Research at the Molecular Oncology Programme (MOP) aims to discover the genetic determinants that contribute to cancer onset and progression, as well as to provide new ideas and tools for the development of innovative therapies for cancer patients. To do so, we have Groups covering a wide range of topics in cancer research, such as DNA and chromosome stability (Maria A. Blasco, Óscar Fernández-Capetillo, Felipe Cortés-Ledesma and Ana Losada), oncogenes and cell cycle kinases (Mariano Barbacid), DNA replication (Juan Méndez), melanoma (Maria S. Soengas), metabolism and cell signalling (Nabil Djouder, Alejo Efeyan and Guadalupe Sabio), immunotherapy (Maria Casanova), epithelial carcinogenesis (Francisco X. Real) and metastasis (Manuel Valiente, Eva González-Suárez and Héctor Peinado). In 2023, Marcos Malumbres’ Group left CNIO to join the VHIO community in Barcelona. I am grateful to have shared many years of science at the MOP with him, and he will certainly be missed. At the same time, Marcos is a very original and creative scientist, and I am confident that he will help to strengthen the importance of basic research at the VHIO.

During 2023, our scientists reported relevant contributions in many areas, and here I provide a few selected examples of their work. For instance, Mariano Barbacid’s Group provided some of the first mechanistic insights as to how resistance to RAS inhibitors might arise and showed that RAS depletion might be more efficacious than its inhibition. On a related theme, and in collaboration with C. Blanpain’s team (Université Libre de Bruxelles, Belgium), Juan Méndez’s Group revealed how the EMT influences the response to genotoxic chemotherapies. The Malumbres’ Group reported on novel roles for phosphatases and kinases, previously related to the cell cycle, to aspects such as pluripotency or nutrient signalling. Eva González-Suárez’s Group keeps making significant advances on the potential of targeting RANK signalling, particularly in the context of breast cancer. In addition, the Group led by Maria A. Blasco presented evidence that supports the potential of targeting telomeres from cells in the tumour microenvironment for cancer therapy. Héctor Peinado and his team identified how a factor related to angiogenesis might be a biomarker and potentially a target for the treatment of some aggressive soft-tissue sarcomas. Ana Losada’s team made important conceptual advances in our understanding of how different cohesin complexes are assembled, critical for overall genome structure. The Group of Francisco X. Real maintains an important focus on elucidating the role that transcription factors play in pancreatic cancer onset and progression and, in 2023, they showed that NFIC is important for coordinating the transcriptional response to ER stress and suppressing the occurrence of pancreatic adenocarcinomas. To end, I would like to single out work done by Manuel Valiente’s Group, since it pioneered in showing how cancer cells in brain metastases interact with neurons and how this can potentially explain their impact on the cognitive decline observed in cancer patients. In summary, scientists at the MOP keep making important contributions to their fields of research, some of which open new areas and directions for others to follow in the future.

Our scientific excellence is exemplified by the recognition that our scientists receive. Notable examples for 2023 include the Premio Nacional de Investigación awarded to Mariano Barbacid, and the Fundación Banco de Sabadell Award to Manuel Valiente. Congratulations to both, once again. In addition, Maria Casanova received an ERC Starting Grant for her research on circadian regulation of immune responses. Best of luck to her in making the most of this important support. To end, and while we, the Principal Investigators, often receive most of the attention, this should not hide the fact that these recognitions largely rely on the hard work done by the students, technicians, postdocs, and investigators in our Groups. Thanks to you all.

“Basic and fundamental research has been behind most cancer therapies that have reached patients. And we, at the MOP, are doing our share to contribute to this effort. Keep it up.”
OVERVIEW

Immortality is one of the most universal characteristics of cancer cells. We study the mechanisms by which tumour cells are immortal and normal cells are mortal. Telomerase is an enzyme present in over 95% of all types of human cancers and absent in normal cells in the body. Telomeres, nucleoprotein complexes located at the ends of chromosomes, are essential for chromosome protection and genomic stability. Telomeres shorten progressively with organism ageing, leading to ageing. If telomeres are altered, adult stem cells have a maimed regenerative capacity.

Our research focuses on:

- Mouse models to validate telomeres and telomerase as therapeutic targets for cancer and age-related diseases.
- Interplay between telomeres and DNA repair pathways.
- Role and regulation of non-coding telomeric RNAs (TERRA).
- Telomerase gene therapy in telomere syndromes and age-related diseases.
- Role of telomerase and telomeres in:
  - adult stem cell biology
  - nuclear reprogramming of differentiated cells to iPS cells.

“Removing immortality from cancer cells by targeting their telomeres is a yet unexploited therapeutic strategy in the fight against cancer that we showed might be effective to treat non-small cell lung carcinoma.”
Telomerase deficiency and dysfunctional telomerases in the lung tumour microenvironment impair tumour progression in lung cancer

Lung cancer is the leading cause of cancer death. The long-term ineffectiveness of current therapies and late-stage diagnosis result in a five-year survival rate of about 20%. Non-small-cell lung cancer (NSCLC) accounts for 85% of lung-cancer-associated deaths. Focusing on the so-called tumour microenvironment, a set of cells and factors surrounding the tumour and playing a crucial role in the development of cancer and the response to therapies, we used a combination of NSCLC mouse models and patient-derived xenografts to address the effects of telomerase deficiency and the anti-tumour activity of 6-thio-2’-deoxyguanosine (6-thio-dG), a nucleoside analogue that leads to telomere dysfunction, genomic instability, and cell death.

