforeseen in the Original Project:
We assessed the specificity of our modular dCas9-based system and investigated the effect of modulating fusion protein expression relative to selection marker expression on offtarget dCas9 activity. We compared the effect of two types of dCas9 fusions (one with weak EFS promoter and another with strong CBh promoter, driving the expression of both dCas9 fusion protein and puromycin resistance gene) on global off-target activity using whole-genome methylation analysis (by Illumina Infinium Methylation EPIC 850K Platform). We concluded that modulation of dCas9

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expression effectively reduced off-target effects while maintaining the desired effect on target regions.

Publications:

Josipović, G., Tadić, V., Klasić, M., Zanki, V., Bečeheli, I., Chung, F., Ghantous, A., Keser, T., Madunić, J., Bošković, M., Lauc, G., Herceg, Z., Vojta, A., Zoldoš, V. Antagonistic and synergistic epigenetic modulation using orthologous CRISPR/dCas9-based modular system. 2019. NAR **47(18)**, 9637-9657

Tadić, V., Josipović, G., Zoldoš, V., Vojta, A. CRISPR/Cas9-based epigenome editing: An overview of dCas9-based tools with special emphasis on off-target activity. 2019. Methods **164-165**, 109-119

HUNGARY

Title: Exosomes in the inflammatory intestinal stem cell niche
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ICGEB Reference No.: CRP/ HUN16-04_EC
Abstract: The intestinal epithelium is renewed by the intestinal stem cells (ISC), residing in a specific microenvironment. This niche is influenced by other cell types, such as fibroblasts, immune cells etc. Inflammation determines the regeneration of the intestinal epithelium. Extracellular vesicles (EVs) are enclosed by membrane, they transport molecules among cells, thus representing a novel cell-cell communication mechanism.
By using 3D intestinal organoids, we show that fibroblast-derived EVs are involved in forming the ISC niche by transmitting Wnt and epidermal growth factor (EGF) activity. With a mouse model that expresses EGFP in the Lgr5+ ISCs we prove that loss in ISC number in the absence of EGF is prevented by fibroblast-derived EVs. Furthermore, we show that fibroblast-derived EVs carry EGF family members, such as amphiregulin. Blocking EV-bound amphiregulin inhibited the EV-induced survival of organoids. Whereas IFNγ and TNFα, molecules acting in the ISC niche, modified organoid apoptosis and morphology, fibroblast-derived EVs had no additional effects. Collectively, we prove the important role of fibroblast-derived EVs as a novel transmission mechanism in the ISC niche.
Objectives:
(i) To test whether important ISC niche factors, such as EGF receptor ligands or Wnt proteins, travel via EVs from intestinal fibroblasts under normal, non-inflammatory condition. Furthermore, we to study the contribution of these EVs to the formation of the ISC niche and thus to controlling the number of stem cells in the presence of the basic niche factors.
(ii) To study the role of fibroblast-derived EVs on changes in the ISC niche in the presence of different pro-/anti-inflammatory mediators. We tested the hypothesis that EVs enhance or inhibit the effects of these cytokines.
(iii)To determine whether EVs induce the niche independence of ISCs in the presence of the tested immune modulators.
Results Obtained:
To study the EV-mediated signal transmission in the intestinal stem cell (ISC) niche, we used normal human colon fibroblasts (HCF) and we isolated intestinal fibroblasts from mice (MIF) as well. We detected EVs from HCFs with anti-CD63 or anti-CD81-coated beads, transmission electron microscopy proved the presence of EVs in the ultracentrifuged pellet of HCF-derived supernatant, furthermore, tunable resistive pulse sensing (TRPS) and Nanoparticle Tracking Analysis (NTA) that are standard methods for EV characterisation, showed the presence of EVs in the preparates. Similarly, MIF cultures produced CD81+ EVs. To prove that EVs can move in 3D Matrigel and thus their effect can be tested in this matrix, we first used colorectal cancer (CRC) cell lines. Importantly, whereas cells cultured 2D produced both CD63+/Annexin V- smaller and Annexin V+ larger EVs, smaller EVs could be detected in 3D cultures as well.
ISCs are responsible for maintaining the crypt structure, leading to the budding of the organoids. Thus, counting the living, budding or dead organoids provides a robust and easy method to detect ISC activity. The exogenously added EGF, the Bmp inhibitor noggin and the Wnt-agonist R-Spondin1 are critical for maintaining the ISC population in small intestinal (SI) organoids as niche factors. Importantly, when fibroblast-derived EVs were added in the presence of all the essential niche factors, they did not influence the number of surviving or budding SI or colonic organoids.
To study whether some of the niche factors travel on EVs, first we focused on the highly hydrophobic Wnt proteins in the ISC niche. In contrast to SI organoids where Paneth cells produce Wnt proteins, colon organoids critically depend on external Wnt factors in cultures due to the lack of this cell type. HCF-derived EVs showed a marked rescue effect in colon organoid survival when Wnt3a lacked from the culture medium, demonstrating that EVs critically contribute to the normal Wnt activity in the ISC niche. To test whether some of the other critical ISC niche factors are transmitted by fibroblast-derived EVs, we removed R-Spondin1, noggin or EGF from culture medium. As expected, the lack of one of these factors led to a markedly reduced organoid survival, showing the disappearance of ISC function. Interestingly, whereas fibroblast EVs did not rescue the reduced survival of organoids when R-Spondin1 or noggin lacked, we observed an extensive rescue effect for EGF when applying HCF or MIF-derived EVs. Similarly, fibroblast-derived EVs restored the proportion of the proliferating and apoptotic cells when EGF lacked from the medium. Importantly, HCF-derived EVs prevented organoid death when EGF lacked in human colon organoid cultures, thus, confirming that EGF activity can be transmitted via EVs in the human ISC niche as well.

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To further prove that EGF activity may be transmitted by EVs in the ISC niche, we used the EGF receptor inhibitor gefitinib. Similarly to the exogenously added soluble EGF, the rescue effect of EVs was blocked in the presence of the inhibitor. We next isolated SI organoids from Lgr5-EGFP-IRES-CreERT2 mice, expressing the green fluorescent protein EGFP in ISCs. We detected a massively reduced organoid budding and a decrease in the proportion of EGFP+ organoids when EGF was removed from culture medium and, importantly, this effect was rescued by fibroblast-derived EVs. Interestingly, EGFP+ cells completely disappeared when EGF lacked, however, we could still detect green cells in SI organoids when the culture medium was supplemented with HCF-derived EVs, further proving that EGF activity is preferentially transmitted via EVs in the ISC niche. EVs isolated from fibroblast cultures were positive for amphiregulin, showing that at least this member of the EGF ligand family is transported by fibroblast-derived EVs in the ISC niche. Importantly, our other experiments demonstrated that recombinant amphiregulin does not simply co-purify with EVs, but the full-length form of this protein is present in EVs. Furthermore, when neutralising amphiregulin in fibroblast-derived EVs before applying them to organoids in the absence of EGF, we observed a marked reduction in the proportion of budding organoids, showing the reduced stem cell activity. Collectively, fibroblast-derived EVs carry active amphiregulin as EGF activity in the ISC niche, thus, EVs provide a novel tool for transmitting niche factors.

Results Unforeseen in the Original Project:

We tested the combinatorial effect of fibroblast-derived EVs and cytokines in the ISC niche. TNFα and IFNγ were selected, since they have published effects on stem cells and/or transit amplifying cells. IFNγ led to the death of SI organoids and TNFα resulted in the appearance of cyst-like organoids without modifying the proportion of active caspase-3+ apoptotic cells. Unexpectedly, the addition of fibroblast-derived EVs did not have a rescue effect on these measured parameters either with IFNγ or in the presence of TNFα. Thus, in contrast to previous expectations, EVs had no additive effects in our model systems when these selected cytokines were present.

Publications:

Oszvald, Á., Szvicsek, Z., Sándor, G.O., Kelemen, A., Soós, A.Á., Pálóczi, K., Bursics, A., Dede, K., Tölgyes, T., Buzás, E.I., Zeöld, A., Wiener, Z. Extracellular vesicles transmit epithelial growth factor activity in the intestinal stem cell niche. 2019. Stem Cells doi: 10.1002/stem.3113

Szvicsek, Z., Oszvald, Á., Szabó, L., Sándor, G.O., Kelemen, A., Soós, A.Á., Pálóczi, K., Harsányi, L., Tölgyes, T., Dede, K., Bursics, A., Buzás, E.I., Zeöld, A., Wiener, Z. Extracellular vesicle release from intestinal organoids is modulated by Apc mutation and other colorectal cancer progression factors. 2019. Cell. Mol. Life Sci. **76**, 2463-2476

MALAYSIA

Title: Mode of action of a new antibiotic Phomopsidione on *Pseudomonas aeruginosa*
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ICGEB Contract No.: CRP/17/008
ICGEB Reference No.: CRP/MYS17-03
Abstract: *Pseudomonas aeruginosa* is an opportunistic Gram-negative bacterium that causes nosocomial and ventilator associated pneumonia. The bacterium forms biofilm that harbours slow growing bacterial populations that are extremely resistant to antibiotics. Data from the United States Nosocomial Infections Surveillance system from year 1986-2003 showed that *P. aeruginosa* is the second most common cause of pneumonia, the third common cause of urinary tract infection and the fourth most frequently isolated pathogen from bloodstream. Furthermore, a growing epidemic of infections due to Gram-negative bacteria resistance to many classes of antibiotics was reported in many countries.
The aim of this project is to determine the mode of action of phomopsidione on *P. aeruginosa* by using ATCC15692 strain as model organism. We successfully purified the compound using reverse phase HPLC. Based on the carboxyfluorescein (CF) leakage assay, phomopsidione caused substantial release of CF. Phomopsidione destabilises the phospholipid bilayer of *P. aeruginosa*. Based on membrane permeability test, phomopsidione rapidly induced SYTOX green uptake in *P. aeruginosa*, which indicates that the inner membrane of the bacteria cell is the target of phomopsidione. The treatment of *P. aeruginosa* cells with phomopsidione caused a significant fluorescence uptake in membrane permeability assay, which also indicates the effect of phomopsdione on the outer membrane of *P. aeruginosa*. The finding is in agreement with fluorescence microscopy analysis, which further corroborates a clear direct effect of phomopsidione on the bacterial membrane. The total RNA was successfully isolated from *P. aeruginosa* culture treated with phomopsidione. Based on the transcriptomic profile, a total of 380 genes were down-regulated, with the treatment of phomopsidione, with 41 out of 380 genes were membrane-protein related. The selected gene (*OprH*) was successfully cloned into *Escherichia coli* competent cells. Two colonies were identified as positive knockout transformants, which introduced by electroporation. The MIC recorded was significantly higher than non-mutant strain of *P. aeruginosa*. We can conclude that phomopsidione exerts antimicorbial effect by influencing the expression of *OprH* A, an outer membrane protein of *P. aeruginosa*. Phomopsidione inhibits *P. aeruginosa* by targeting bacterial membrane.
Objectives:
In previous studies we discovered a new antibiotic, Phomopsidione, that exhibited excellent bactericidal activity on XDR *P. aeruginosa*. We are the first group who reported the isolation of this compound. Phomopsidione was isolated from *Diaporthe fraxini* ED2, an endophytic fungus residing in the medicinal herb *Orthosiphon stamineus*. However, the mode of action of this compound remains unknown. The specific objectives of our project are: (i) to identify the antibiotic mode of action of phomopsidione which result in the degeneration of XDR *P. aeruginosa* cells, and (ii) to validate the proposed mode of action.
Results Obtained:
Yeast extract sucrose broth was used to cultivate the fungal isolate in the shake-flask system. After 20 days of incubation the culture was extracted with an equal volume of ethyl acetate. The ethyl acetate extract was first fractionated on silica gel 60 column chromatography by using chloroform/methanol at a ratio of 2:3 (v/v) as mobile phase. The fourth fraction, which is yellow in colour, was collected and subjected to High Performance Liquid Chromatography (HPLC) to obtain the purified phomopsidione. The fraction of ethyl acetate extract was separated into 3 peaks by reverse phase HPLC, with retention time of 2.43, 5.64 and 7.39. The peak with retention time of 5.64 was previously identified as phomopsidione. The isolation of phomopsidione was evident by the observation of a single peak with retention time of 5.64.
Carboxyfluorescein (CF) leakage assay was performed to study the interaction of phomopsidione with phospholipid bilayer. CF-loaded liposome was used to mimic the phospholipid bilayer composition of *P. aeruginosa*. The integrity of liposome remained intact throughout the experimental period for control treatment (Dimethylsulfoxide). However, at concentration of minimal inhibitory concentration (MIC), intermediate release of CF was observed. The results showed that phomopsidione destabilises the phospholipid bilayer of *P. aeruginosa*. The permeabilisation of *P. aeruginosa* membrane was examined using SYTOX green dye, which only enters the cytoplasm if the bacterial membrane is damaged. At the concentration of MIC, the membrane permeabilisation was significantly increased compared to control. Phomopsidione rapidly induces SYTOX green uptake in *P. aeruginosa*, which indicates that the inner membrane of