We showed in mice that telomerase deficiency and 6-thio-dG-induced telomere dysfunctionality reduced lung tumour implantation and vascularisation, and increased DNA damage response, cell cycle arrest and apoptosis, while it reduced proliferation, inflammation, and lung tumour immunosuppression and invasion. 6-thio-dG-treated human NSCLC xenografts exhibited increased telomere damage, cell cycle arrest and apoptosis, as well as reduced proliferation, resulting in reduced tumour growth. Targeting telomerases might thus be an effective therapeutic strategy in NSCLC.

A link between short telomerases in alveolar type II cells and lung fibrosis in post-COVID-19 patients with cancer

The severity of COVID-19 increases with each passing decade of life, suggesting that organisational ageing contributes to the fatality of the disease. We and others previously showed that COVID-19 severity correlates with shorter telomeres, a molecular determinant of ageing, in patients’ leukocytes. Lung injury, a predominant feature of acute SARS-CoV-2 infection, can further progress to lung fibrosis in post-COVID-19 patients. Short or dysfunctional telomerases in alveolar type II (ATII) cells suffice to induce pulmonary fibrosis in mouse and humans. Our analyses of telomere length and histopathology of lung biopsies from a cohort of alive post-COVID-19 patients revealed a loss of ATII cellularity and the presence of shorter telomerases in ATII cells, concomitant with a marked increase in fibrotic lung parenchyma remodelling in post-COVID-19 patients. Our findings uncovered a link between presence of short telomerases in ATII cells and long-term lung fibrosis sequelae in post-COVID-19 patients.

Expanding the hallmarks of ageing

Aging research explores the decline in function of organisms during adulthood. In a joint effort with 4 other groups, in 2013 we suggested 9 molecular, cellular, and systemic hallmarks of ageing: telomere attrition, DNA instability, epigenetic alterations, loss of stress resistance and homeostasis, mitotic catastrophe, accumulation of damage, and cellular senescence. Since then, several studies have extended and updated these hallmarks of ageing. To address the effects of telomerase deficiency and the anti-tumour activity of 6-thio-dG on the biology of cancer cells, we performed a comprehensive analysis of telomere length and histopathology of lung tissue from a cohort of age-matched controls with lung cancer and patients with both NSCLC and lung fibrosis in post COVID-19 patients with cancer.

Our analyses of telomere length and histopathology of lung biopsies from a cohort of alive post-COVID-19 patients and a cohort of age-matched controls with lung cancer have now revealed a loss of ATII cellularity and the presence of shorter telomerases in ATII cells, concomitant with a marked increase in fibrotic lung parenchyma remodelling in post-COVID-19 patients. Our findings uncovered a link between presence of short telomerases in ATII cells and long-term lung fibrosis sequelae in post-COVID-19 patients.
The main thrust of our laboratory is to identify therapeutic strategies against KRAS mutant lung and pancreatic tumours. In recent years, inhibitors against KRAS oncoproteins selective for some of their mutations such as G12C and G12D, as well as pan-KRAS inhibitors active against all mutations, have been either approved by the FDA (sotorasib and adagrasib) or are undergoing clinical trials. Yet, their clinical efficacy is far from what was expected. In lung cancer patients, sotorasib does not increase overall survival compared to standard chemotherapy regimens due to the rapid appearance of tumour resistance. We have used genetically engineered mouse models of lung and pancreatic tumours to compare the therapeutic efficacy of KRAS ablation with that of KRAS inhibition, and to interrogate the molecular mechanism responsible for tumour resistance. Whereas ablation of KRAS oncogenes eliminates both lung and pancreatic tumours completely with no signs of tumour resistance, KRAS inhibition results in the rapid appearance of resistance as previously observed in human tumours. We are currently exploring whether inhibiting KRAS signalling at independent nodes within its downstream or upstream signalling pathways, as well as in orthogonal pathways, will not only increase tumour responses but also prevent the appearance of tumour resistance.
Kras oncogene ablation induces complete regression of advanced Kras/Trp53-driven lung adenocarcinomas