the bacteria cell is the target of phomopsidione. Due to the lipopolysaccharides present in the outer membrane, 1-N-phenylnaphthylamine (NPN) probe is excluded from intact bacterial membrane. If the outer membrane is permeabilised by phomopsidione, NPN can penetrate the membrane and causes an increase in fluorescence. The treatment of *P. aeruginosa* with phomopsidione caused a significant fluorescence uptake, which indicates the effect of phomopsdione on the outer membrane of *P. aeruginosa*. Besides, the bacterial cells were observed under fluorescence microscope. Treatment of exponentially growing cells *P. aeruginosa* with control, resulted in uniformly stained membranes. In contrast, treatment of bacterial cells with phomopsidione at concentration MIC resulted in specific membrane accumulations. Overall, the results further corroborate a clear direct effect of phomopsidione on the bacterial membrane.
The 24-hour-old culture of *P. aeruginosa* was treated with phomopsidione at concentration of MIC for 5 hours. Subsequentely, the total RNA of the cells was isolated using RNeasy mini kit (Qiagen). The rRNA was removed by using Illunima Ribo-Zero rRNA removal kit. The total RNA obtained was 381 ng/µl. Using random hexamers as primers the mRNA fragments were reverse transcribed to single stranded cDNAs. After the synthesis of single stranded cDNA, buffer, dNTPs, DNA polymerase I and RNase were applied to synthesise the complementary cDNA strands. The double stranded cDNAs were purified using AMPure XP beads. The double stranded were end-repaired, polyadenylated, ligated with sequences and size selected using AMPure XP beads. Then, the uracil containing strands were degraded by USER enzyme, and the remained strands were amplified using PCR and purified using AMPure XP beads. Next generation sequencing was performed to study the transcriptomic profile of the *P. aeruginosa* treated with phomopsidione. By comparing the transcriptomic profile compared to control, a total of 380 genes were down-regulated, with the treatment of phomopsidione. A total of 41 genes were membrane-protein related. The gene *Opr*H was cloned with StrataClone PCR Cloning kit by using pB plasmid. This is important to study the interaction of gene with phomopsidione. *Opr*H is the outer membrane of *P. aeruginosa* and we noticed a significant difference in the transcriptome of the gene, after treatment with phomopsidione. A targeted mutagenesis was performed using Targetron Gene Knockout System. We successfully disrupted the *Opr*H gene in *E. coli* competent cell by inserting Group II intron to the target gene. The mobile group II intron expressing vectors that were re-targeted for *Opr*H A was introduced through electroporation. Cell transformed with these vectors produced erythromycin-resistant colonies. Two colonies were identified as positive knockout transformants. The antimicrobial activity of the mutants was analysed via broth microdilution assay according to CLSI guidelines. The MIC recorded for the two mutants was significantly higher than non-mutant strain of *P. aeruginosa* (50 ug/ml). We can conclude that phomopsidione influences the expression of *Opr*H A, an outer membrane protein of *P. aeruginosa*.

Developing knowledge

MEXICO

Title: Metabolic regulation of chromatin dynamics in obesity
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ICGEB Contract No.: CRP/16/009
ICGEB Reference No.: CRP/MEX16-05_EC
Abstract: Metabolic diseases and obesity are emerging as a XXI Century epidemic, promoted by modern society's lifestyles, including unhealthy intake of high-calorie content meals and dysregulation of biological rhythms. The molecular mechanisms underlying these processes are the subject of study in this proposal. Precisely, we want to investigate how chromatin is influenced by cellular metabolism to determine expression of circadian genes, which are crucial for the maintenance of metabolic homeostasis. Spatial positioning of genes inside of the nucleus is determinant of its transcriptional state. Here we will assess a potential role for metabolic cues in relocating circadian genes in the nuclear space, thereby influencing its regulation and transcriptional levels. For these studies, we will use a diet induced obesity (DIO) mouse model combined with cell culture, microscopy and molecular biology techniques.
Objectives:
In this proposal, we want to determine whether metabolic cues and the circadian clock can shape nuclear architecture by influencing gene positioning and function. Our specific aims are:
(i) To investigate dynamics in spatial positioning of metabolic genes in the liver during diet-induced obesity: We plan to use a mouse model of diet-induced obesity to address reorganisations in gene positioning and chromosomal interactions triggered by metabolic changes.
(ii) To elucidate if NAD+ metabolism can affect the subnuclear localisation of clock-controlled gene transcription: Given the functionality of the nuclear sirtuin SIRT1 in the control of the circadian epigenome, the nuclear dynamics of the tandem NAD+-SIRT1 appear as a potential regulatory layer for circadian control of genome organisation.
Results Obtained:
We generated a model system in the laboratory for diet-induced obesity and associated pathologies. Age matched, male C57BL/6(Harlan) mice were maintained on a 12 hour light/12 hour dark cycle. At 8 weeks of age, mice were placed on a normal control diet (CD) (2018 Teklad), or a High fat diet (HFD) (55% kcal from fat, Teklad Diet 07011) for 12 weeks. Mice on a HFD gained 31% (average) more body weight than those on a CD. Mice on a HFD showed high circulating glucose levels, display poor response to insulin and low glucose tolerance, and these differences are statistically significant when compared mice fed a CD. These data demonstrate that our diet induced obesity mouse model worked as expected, displaying impaired glucose metabolism and insulin resistance at the end of the feeding protocol. These mice also showed differential gene expression in the liver, which was coherent with previous reports. Then, we performed chromatin conformation capture (4C-seq) experiments in livers from lean and obese mice sacrificed at two circadian time points: ZT6 (day, resting period) and ZT18 (night, active phase). We selected baits located within regulatory regions from selected circadian genes (*Dbp*, *Nampt*) or metabolic genes (*Ppara*, *Pparg* and *Srebp1c*). We found that long-range interactions enhance transcriptional levels for all these genes. Interestingly, analyses from the *Dbp* bait showed a highly robust circadian interactome in livers from lean mice, while in obese mice, rhythmicity is partially lost and *Dbp* locus engage in specific long-distance interactions shaping a new transcriptional program involving expression of adjacent genes such as *Fgf21* or *Irf3*, which are know to be overexpressed in fatty liver disease. Remarkably, *Dbp* mRNA expression itself does not show significant variations between both dietary conditions, indicating that post-transcriptional mechanisms take action to keep steady *Dbp* mRNA levels, while *Dbp* locus engages in multiple interactions with neighboring genes, as has been described for known e-promoters. 4C-seq analyses selecting regulatory elements from metabolic genes as baits reveals that specific long-range interactions are established depending on the nutritional status, and correlate with dynamic variations in specific histone marks associated to regulatory elements, such as H3K27ac. When analysing the role of clock proteins in chromatin conformational dynamics triggered by nutritional cues, we saw that the nuclear receptor and transcriptional repressor REV-ERBa was mostly located at the dynamic enhancers, accounting for most of the topological changes in selected baits. Additionally, certain transcription factors cooperate with the clock molecular machinery to reorganise the three-dimensional architecture at the studied loci, such as is the case for the CCAAT/enhancer-binding protein (C/EBP) transcription factor, C/EBPa.
Then, we designed a pharmacological strategy to induce high nicotinamide adenine dinucleotide (NAD+) levels in the liver from high fat diet fed mice. We analysed the hepatic transcriptome from

Developing knowledge

these mice and compared to those of the lean and obese littermates. Interestingly, we observed a set of genes whose transcriptional levels were fully recovered to normal conditions upon the intervention. Whole transcriptome analyses precisely identified these genes as members of regulatory pathways involved in the immune response and the control of inflammatory processes, as well as a number of genes involved in lipid homeostasis. Interestingly, most of these pathways were segregated to a specific time of the day after rising hepatic NAD+. We performed a motif analyses to elucidate possible master regulators driving transcriptional responses to NAD+ levels, and found that the peroxisome proliferator-activated receptor gamma (PPARg) and the hepatocyte nuclear factor 3-beta (HNF-3B), also known as forkhead box protein A2 (FOXA2) were mostly involved in this transcriptional reprogramming triggered by NAD+.

Results Unforeseen in the Original Project:

In this project we revealed a previously unappreciated role of nuclear topology in the development of high-fat diet induced obesity and fatty liver disease. Moreover, we described specific transcriptional responses to the metabolite NAD+, which contribute to the correction of fatty liver disease. Altogether, our data provides new insights to further understand the coordinated action of gene expression and chromatin topology during the development of high-fat diet induced obesity and describes new gene regulatory elements involved in fatty liver disease. Finally, an unexpected and significant reorganisation of the clock function in response to high NAD+ levels in mouse fatty liver was evidenced.

Publications:

Escalante-Covarrubias, Q., Aguilar-Arnal, L. Environmental regulation of metabolism through the circadian clock. 2018. Current Opin. Toxicol., **8**, 93-101

Pacheco-Bernal, I., Becerril-Pérez, F., Aguilar-Arnal, L. Circadian rhythms in the three-dimensional genome: implications of chromatin interactions for cyclic transcription. 2019. Clinical Epigenetics **11**, 79

MEXICO

Title: Antibody facilitated Zika virus infection of primary cultures of Hofbauer cells and study of the mechanism used by the virus to cross the transplacental barrier

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ICGEB Reference No.: CRP/MEX 17-02

Abstract: Zika virus (ZIKV) infection may severely affect the newborn. Yet, dengue virus (DENV) infections or the inadvertent application of the Yellow Fever vaccine 17D during pregnancy, have not been associated with teratogenic effects.

Objectives:

Given the biological similarities among these 3 flaviviruses but the very different outcomes for the newborn, the objective of this project was to compare ZIKV, DENV and YFV-17D infections in human macrophage (U937) and placental trophoblast (HTR8) cell lines

Results Obtained:

Results showed that ZIKV grow faster in macrophages and infect trophoblast with higher efficiency that either DENV or YFV-17D. In addition, macrophages infected with ZIKV, but not with YFV-17D, showed a tendency to express a cytokine pro-inflammatory profile, with a significant higher expression of IL-1β. Meanwhile, all virus infections equally activate the production of inflammosome related cytokines (IL-1β, IL-8, TNF-α) and INF-2α in HTR8 cells. In contrast, the chemokines MIP-1α, MIP-1β, IP-10 and RANTES response in HTR8 cells seems to be induced only by the vaccine strain. However, no changes in the trans-epithelial resistance of placental syncytiotrophoblast (BeWo) monolayers exposed to the conditioned supernatants was observed. These results corroborate that trophoblast are susceptible to ZIKV infection and may act as efficient centers for ZIKV multiplication in placenta.

Developing knowledge

NIGERIA

Title: Establishment of a laboratory facility for the diagnosis and surveillance of Arbovirus infections in Nigeria
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ICGEB Contract No.: CRP/16/010
ICGEB Reference No.: CRP/NGA16-03
Objectives:
Absence of systematic surveillance for arbovirus infections in Nigeria is largely due to affordable, accessible and available appropriate diagnostic facilities. This often leads to under-recognised, under estimated and under reported cases of these viral infections. The project aimed at establishing a facility for routine diagnosis of arboviral diseases in Maiduguri, Nigeria
Results Obtained:
Of 200 serum samples from Borno State, 26 were positive for flaviviral RNA and sequence analysis indicated 13 West Nile (one matching WNV Italian isolate 358 (FJ472944.1) and another Indian isolate Gwl-01 2015 (MG516600.1), 1 Zika, 1 DENV-2 and 2 DENV-4. The RNA sequences of the 16 samples have been deposited in GeBank and assigned the accession numbers (code to be provided). YF neutralising antibody, which marks protection against the disease, was highest in Borno (11.5%) and Bauchi (10%) compared to Adamawa (6%). The high level of cross reactivity of IgM among Flaviviruses limits its specificity to YFV. CHIKV neutralising antibody was significantly different in the three states studied with the highest in Borno (49%) and Adamawa (27.0%) compared with Bauchi (10.6). Neutralising antibody to WNV was significantly higher in Bauchi (18.8%) compared to Borno (13.5%) but least in Adamawa (1.5%). Antibodies to DENV 2,3,4 were significantly different in the three states. DENV-4 was predominantly higher in Adamawa (76.5%) compared to Bauchi (38.8%) and Borno (26.0%). However, DENV-2 was more in Bauchi (68.4%) and Adamawa (66.5%) than Borno (23.8%). More DENV-3 was detected in Bauchi (43%) than Adamawa (36.5%) and Borno (26.0%). Neutralising antibody to ZKV was significantly higher in Borno (12.0%) compared to Adamawa (3.0%). None of Bauchi samples was tested for ZIKV. The seroprevalence of the five arboviruses studied was significantly different from the ages of the patients but not the gender. Neutralising antibodies to DEN-4, CHIKV and ZIKV significantly different among samples collected 1-7 days after onset of symptoms compared to those collected thereafter. At the point of sample collection 56% of 600 patients were not vaccinated against YFV, while 22% were vaccinated and YFV neutralising antibody was more among the unvaccinated than vaccinated. A significant number of the patients had antibiotics/antimalarial before seeking for laboratory test for malaria parasites. Newly produced serodiagnostic kits for arboviruses (rNS1 IgM and IgG) produced from the Molecular Virology Laboratory, ICGEB Trieste, Italy tested on Nigerian samples showed comparable sensitivity and specificity with commercially available kits.
Conclusion: Prevention programs (or in the case of an outbreak investigation and intervention efforts) are best virus or genus-specific than otherwise. Furthermore, mitigation efforts may be most effective if targeted at a state level as opposed to country-wide, which would be too broad or village-specific, which might be too narrow. Embarking on urgent YF mass vaccination along with National Program on Immunisation campaigns is urgent important and urgent to prevent future epidemics.
Results unforeseen in the original project: Different virus isolates were obtained from the culture of 299 (49.3%) patients who thought that they had malaria infections. Although these virus isolates were not identified due insufficient funds, the difference in their characteristic CPEs signals the diversities of the species involved.
Publications:
Oderinde, B.S., Mora-Cárdenas, E., Carletti, T., Baba, M.M., Marcello, A. Prevalence of locally undetected acute infections of Flaviviruses in North-Eastern Nigeria. 2020. Virus Res. **(286)** 198060