We have interrogated whether continuous expression of the Kras oncogene was essential for tumour progression and maintenance as well as for the appearance of tumour resistant cells. To this end, we modified the Kras conditional allele by inserting loxP sites flanking the first exons sequence that encompass the G12V activating mutation. In addition, we added 2 independent loci encoding the inducible CreERT2 recombinase. The resulting mice, Kras<sup>Flox/delta</sup>-CreERT2<sup>Flox/s</sup>/<sup>Flox/s</sup>;Ty<sub>SUBC-CreERT2<sup>Flox/s</sup>/<sup>Flox/s</sup></sub> (Kras<sup>Flox/delta</sup>-CreERT2<sup>Flox/s</sup>/<sup>Flox/s</sup>;Ty<sub>SUBC-CreERT2<sup>Flox/s</sup>/<sup>Flox/s</sup></sub>) developed lung tumours with the same incidence and latency as their parental strain when exposed to Adeno-FLPo particles. Exposure of 76 Kras<sup>Flox/delta</sup>-CreERT2<sup>Flox/s</sup>/<sup>Flox/s</sup> mice harbouring 156 lung tumours ranging in size from 0.13 to 43 mm<sup>3</sup> to a tamoxifen (TMX) diet for 1 month resulted in increased percentage of tumour burden after 1 month of TMX exposure, reaching 64.9% of the resulting mice, Kras<sup>Flox/delta</sup>-CreERT2<sup>Flox/s</sup>/<sup>Flox/s</sup>;Ty<sub>SUBC-CreERT2<sup>Flox/s</sup>/<sup>Flox/s</sup></sub> mice. Continuous exposure of these tumour-bearing mice to sotarolab 1 for 1 month (100 mg/kg), a dose equivalent to that used in the CellBreak100 clinical trial, resulted in the appearance of resistant tumours in all treated animals after 4 to 12 weeks of treatment. Resistant tumours did not differ from untreated controls, with the exception of the degree of apoptotic cells that remained elevated. Finally, resistant tumours displayed a clear trend towards higher histological grades.

Sotarolab resistant tumours display amplification of the Kras<sup>G12V</sup> allele and elevated levels of drug metabolism pathways

To identify the mechanisms associated with resistance to sotarolab, we submitted resistant tumours to WES analysis. We could not detect any of the driver mutations described in a fraction of human resistant tumours. However, we identified robust amplifications of the genomic region chromosom 6 encompassing the Kras locus in the resistant tumours analysed, suggesting that amplification of the mutant Kras<sup>G12V</sup> allele is a major driver of sotarolab resistance in this experimental model.

To further characterise these sotarolab-resistant tumours, they were submitted to RNAseq analysis. Gene Set Enrichment Analysis (GSEA) of differential gene expression revealed upregulation of gene sets involved in the metabolism of drugs by cytochrome P450 (CYP450) and glutathione S-transferases (GSTs), as well as proliferation-related pathways. These results suggest that resistance could, at least partially, emerge as a consequence of an altered metabolism of sotarolab, resulting in its detoxification and reducing its effect in tumour cells. To determine whether these results could be translated to human tumours, we implanted pieces derived from a patient-derived Kras<sup>G12V</sup>-positive xenograft (PDX) lung tumour model in immunocompromised mice. Four mice carrying a PDX piece in each flank were treated with vehicle, whereas 3 mice implanted with 6 pieces were treated with 100 mg/kg of sotarolab. These PDX tumours became resistant to the drug at about 150 days of treatment and were subsequently submitted to WES analysis. Interestingly, we did not identify amplifications in the Kras oncogene or additional driver mutations as previously described in clinical samples. Yet, RNAseq analysis also revealed upregulation of a pathway related to the metabolism of xenobiotics. In sum, our results revealed that inhibition of the molecular events responsible for tumour resistance, at least in this experimental model, will be difficult to be overcome using pharmacological strategies. Instead, they suggest that resistance to KRAS inhibitors might be prevented, or at least ameliorated, by achieving a more robust inhibition of KRAS signalling, mimicking the results obtained upon Kras ablation.
The Genomic Instability Laboratory is interested in understanding the molecular mechanisms causing cancer and other age-related diseases, in order to provide the knowledge needed to develop novel treatments for these diseases. Initially, we focused on the study of replicative stress, a type of DNA damage that fuels genomic instability and is present in many types of cancer. Those studies led to important contributions to basic research and also led to the development of potent and selective ATR inhibitors that were transferred to the pharmaceutical industry for clinical development. Subsequent to elucidating the mechanisms of resistance to ATR inhibition by genetic screens, our Group gradually developed an interest in understanding how cancer cells develop resistance to therapies, and how we can target therapy-resistant cancer cells. In addition, we are actively involved in exploring the contribution of nucleolar stress to cancer and neurodegeneration.

“In 2023, we discovered new biomarkers that predict sensitivity to SETD8 inhibitors and significantly advanced in our understanding of nucleolar stress as a driver of ageing and neurodegeneration.”
RESEARCH HIGHLIGHTS

Targeting SETD8 in tumours with high rates of ribosome biogenesis

A large number of the driver mutations found in tumour cells occur in genes related to chromatin regulation, a fact particularly relevant for paediatric tumours, which frequently harbour mutations linked to cell fate and differentiation. These findings have revitalised the efforts to develop drugs targeting epigenetic regulators ("epi-drugs") and today, epigenetics is a very active area in the development of cancer therapies. In this regard, SETD8 is a histone methyltransferase known to play important roles in DNA replication and repair, and is overexpressed in a wide range of cancers. Moreover, SETD8 has been identified as a specific vulnerability of several tumours of bad prognosis, such as neuroblastoma or MYC-driven medulloblastoma. This research triggered additional efforts to develop SETD8 inhibitors, and several compounds have already been generated. However, the available molecules present poor pharmacological properties and none have progressed to clinical development. In our Group, we have discovered novel SETD8 inhibitors (SETD8i) and performed the first steps to characterise them. In 2023, we completed several CRISPR screens using both chemical and genetic strategies to target SETD8. These efforts revealed that the toxicity of SETD8 inhibitors is highest in cells with increased levels of nucleolar activity. Accordingly, these compounds are particularly efficacious for the killing of cancer cells with high MYC or mTOR activity. Conversely, their toxicity is alleviated upon MYC depletion or rapamycin treatment (FIGURE 1).