Developing knowledge

PERU

Title: Molecular mechanism of *T. cruzi* virulence factors
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ICGEB Contract No.: CRP/16/012
ICGEB Reference No.: CRP/PER16-05_EC
Abstract: American trypanosomiasis also known as Chagas' disease is considered "the archetypal neglected disease" due to the lack of effort displayed worldwide in the discovery of new less toxic drugs for its treatment. An approach not yet exploited in this arena is the Macrophage Infectivity Potentiator (TcMIP) protein, recognised in 1995 to play an important role in host cell invasion. Macrophage Infectivity Potentiator proteins are proline cis-trans isomerases FKBP-like proteins secreted by the pathogenic microorganisms in order to promote cell invasion, however very little is known about its molecular mechanism. The research project comprised the following general aspects. First we performed several *in vitro* and *in vivo* assays in order to try to understand the molecular mechanism of TcMIP and its interaction with parasite collagenases. Second, in an effort to discover new inhibitors against TcMIP, we performed a large in silicon drug screening where most of our best ligand candidates were small drugs that are currently used as therapeutic agents with relatively low toxicity in humans. Finally, we performed a first NMR-drug screening with 500 molecules.
Objectives:
(i) Development of enzymatic assays in other to prove that Tc80 collagen protease activity is potentiated by TcMIP prolyl cis-trans isomerisation activity;
(ii) To use NMR spectroscopy to characterise the interaction and catalytic activity of TcMIP against collagen;
(iii) To use NMR spectroscopy to investigate the importance of TcMIP terminal helixes in collagen Binding;
(iv) To develop a new inhibitor again TcMIP.
Results Obtained:
(i) We successfully produced and purified recombinant TcMIP protein;
(ii) We set up a NMR assay to characterise TcMIP catalytic activity and interactions;
(iii) In collaboration with Dr. Javier Navarro (UTMB) we performed a TcMIP- cells interaction study;
(iv) We completed and published the TcMIP backbone NMR assignment;
(v) We performed structural analysis of TcMIP and TcMIP-drug complex;
(vi) We performed an in silicon TcMIP drug screening in collaboration with a member of our team Dr. W. Evangelista;
(vii) In collaboration with Novalix Pharma and Dr. Guy Lippens (CNRS France), we set up a first NMR drug screening (500 compounds);
(viii) We successfully produced and purified recombinant Tc80 protein;
(ix) We set up a fluorescence assay to characterise Tc80 enzymatic activity;
(x) We succeeded in setting up a real time collagen folding experiment by NMR;
(xi) We set up a convenient protocol to evaluate collagen degradation assay by electrophoresis;
(xii) We developed a new NMR method to accurate measure torsion angles on a protein.
Results Unforeseen in the Original Project:
One of our hypotheses is that the N-terminal and C-terminal TcMIP domains serve as anchors for collagen binding. We tried three different approaches. First, we investigated the interaction between recombinant TcMIP and a collagen like peptide by NMR. Second, we investigated the interaction against a rat tail collagen IV by electrophoresis. Finally, we performed a TcMIP - host cell interaction with two different human cells lines (skin fibroblast and promyelocytic HL60). Neither of our experiments revealed a clear interaction between TcMIP and the collagen, even more TcMIP does not seem to interact with the cells, in contradiction with Moro *et al*. data. In the collagen triple helix structure it has been reported that all prolines are in trans conformation, in consequence the slow cis-trans prolyl bond isomerisation is the kinetic limiting step for collagen folding. One of our main hypotheses is that TcMIP prolyl cis-trans isomerase activity accelerates the folding and defolding of collagen making it more accessible to collagenase catalytic pockets of Tc80. First, we set up a convenient protocol to evaluate collagen degradation assay by electrophoresis. Our results shown that TcMIP plays an important role in collagen degradation, enhancing the catalytic activity of Tc80. In order to understand the TcMIP molecular mechanism we have implemented a collagen refolding experiment on a collagen-like peptide by real time NMR. However, the experiment showed only marginal acceleration of the folding process. This result could not explain the observed enhancement of the Tc80 catalytic activity.
Publications:

Lopez, J.M., Antiparra R., Lippens, G., Zimic, M., Sheen, P., y Maruenda, H. Backbone chemical shift assignment of macrophage infectivity potentiator virulence factor of *Trypanosoma cruzi*. 2019. Biomol. NMR Assign. **13(1)**, 21-25

POLAND

Title: Mesenchymal stem cells and myxoma virus in oncolytic melanoma therapy
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Abstract: Oncolytic viruses are capable of targeting neoplasms triggering tumour-lytic action and immune effects. However, systemic delivery of benevolent viruses to tumours has remained a challenge. Therapeutic myxoma virus (MYXV) constructs were thus delivered by human bone marrow-derived mesenchymal stem cells (MSCs). We showed that MSCs were permissive enough to MYXV infection *in vitro* and transferred MYXV to co-cultured melanoma cell lines caused killing of cancer cells. Intravenous injection of MYXV-infected (both directly and *via* MSCs) B16-F10 cells strongly reduced development of pulmonary melanoma foci in naïve mice. Systemic administration of MSCs loaded with luciferase-expressing MYXV showed accumulation and persistence of virus in lungs of lesion-bearing mice, unlike administration of naked MYXV. Therapy of experimental pulmonary melanoma foci with IL-15-encoding MYXV shielded by MSCs resulted in pronounced lesion regression and extended survival; NK cell levels in blood of treated mice confirmed innate immune response against unprotected virus, absent when MSC-protected virus was used. Increased lung infiltration by NK cells early during therapy correlated with significant adaptive response and influx of CD8+ cytotoxic T cells into treated lesions. MSCs enable efficient systemic ferrying of therapeutic MYXV to experimental pulmonary melanoma foci in treated animals.
Objectives:
The overall scientific objective of this project was to seek answers about the feasibility of constructing a working mesenchymal stem cell-based delivery system shielding an oncolysis-triggering and immune response-enhancing myxoma virus constructs allowing systemic oncovirotherapy of metastatic melanoma. The specific objectives were: (i) to develop a working protocol for isolation and characterisation of MSCs from human bone marrow material; (ii) to verify infectiveness and permissiveness status (for vMyxEGFP reporter construct) of all types of cells employed in preparation and performance of the investigated therapeutic system (MSCs and melanoma; (iii) to assess characteristics of MSC-entrapped reporter MYXV construct transfer to various cultured melanoma cell lines; (iv) to characterise in more detail the prerequisites for MSC-entrapped therapeutic MYXV construct (i.e. infected MSCs) systemic delivery to disseminated melanoma foci; (v) to assess by *in vivo* imaging virus accumulation and persistence in lungs of lesion-bearing mice administered intravenously with MSCs carrying luciferase-encoding reporter MYXV construct; (vi) to verify the extent of preventing formation and growth (inhibitory effect) of melanoma foci in murine lungs when infusing melanoma cells that had been previously co-cultured with MYXV-infected MSCs; (vii) to carry out the therapeutic experiments in order to substantiate the actual impact of MSC-entrapped MYXV oncovirotherapy on growth kinetics of experimental melanoma lung lesions as well as to examine animal survival; (viii) to investigate the parameters of early and late antitumor immune response triggered by systemic administration of MSC-shielded therapeutic MYXV construct; (ix) to correlate, using transmission electron microscopy (TEM), possible myxoma virus physical presence in lung tissues from mice burdened with experimental melanoma foci following systemic administration of MSC-shielded therapeutic MYXV construct.
Results Obtained:
As a prelude to the proposed therapy we streamlined isolation and culture of human bone marrow-derived material, characterised obtained MSCs and tested infectiveness and permissiveness of human and murine cell lines explored in the generation of the proposed therapeutic system. Transfer of MYXV into cultured murine or human melanoma cells (i.e. infection) was examined using recombinant viral constructs encoding reporter fluorescent proteins (vMyx-EGFP or vMyx-EGFP/tdTr). We further investigated the ability of infected MSCs to transfer MYXV constructs to co-cultured human or murine melanoma cells and tested cytopathic effects exerted by MYXV constructs.
Our data show that both human MSCs and the tested human/murine melanoma cell lines are permissive to MYXV infection. However, melanoma cells were destroyed rapidly by the viral infection whereas MSCs remained viable (~90%) for at least 2 days post-infection (which largely exceeds transit time in bloodstream that is anticipated for therapeutic constructs under investigation).
We found out that MSCs can indeed pass infection to target melanoma cells and thus serve as suitable cell carriers (i.e. “Trojan horse”) to ferry more advanced therapeutic MYXV constructs to sites of disseminated cancer such as metastatic melanoma.

Developing knowledge

Inhibitory effect on melanoma foci formation in murine lungs was examined and demonstrated using infused melanoma cells co-cultured with MSCs that had been pre-infected with MYXV.
Using luciferase-encoding reporter MYXV we demonstrated virus accumulation and persistence in lungs of lesion-bearing mice administered intravenously with MSCs pre-infected with this construct.
Presence of MYXV in melanoma foci in lung tissues excised from mice administered MSC-shielded MYXV construct was confirmed by transmission electron microscopy (TEM).
Treatment of experimentally-induced melanoma lung lesions with IL-15-carrying MYXV therapeutic construct delivered by MSCs led to their marked regression. Immune response was assessed by examining levels of NK, CD4+ and CD8+ cells in blood and lungs. Increased NK cell levels in blood indicated robust innate responses only against unshielded virus. Infiltration of lungs by NK cells was subsequently followed by inflow of CD8+ T lymphocytes and led to further elimination of melanoma lesions. We also noted extension of survival of the treated mice as compared to control animals.

Results Unforeseen in the Original Project:

Two main unforeseen results have been obtained: (i) An increased number of foci in mice injected with B16-F10 cells first co-cultured with uninfected MSCs seemed to confirm some reports that MSCs might help sustain tumour growth and contribute to metastasis. What is relevant, however, is that development and growth of lung foci in mice injected with melanoma cells was strongly inhibited both when melanoma had been first pre-loaded *ex vivo* directly with MYXV or when they had been first co-cultured with MYXV-infected MSCs. Indeed, virus confinement to MSCs did not abrogate tumour growth-inhibitory power of oncolytic MYXV.
(ii) The confirmation, by transmission electron microscopy (TEM), of MYXV presence in melanoma foci in lung tissues excised from mice administered MSC-shielded MYXV construct.

Publications:

Hadryś, A., Sochanik, A., McFadden, G., Jazowiecka-Rakus, J. Mesenchymal stem cells as carriers for systemic delivery of oncolytic viruses. 2020. Eur. J. Pharmacol. **874**, 172991