Nucleolar stress as a driver of ageing

Ribosome biogenesis is the most energy-demanding activity in a cell and takes place in the nucleolus. Accordingly, abnormalities in nucleolar activity or structure, collectively known as nucleolar stress (NS), have often been found in patients with several human diseases such as cancer or neurodegeneration. Although P53 contributes to NS toxicity, this stress ultimately kills cells by P53-independent mechanisms that remain to be deciphered. To investigate how NS triggers cellular toxicity, our Group used (PR)n arginine-rich peptides, found in patients with amyotrophic lateral sclerosis (ALS) and other neurodegenerative pathologies, as inducers of this perturbation. We previously showed that PR-peptides accumulate at nucleoli and impair rRNA processing. These observations led us to hypothesise that reduced amounts of mature rRNA molecules could trigger an accumulation of free r-proteins. Indeed, proteomic analyses of the ribosome free-fraction of cells expressing (PR)97 peptides allowed us to confirm a significant increase in the levels of free r-proteins. Furthermore, targeting r-protein synthesis by mTOR inhibition or MYC depletion, the 2 main known regulators of ribosome biogenesis, alleviates (PR)n toxicity in several cell lines. In mice, systemic expression of (PR)97 drives widespread NS and accelerated ageing (FIGURE 2), which is alleviated with rapamycin. Importantly, we discovered that the generalised accumulation of free r-proteins is a common outcome of NS, independent of its source. Overall, our work reports a unifying model to explain how NS kills cells independently of P53 and provides the first in vivo evidence to illustrate that NS accelerates ageing in mammals.
OVERVIEW

We have a general interest in understanding how DNA topoisomerase activity is regulated to integrate different aspects of genome dynamics, how an imbalance in these processes can lead to the appearance of pathological DNA breaks, and how cells specifically respond to these lesions to maintain genome stability. In this sense, topoisomerase-induced DNA breaks are emerging as important drivers of oncogenic transformation. Moreover, since drugs that target topoisomerase activity are widely used chemotherapeutic agents, our discoveries have direct implications in cancer treatment.

Recently, we opened a new research line aimed at developing novel methods for sequence-specific nucleic-acid detection based on CRISPR-Cas technology, with the idea of producing sensitive and versatile genetic diagnostic kits and devices that can be implemented in a point-of-care setting.

“We have developed 2 different technologies with unprecedented sensitivity for nucleic acid detection, which will constitute the basis for future point-of-care testing of genetic cancer biomarkers.”
A topological model for the estrogen transcriptional response

Estrogen response is a well-characterised mechanism of transcriptional regulation with important implications in breast and ovarian cancer that involves an acute response with strong changes in chromatin organisation. We therefore reasoned that it could be an interesting process to study the contribution of DNA topoisomerases. By applying novel methodology developed in the laboratory to measure topoisomerase activity, we uncovered the topological principles regulating estrogen response (FIGURE 1). Thus, under non-induced conditions, topoisomerases maintain low levels of topological stress (supercoiling) in enhancer regions. However, upon exposure to estrogen, recruitment of the estrogen receptor (ER) to target enhancers results in a local inhibition of topoisomerase activity and an accumulation of supercoiling that increases the contacts with their target promoters and, therefore, subsequent transcriptional stimulation. This provides a first example in which topoisomerase activity is physiologically downregulated to promote long-range regulatory chromatin contacts and gene expression. The manuscript reporting these findings is currently under review. We will now explore the direct implications of this model in breast and ovarian cancer, with the aim of developing more potent and safer therapeutic treatments.

Novel method for point-of-care genetic testing

The capacity of CRISPR-Cas systems being programmed to recognise specific nucleic acid sequences has boosted their biotechnological applications. One of them is the detection of the genetic material of pathogens or genetic markers in diagnosis. Systems for the detection of specific nucleic acid sequences based on CRISPR-Cas technology have recently been developed and promise to revolutionise point-of-care diagnostics in the near future. These systems rely on the fact that, upon recognition and cleavage of the desired target, which is highly specific and easily programmable, the Cas protein becomes activated with a sequence-independent, unscheduled nucleolytic activity that can be easily detected with nuclease reporter substrates, whose signal can therefore be used as a readout for the presence of the given nucleic acid of interest. These CRISPR-Cas diagnostics, however, despite their great specificity and versatility, are currently limited by their levels of sensitivity, which are outside the range of the concentrations required for diagnostic purposes, and currently rely on pre-amplification of the target sequences by methods such as PCR or LAMP. This introduces a complication to the reactions, limiting their current use in point-of-care applications. We developed and patented a conceptually novel solution that, instead of amplifying the target nucleic acid, focuses on boosting Cas activation, so that the reaction is carried out in a single step at room temperature, providing an ideal setting for point-of-care diagnostics. The trick is to establish a chain reaction of Cas endonuclease activity (Endonuclease Chain Reaction, ECR; read “easier”) that reaches high levels of signal, even in the presence of very low amounts of the target sequence of interest. In analogy to PCR, one can use end-point measurements for qualitative detection of a nucleic acid of interest, or real time reactions for quantitative analysis, as shown in FIGURE 2. Due to its versatility in the detection of any nucleic acid of interest, this invention should constitute the platform for the development of a wide range of specific genetic testing kits and devices, including pathogen and genetic marker detection. Our next steps will be to adapt the methodology to the detection of genetic cancer biomarkers.

![FIGURE 1 Model to explain the role of topoisomerases in estrogen response. TOP2B removes supercoiling caused by basal transcription. Upon estrogen exposure, ER, together with TOP2A and ZAT1, inhibits TOP2B, allowing the accumulation of supercoiling that mediates enhancer-promoter contacts.](Image)

![FIGURE 2 ECR method for sensitive nucleic-acid detection with CRISPR-Cas. Direct detection of decreasing concentrations of a nucleic acid sequence of interest with CRISPR-Cas (left), compared to our improved method with chain amplification of Cas endonuclease activity (ECR) (right).](Image)
Our research focuses on a protein complex named cohesin that embraces DNA to mediate sister chromatid cohesion, a process essential for chromosome segregation and faithful DNA repair by homologous recombination. Cohesin also plays a major role in the spatial organisation of the genome by promoting long-range DNA looping, which in turn contributes to transcriptional regulation, DNA replication and recombination. Two variant cohesin complexes, carrying either the STAG1 or the STAG2 subunit, are present in all somatic vertebrate cells. While cells require a single complex for viability, both are necessary to fulfill embryonic development. Mutations in cohesin genes, most prominently in STAG2, have been found in several tumour types, including bladder cancer, Ewing sarcoma, and acute myeloid leukaemia. Germline mutations in cohesin and its regulatory factors are also at the origin of developmental syndromes collectively known as cohesinopathies, such as Cornelia de Lange Syndrome (CdLS). Our goal is to understand how cohesin works, how it is regulated, and how its dysfunction contributes to cancer and other human diseases.

“We identified a 13-gene signature that recognises cases with poor prognosis among Ewing sarcoma patients without detectable metastases at the time of diagnosis.”
Different NIPBL requirements of cohesin-STAG1 and cohesin-STAG2

Cohesin mediates 3D genome organisation by binding to chromatin and extruding DNA loops that become stabilised at several locations along the genome, most notably at sites bound by CTCF. In this way, the complex facilitates contacts between promoters and distal enhancers while restricting such interactions within topological associated domains (TADs). In recent years, we reported that the two versions of cohesin carrying either STAG1 or STAG2 make some specific contributions to 3D genome architecture. Moreover, we proposed that their different chromatin association dynamics underlie this specificity. STAG2 is more often found to be associated with the cohesin unloading factor WAPL, while cohesin-STAG1 is more stably retained at CTCF-bound sites. We have now found that the two complexes also respond differently to limited availability of NIPBL, the putative cohesin loader.

NIPBL activates the cohesin ATPase and is essential for loop extrusion by cohesin in vitro. Using a flow cytometry assay to measure chromatin-bound proteins and chromatin immunoprecipitation (ChIP) to map cohesin binding sites along the genome, we found that cohesin-STAG1 increases on chromatin and further accumulates at CTCF positions after NIPBL knock down, while cohesin-STAG2 diminishes genome-wide. Despite the presence of cohesin-STAG1 on chromatin, in situ Hi-C analyses reveal that loop formation is severely impaired. Based on these data, we propose that NIPBL is not required for initial association of cohesin with chromatin, as currently thought. Instead, NIPBL is an essential processivity factor for loop extrusion by cohesin. Given the more dynamic behaviour of cohesin-STAG2, this complex would have a stronger requirement of NIPBL-dependent loop extrusion activity to reach CTCF-bound stabilisation sites. Cohesin-STAG1 would get stabilised at CTCF sites even under low NIPBL levels, although in that condition is unable to form long loops. These results add to our understanding of the different behaviour of cohesin-STAG1 and cohesin-STAG2. More importantly, they provide a new perspective on the role of NIPBL on cohesin dynamics that needs to be considered when thinking of potential therapies for CdLS patients, most of which carry mutations in NIPBL (FIGURE 1).

A STAG2 dependent gene signature to predict aggressive Ewing sarcoma

Ewing sarcoma (EWS) is the second most frequent type of bone cancer in children and young adults. It is driven by a fusion protein, most often EWS-FLI1, which encodes a neomorphic transcription factor that rewire the transcriptome of the cell initiating the tumour. It is a highly aggressive cancer with a 5-year survival below 30% in patients that present metastasis. The prognosis is generally better for patients with localised tumours at diagnosis, but around 25% of these patients do not respond well to therapy and show poor survival. Among the few recurrent mutations identified in EWS, in addition to oncogenic fusion, are those that inactivate STAG2. These mutations are often present in the most aggressive EWS tumours, suggesting that loss of cohesin STAG2 facilitates the acquisition of the aggressive phenotype. We have therefore asked whether we can identify a STAG2-dependent gene signature to predict the prognosis of patients that do not present detectable metastases when they are first diagnosed.