ROMANIA

Title: Iba1 macrophages contribution to peripheral neuropathic pain development
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ICGEB Contract No.: CRP/16/014
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Abstract: Neuropathic pain is a complex syndrome caused by a primary lesion or dysfunction in the peripheral or central nervous system. Injury to peripheral nerves triggers activation of endogenous macrophages and infiltration of hematogenous macrophages at the nerve and dorsal root ganglia (DRG) level and activation of microglia at the central level. At the DRG level, after spinal nerve ligation (SNL) as a model of peripheral nerve lesion, Iba1 (+) endogenous macrophages proliferate, enlarge their cell bodies, exhibit extensions and cluster as peri-neuronal rings around neurons, possibly contributing to neuronal sensitisation and neuropathic pain development. In this study we showed that specific silencing of Iba1 protein with siRNA technology significantly reduced the SNL-induced hypersensitivity to mechanical and thermal stimuli, by disorganising the peri-neuronal ring around DRG neurons and by promoting the Iba1 (+) macrophages switch from M1 pro-inflammatory, to M2 anti-inflammatory phenotype. Our data show that Iba1 protein from dorsal root ganglia macrophages could represent a new therapeutic target for neuropathic pain associated with peripheral lesions.
Objectives:
(a) Determine the consequences of intra-ganglionic delivery of Iba1 siRNA on the SNL-induced neuropathic pain.
(a.i) Is the intra-ganglionic delivery of naked Iba1 siRNA reducing SNL-induced pain behaviour?
(a.ii) Is the activation profile of Iba1 (+) macrophages altered after siRNA delivery?
(b) Investigate how the Iba1 (+) macrophages communicate with the DRG neurons after SNL and if this is altered by the Iba1 silencing.
(b.i) Do Iba1 (+) macrophages develop special contact zones between them, with the adjacent neurons or satellite cells?
(b.ii) Do ATP and P2 receptors mediate the communication between Iba1 (+) macrophages and DRG neurons?
Results Obtained:
The results showed that intra-ganglionic delivery of naked Iba1 siRNA at the level of L5 DRG was associated with silencing efficacy to 76% at the mRNA level, and 88% silencing efficacy at the protein level. Silencing Iba1 was associated with a significant reduction of SNL-induced mechanical and cold allodynia 5 days after surgery, confirming our initial hypothesis that endogenous Iba1 (+) macrophages contribute to pain by activating/sensitising DRG neurons. In preliminary experiments we showed that Iba1 macrophages activated after SNL surgery cluster mainly around large A-neurons, which are mechano-sensitive, and also around IB4 (+) neurons which are thermo-sensitive (Ton et. al., 2013), although in a more reduced number. Our behavioural data confirm this observation, with both mechanical and cold sensitivity being significantly improved after Iba1 silencing.
To better understand how the peri-neuronal ring forms around DRG neurons, and if Iba1 (+) macrophages develop special contact zones between them, with the adjacent neurons or satellite cells, we investigated its structure with electron microscopy and immunohistochemistry. The electron microscopy data showed that when macrophages clustered around neurons they did not establish direct contact between them: they did come in very close vicinity between them, but they did not actually touched. More specifically, after SNL, Iba1 (+) macrophages may form a peri-neuronal ring outside the neurons, locate very close to DRG neurons, in a pocket formed by satellite cells, or locate inside the cytoplasm of DRG neurons. After Iba1 silencing the peri-neuronal ring was loosely disorganised, with macrophages located only under the satellite cells sheath and not inside the cytoplasm of neurons. This altered structure of the peri-neuronal ring was additionally confirmed by immunohistochemistry, which showed that mainly the ring around large, NF200 (+) DRG neurons is disorganised, while the data for CGRP (+) neurons were not so conclusive, yet.
The analgesic effect of Iba1 silencing was not due only to a disorganised structure of the peri-neuronal ring, but also to a molecular switch of Iba1 (+) macrophages from M1 pro-inflammatory, to M2 anti-inflammatory phenotype, as confirmed by the genic expression of specific markers and similar amplitude of P2x7 receptors at their level. The uncoupling between P2x7 receptors and the inflammasome as an additional confirmation of the molecular switch from M1 to M2 phenotype was not confirmed at this stage, thus requiring additional investigations.

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In conclusion, we showed that Iba1 protein is a new molecular target for neuropathic pain associated with peripheral lesions. Its specific silencing by siRNA technology reduces the sensitising effect of activated macrophages by dispersing them from around the neurons, and by changing their phenotype from M1 pro-inflammatory to M2 anti-inflammatory.

Results Unforeseen in the Original Project:

The unexpected results of this project was the engulfed position of macrophages inside DRG neurons cytoplasm after spinal nerve ligation lesion, quite similar to emperipolesis, a process in which an intact cell is present within the cytoplasm of another cell. We don't know yet its functional significance.

SERBIA

Title: New synergistic strategy to treat chronic wound infections
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Abstract: Chronic wounds are frequent complications in individuals with diabetes mellitus. The leading cause of chronic wound infections is *Pseudomonas aeruginosa,* which exhibit low sensitivity to antibiotics due to formation of biofilms. Current treatments of diabetic chronic wounds remain unsuccessful and novel therapeutics are needed. We screened bacterial collections aiming to discover new anti-virulence therapeutics. Soil bacteria were poor producers of small molecules with anti-quorum sensing (QS) activity, but appeared rich source of quorum quenching enzymes that could be used for developing bioactive materials for chronic wounds prevention/treatment. We also synthetised a library of long-chained aminoquinolines (Aq) with strong anti-QS activity in *P. aeruginosa*. The most potent candidates inhibited pyocyanin production, biofilm formation and motility through competitive inhibition of PqsR. Further, 21 Aq-containing nanocomposites were synthetised and tested against *P. aeruginosa* biofilms in microtiter plates, 3D human lung infection model and artificial wounds. Anti-biofilm activity of nanocomposites depended on particular Aq derivative and on Aq-to-nanoparticles ratio. In murine model of infected diabetic wound Aq therapy caused rapid wound closure, and increased the number of phagocytic macrophages and regulatory T cells that aided wound closure.

Objectives:
The aim of this project was to develop effective therapeutic strategy for the treatment of infected chronic wounds such as diabetic wound ulcers. The main idea was to identify new anti-infective drug candidates that target bacterial virulence, particularly biofilm formation, and to combine them with bactericidal two-component nano-carriers to improve their therapeutic index and achieve effective clearance of deep tissue infection. To identify molecules with potent anti-virulence activity we planned to use two strategies: (i) screening of bacterial collection from the Laboratory for Microbial Molecular Genetics and Ecology (LMMGE) for the producers of compounds that inhibit QS system and biofilm formation in *P. aeruginosa* and (ii) chemical synthesis of compound library with anti-QS activity and detailed structure-activity study that would enable identification of structural parameters required to achieve anti-virulence activity. Once the potent anti-virulence agents were identified, our objective was to couple selected most active anti-virulence molecules with multifunctional composite nanomaterials composed of bioactive metal oxide nanoparticles and to evaluate their effectiveness in murine model of chronic wounds. Finally, we aimed to address effects of combined therapy, exhibiting both antibacterial and anti-QS activity, on wound healing and on immune response within infected wounds.

Results Obtained:
During the three years of the project we screened crude extracts obtained from 150 *Streptomyces* spp. isolates and mycelium activity of 122 *Streptomyces* spp. isolates from the LMMGE bacterial collection and identified one isolate with anti-QS activity in *Chromobacterium violaceum* CV026 disk assay. However, cultivation of this isolate on the larger scale for the active principle purification and identification revealed unstable and weak production of QS inhibitor. We have been working on optimisation of cultivation conditions and extraction process in order to gain stable production with sufficient yield of the molecule of interest for its identification and chemical characterisation. We generated two new microbial collections during the course of the project, one with bacteria from soda lake and the other with bacteria from agricultural soil. Out of 76 isolates from soda lake we found three strains with anti-QS activity and identified them as *Pseudomonas* sp., while one isolate with QS-independent anti-biofilm activity was identified as *Bacillus* sp. Chemical analysis of *Bacillus* sp. supernatant showed that the activity was combined effect of more than one compound and their activity decreased after separation. Among the isolates from agricultural soil, five bacteria showed quorum quenching (QQ) activity and ability to grow in the minimal media supplemented with acyl homoserine lactone as the sole carbon source. The genome sequencing results revealed the presence of lactonase and acyl-homoserine lactone acylase with quorum-quenching activity.
In parallel, the library of 53 novel long chain Aq derivatives was synthetised and detailed quantitative structure activity relationship (QSAR) analysis performed. All compounds have been tested for inhibition of violacein production using *C. violaceum* CV026, prodigiosin production by *Serratia marcescens*, and biofilm formation and the production of virulence factor pyocyanin in *P. aeruginosa*. In addition, all derivatives have been tested for the potential to interact with and inhibit activity of *P. aeruginosa* QS receptors (LasR, RhlR, and PqsR) using specific biosensor

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strains. All Aq8 derivatives with aromatic substitution on C8 aliphatic chain exhibited strong inhibition of pyocyanin production and PqsR activity. Only four Aq8 derivatives showed anti-biofilm formation activity.