We generated isoionic EWS cell lines with and without STAG2 and compared their transcriptomes with those of EWS patients carrying or not STAG2 mutations. Out of 235 genes commonly deregulated after STAG2 loss, we selected 68 genes that showed significant correlation with patient survival. The gene list was further reduced based on proteomic data and additional features such as drugability. The final gene signature consists of 13 genes and identifies cases with worse prognosis among patients that present localised disease at diagnosis (FIGURE 2). We are currently exploring the contribution of these genes to the metastatic phenotype of EWS cells. ■

**FIGURE 1** Model for the consequence of NIPBL mutation in CdLS. Proper balance between loop-extruding cohesin-STAG2, bound by NIPBL, and WAPL-mediated release of this complex is important for transcriptional regulation (healthy, left). In CdLS, NIPBL function is impaired (dashed arrow), resulting in reduced gene expression (CdLS, right).

**FIGURE 2** A 15-gene signature to predict outcome of Ewing sarcoma patients. Overall survival for patients with localised Ewing sarcoma stratified by expression of the 13-gene signature comprising 9 up-regulated genes (in blue) and 4 down-regulated genes (in red) whose expression depends on cohesin-STAG2.
OVERVIEW

Our Group studies the fundamental mechanisms of DNA replication and how the replicative process adapts to cell type-specific transcriptional programmes and chromatin organisation. We also investigate the cellular responses to replication stress (RS), a phenomenon caused by endogenous or exogenous factors that slow down DNA replication forks and may result in DNA damage and genomic instability. Our long-term goal is to develop strategies to minimise RS in normal cells and enhance it in cancer cells to increase their vulnerability. In 2023, we addressed the molecular changes that underlie the rewiring of DNA replication during pluripotency transitions in mouse embryonic stem cells. We also described how regulation of replication origin activity influences the acquisition of chemotherapy resistance in tumour cells undergoing epithelial-to-mesenchymal transition. Finally, we showed how DNA replication is blocked by a new combination therapy for diffuse large B cell lymphoma.

“We have described how the activation of new replication origins in response to chemotherapy mediates the acquisition of resistance in epithelial tumour cells.”
**RESEARCH HIGHLIGHTS**

A rewiring of DNA replication during cell pluripotency transitions

In previous years, we had mapped the positions of replication origins in mouse embryonic stem cells (mESCs) in the primed pluripotency state that resembles the post-implantation epiblast (Jodkowska K et al., 2022, Nucleic Acids Res 50, 12149-12165). We are now studying the adaptation of the DNA replication programme when primed mESCs are de-differentiated to the naive state, which resembles the pre-implantation inner cell mass. We have found that the primed-to-naive mESC transition, triggered in cultured cells by MEK and GSK3 inhibitors (23), entails a significant slowdown of replication forks, a higher frequency of asymmetric fork progression, and the compensatory activation of dormant origins. Using iPOD (“isolation of proteins on nascent DNA”) coupled to mass spectrometry, we identified key changes in replisome composition that are likely responsible for these effects. For instance, naive mESC forks are enriched in proteins involved in DNA recombination and repair, notably MRE11 nuclease, while primed mESC forks are enriched in factors related to translation initiation, ubiquitin-dependent protein metabolism and cell cycle progression. We are investigating the causal links between the alteration in DNA replication dynamics and the capacity of mESC to be reprogrammed into earlier pluripotency states.

**Activation of dormant replication origins in response to cisplatin/5-FU facilitates chemotherapy resistance**

The development of resistance by tumour cells constitutes a major problem in anticancer therapy. Epithelial-to-mesenchymal transition (EMT) is one of the cellular processes that has been linked to chemotherapy resistance by mechanisms that are not well understood. We collaborated with C. Blanpain (Université Libre de Bruxelles, Belgium) to report that skin squamous carcinoma cells undergoing EMT are highly resistant to anti-cancer therapy both in vivo and in vitro. RhoJ, a small GTPase that is preferentially expressed in EMT-highly resistant to anti-cancer therapy both in-vitro and in-vivo. The development of resistance by tumour cells constitutes a major problem in anticancer therapy.

**DNA replication and RS in other cellular contexts**

Other current projects include: (i) a structural and functional characterisation of PRIMPOL primase, specialised in re-initiation of DNA synthesis at stalled forks; (ii) the genome-wide identification of pre-replicative complexes in human cells using CUT&RUN; (iii) the identification of molecular mechanisms that prevent DNA over-replication and gene amplification; and (iv) the participation of mitotic kinase AURKA in the regulation of S phase progression.

**A combination therapy in large diffuse B-cell lymphomas effectively blocks DNA replication**

Diffuse large B cell lymphoma (DLBCL) is the most common aggressive B cell lymphoma. DLBCL is normally treated with chemotherapy, but a substantial proportion of patients do not respond or relapse after treatment. In a collaborative study with V.G. de Yébenes and A. Ramiro (CNIC, Madrid), we have observed that the combination of tumour suppressor microRNA miR-28 with BTK inhibitor ibrutinib induces a specific transcriptional cell-cycle arrest programme that impairs DNA replication in DLBCL cells. Single-molecule analysis revealed that miR-28 was sufficient to reduce replication origin activation, while ibrutinib restricted the compensatory acceleration of replication forks (FIGURE 2). Notably, the same transcriptional signature repressed by miR-28 plus ibrutinib combination therapy appears downregulated in DLBCL patients with better survival (Fortes T et al., 2023).