The most active derivative inhibited biofilm formation ($BFIC_{50}$ at 50 μM), pyocyanin production (BIC_{50} at 12.5 μM) and motility in *P. aeruiginosa*. Mechanistic studies showed that most active Aq8 derivatives dose dependently reduced PqsR activity, with weak effect on RhlR activity and did not significantly affect LasR activity. Analysis of the compounds' effects on the receptors' level (*lasR*, *rhlR*, and *pqsR*), as well as synthases' transcription (*lasI*, *rhlI*, and *pqsA*) suggested that reduced levels of PQS/HHQ in the supernatants of treated *P. aeruginosa* cultures could be a consequence of reduced functionality of PqsR and/or enzymes required for quinolones' biosynthesis. Both experimental and modelling results showed that the most active compounds affected PqsR activity by competing with natural ligands for PqsR ligand-binding site. Compounds Aq12, Aq8, and two most active Aq8 derivatives were selected for conjugation with nano-carriers. Magnetic nanoparticles (NPs) formed aggregates in the presence of Aq derivatives, thus composite nanomaterials composed of bioactive metal oxide NPs and selected Aq derivatives were produced. Twenty-one nanocomposites comprising different ratio of Aq and metal oxide NPs have been prepared and their antibacterial and anti-biofilm activity tested. Neither pure NPs nor nanocomposites affected *P. aeruginosa* growth, while anti-biofilm activity of nanocomposites depended on the type of Aq derivative, as well as on Aq-to-NPs ratio. The efficacy of the most active nanocomposites combinations was further analysed in 3D human lung infection model composed of A549 cells and in artificial wound model. These results showed that pure Aq12 was the most promising compound to be used in *in vivo* chronic wound model.

In the murine model of diabetic wounds infected with *P. aeruginosa,* Aq12 had similar effects as the well-known antibiotic tobramycin but was not able to potentiate antibiotics activity when applied in combination. Along with inducing rapid wound closure, Aq12 increased the number of macrophages that phagocytised bacteria and the number of regulatory T cells that aided wound closure.

The results of this research were the major part of one diploma and two master theses, and contributed to one PhD thesis that were all defended during the course of the project. During the project lifespan two new collaborations were established and they will be continued after the project completion through new grant applications.

Results Unforeseen in the Original Project:

Biofilms that form during chronic infections are usually composed of different microorganisms including both bacteria and fungi. The species that are most commonly encountered within mixed biofilms are *P. aeruginosa* and *Candida* sp. We found that several Aq derivatives inhibited *C. albicans* biofilm formation and also showed inhibitory activity against pre-formed biofilms. Certain Aq derivatives also inhibited formation of mixed *P. aeruginosa* and *C. albicans* biofilms. These results set the bases for the future research on developing novel therapy against mixed species infections.

Publications:

Aleksic, I., Jeremic, J., Milivojevic, D., Ilic-Tomic, T., Šegan, S., Zlatović, M., Opsenica D., Senerovic, L. N-benzyl derivatives of long-chained 4-Amino-7-chloro-quionolines as inhibitors of pyocyanin production in *Pseudomonas aeruginosa*. 2019. ACS Chem. Biol. **14(12)**, 2800-2809

SLOVENIA

Title: Lysosomal proteases in semaphorin signalling and cell polarity
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Abstract:
Protease signalling is an irreversible way of signal transduction, where the signal is transmitted through proteolytic processing of their substrates, resulting in their activation, inactivation or modulation of their function. Proteases thus control a great variety of physiological processes that are critical for life, including the immune response, cell cycle, cell death, wound healing and protein and organelle recycling. They have been also found to be critical in a number of pathologies, including cancer and inflammation. However, we are still very far from a clear understanding of the molecular signalling pathways including those leading to disease progression, which is largely linked with very limited information available on the physiological substrates of proteases.
In cancer, proteases are secreted from cancer and immune cells, thereby modulating tumour microenvironment including extracellular matrix and contributing to tumourigenesis. One of the processes associated with protease secretion into the extracellular milieu is proteolytic removal of the extracellular domains of transmembrane proteins, known also as ectodomain shedding. The process has been shown to have a major role in homeostasis and in a number of diseases, including cancer. Recently we have been able to demonstrate that cysteine cathepsins B, L and S, a group of proteases normally residing within the lysosomes, are critically involved in this process, and identified a subset of cca 15 transmembrane or membrane-anchored proteins as cathepsin substrates. Furthermore, since cathepsins are known to be major contributors to cancer progression and metastasis with relatively unknown molecular mechanisms of their involvement, they were thereby excellent targets for our project.
In our project, we have identified a number of membrane-anchored proteins that are shed from cancer cells by cathepsin K and legumain. A vast majority of cathepsin K targets are identical to those of cathepsins S and L, whereas legumain was not found to cleave any Cell Adhesion Molecule (CAM). Among the targets, MMP14 seems to be a unique cathepsin K target and physiological relevance of this process is currently further investigated as it may present an important novel link between extracellular cathepsins and metalloproteases in cancer. In addition, cathepsin involvement in the regulation of complement activation in cancer seems extremely exciting. Cathepsins, in particular cathepsins S and L, were thus found to blunt complement activation already at the level of C1q binding, thereby primarily affecting the classical complement activation pathway, which has a major role in cancer. Several potential binding partners of C1q have been identified by proteomic approaches and are currently under validation. On the basis of our results it is possible to speculate that secreted cathepsins in the tumour microenvironment may have an important role in blunting complement activation that may have important consequences not only for understanding of cancer biology, but potentially also for cancer immune therapy, as a number of membrane anchored proteins are targeted that way. Finally, we were able to identify the primary cathepsin cleavage site in EGFR and are evaluating the consequences of this shedding, which seems to affect the MAP kinase pathway. These findings have thus revealed some completely new and unexpected roles of extracellular cathepsins in cancer and present excellent opportunity for further studies.
Publications:
Vidak, E., Javoršek, U., Vizovišek, M., Turk, B. Cysteine cathepsins and their extracellular roles: Shaping the microenvironment. 2019. Cells **8(3)**, pii: E264
Vizovišek, M., Fonović, M., Turk, B. Cysteine cathepsins in extracellular matrix remodelling: Extracellular matrix degradation and beyond. 2019. Matrix Biol. **75-76**, 141-159

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