**FIGURE 1** RhoJ promotes activation of new origins after chemotherapy. (A) Schematic of DNA fibre assay in cells treated with EPAC1+ (EPAC1− and EPAC1+ RhoJ KO cells treated with cisplatin).

**FIGURE 2** Efficient block of DNA replication by miR-28 + ibrutinib combination. (A) Schematic of DNA fibre assay in cells treated with miR-28, ibrutinib (ib) or the combination of both. (B) Frequency of origin activation. (C) Distribution of fork rate in the different conditions. Adapted from Fuertes T et al. (2023).

**PUBLICATIONS**

Debaugnies M, Rodriguez-Arèoles S, Blanpain C, Blon- deau J, Parent MA, Zucca M, Song Y, de Maertelaer V, Moore V, Latil M, Dubuisson C, Coulomna R, Impens F, Van Haever M, Dufour S, Liemone A, Solominovsky PA, Méndez J, Blanpain C (2023). RhoJ con- tra- MRE11 nuclease, while primed mESC forks are enriched in factors related to translation initiation, ubiquitin-dependent protein metabolism and cell cycle progression. We are investigating the causal links between the alteration in DNA replication dynamics and the capacity of mESC to be reprogrammed into earlier pluripotency states.

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The development of resistance by tumour cells constitutes a major problem in anticancer therapy. Epithelial-to-mesenchymal transition (EMT) is one of the cellular processes that has been linked to chemotherapy resistance by mechanisms that are not well understood. We collaborated with C. Blanpain (Université Libre de Bruxelles, Belgium) to report that skin squamous carcinoma cells undergoing EMT are highly resistant to anti-cancer therapy both in vivo and in vitro. RhoJ, a small GTPase that is preferentially expressed in EMT-highly resistant to anti-cancer therapy both in-vitro and in-vivo. The development of resistance by tumour cells constitutes a major problem in anticancer therapy.

**DNA replication and RS in other cellular contexts**

Other current projects include: (i) a structural and functional characterisation of PRIMPOL primase, specialised in re-initiation of DNA synthesis at stalled forks; (ii) the genome-wide identification of pre-replicative complexes in human cells using CUT&RUN; (iii) the identification of molecular mechanisms that prevent DNA over-replication and gene amplification; and (iv) the participation of mitotic kinase AURKA in the regulation of S phase progression.

**A combination therapy in large diffuse B-cell lymphomas effectively blocks DNA replication**

Diffuse large B cell lymphoma (DLBCL) is the most common aggressive B cell lymphoma. DLBCL is normally treated with chemotherapy, but a substantial proportion of patients do not respond or relapse after treatment. In a collaborative study with V.G. de Yébenes and A. Ramiro (CNIC, Madrid), we have observed that the combination of tumour suppressor microRNA miR-28 with BTK inhibitor ibrutinib induces a specific transcriptional cell-cycle arrest programme that impairs DNA replication in DLBCL cells. Single-molecule analysis revealed that miR-28 was sufficient to reduce replication origin activation, while ibrutinib restricted the compensatory acceleration of replication forks (FIGURE 2). Notably, the same transcriptional signature repressed by miR-28 plus ibrutinib combination therapy appears downregulated in DLBCL patients with better survival (Fortes T et al., 2023).

**FIGURE 1** RhoJ promotes activation of new origins after chemotherapy. (A) Schematic of DNA fibre assay in cells treated with EPAC1+ (EPAC1− and EPAC1+ RhoJ KO cells treated with cisplatin).

**FIGURE 2** Efficient block of DNA replication by miR-28 + ibrutinib combination. (A) Schematic of DNA fibre assay in cells treated with miR-28, ibrutinib (ib) or the combination of both. (B) Frequency of origin activation. (C) Distribution of fork rate in the different conditions. Adapted from Fuertes T et al. (2023).

**PUBLICATIONS**

The main objective of our Group is to identify and validate new drivers and therapeutic targets in melanoma, the most aggressive form of skin cancer. We are particularly interested in mechanisms that, being selectively deregulated in melanoma, may account for the unique ability of this tumour type to bypass immune recognition and generate metastasis already from lesions barely over one millimetre in depth (publications in *Nature*, *Cancer Cell*, *Nature Cell Biology*, *Nature Communications*, among others). Our laboratory has also reported the first-in-class lymphoreporter (*MetAlert*) mice for non-invasive imaging of pre-metastatic niches in melanoma (*Nature*). These systems led to the identification of new mechanisms of immune resistance (*Nature Medicine*) and the generation of nanoparticle-based treatments (*Cancer Cell, EMBO Mol Med*), with derivatives now being tested in clinical trials. These studies are performed in the context of large cohorts of patient-associated datasets, with the ultimate goal of defining physiological relevance.

“The performing single cell analyses, we have identified different cellular states and new mechanisms of immune suppression in melanoma that will pave the way for potential diagnostic markers and therapeutic targets.”
RESEARCH HIGHLIGHTS

The long-term goals of our Group are to:

1. Define the “fingerprint” that distinguishes melanomas from other cancer types.
2. Visualise and target melanoma progression at the whole-body level in vivo.
3. Determine and target signalling cascades that turn immunologically “hot” melanomas into “cold” and refractory tumours.
4. Develop new therapeutic strategies to overcome immune suppression and immune tolerance in melanoma.

New drivers of melanoma progression

A main objective of our Group is to understand and target mechanisms that define the inherent aggressiveness of malignant melanoma. We address this unmet need through genetic and functional studies in melanocytic cell lines, mouse models, and tissue specimens, but also by performing cross-cancer type analyses. We previously identified mechanisms of vesicular trafficking, autophagy, and RNA-associated metabolism with protumorigenic functions in this disease that are not shared by over 25 malignancies (Alonso-Curbelo et al., Cancer Cell 2014; García-Fernández et al., Autophagy 2016; Perez-Guijarro et al., Nat Commun 2016; Cifdaloz et al., Nat Commun 2017; Karras et al., Cancer Cell, 2019). In addition, in collaboration with Sagrario Ortega at CNIO, we developed the first ‘Melanoma-MetAlert’ murine strain for spatio-temporal analyses of premetastatic niches in vivo temporal analyses of premetastatic niches in vivo. ‘Melanoma-MetAlert’ murine strain for spatio-temporal analyses of premetastatic niches in vivo. ‘Melanoma-MetAlert’ murine strain for spatio-temporal analyses of premetastatic niches in vivo.

One of the challenges in the rational design of new therapies in melanoma is the marked inter- and intra-tumoural heterogeneity of these lesions. Recent studies have identified various cellular states in melanomas, but the underlying drivers are not well understood. Using scRNAseq, we recently addressed the impact of MDK at the cellular level, both in cutaneous melanoma. Combination of MetAlert mice and functional studies in patient biopsies for the discovery of lymphangiogenic factors, with roles in tumour cell metastasis and immune suppression, here illustrated for the growth factor MDK.

Single cell analyses of tumour and immune compartments in melanoma

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MDK associates with tumour progression and immune suppression

We previously reported dsRNA mimetics that repress MDK mRNA expression (Olmeda et al., EMBIO Mol Med 2021) and are now developing small molecule inhibitors and blocking antibodies.

FIGURE 1 Identification of tumour drivers and immune modulators in melanoma. Combination of MetAlert mice and functional studies in patient biopsies for the discovery of lymphangiogenic factors, with roles in tumour cell metastasis and immune suppression, here illustrated for the growth factor MDK.
OVERVIEW

We focus on the molecular pathophysiology of pancreatic ductal adenocarcinoma (PDAC) and bladder carcinoma taking a disease-oriented approach. These tumours present very distinct clinical challenges. We learn from patient samples, cultured cells/organoids, and genetically modified mice. To translate the findings, we bring this knowledge to a “population” level — leveraging on information and samples from large patient cohorts — together with Núria Malats (CNIO).

PDAC has a dismal prognosis even when diagnosed early. We aim at dissecting the molecular mechanisms involved in very early steps of tumour development, harnessing the power of mouse genetic editing. A main hypothesis is that cell differentiation is an early and potent tumour suppressor mechanism. Understanding the contribution of early molecular events is crucial to design better strategies for prevention and early tumour detection.

Bladder cancer presents with a very wide clinical and pathological heterogeneity. We aim at acquiring knowledge about the underlying biology that might be leveraged towards improved tumour subclassification, prediction of outcome, and therapy.

“A new mouse strain carrying an exon 6 deletion in Ctrb1 recapitulates the human variant associated with PDAC. A mutant, insoluble, CTRB1 truncated protein is present in the pancreas of these mice, associated with a dramatic ER stress phenotype.”
PANCREAS CANCER MORPHOLOGICAL PATHOLOGY

Genome-wide association studies (GWAS) have identified common genetic variants associated with PDAC risk. Several of them are associated with genes involved in acinar cell biology, including NRAS and HNF4A coding for transcription factors required for full acinar differentiation. A few other GWAS hits associate with genes involved in acinar function, such as CTRB1 and XRPI. These observations have strengthened the notion, pioneered by our lab, that cell differentiation is the first tumour suppressor mechanism in the pancreas. Among the processes participating therein are inflammation and ER stress response. We have extensively studied the role of NRAS using heterozygous mice. Recently, we focussed on a risk variant in CTRB2 that has been finally mapped as a deletion in exon 6. We, however, generated with Sagararo Ortega (CNIO), a new mouse strain carrying the corresponding exon 6 deletion in the mouse, the orthologue of the human gene. CTRB2+/- mice are viable and display the normal growth pattern until adulthood. The mutant CTRB2 protein has a smaller molecular mass and is largely present in the insoluble fraction of pancreatic lysates, unlike the wild type protein. While the pancreas of 3-month-old homozygous mutant mice has a normal histological appearance, ultrastructural analysis shows dramatic alterations of the endoplasmic reticulum, with extensive cisternal dilatation, abundant cytoplasmic aggregates, partial loss of zymogen granules, and even some nuclear inclusions. Strikingly, some acinar cells appear spared despite the germline nature of the mutation introduced. Similar, but less dramatic, changes occurred in heterozygous mice. RNA-Seq analysis of the pancreas of CTRB2+/- mice revealed a down-regulation of the acinar programme and an up-regulation of ER stress pathways, in agreement with the morphological changes. In addition, we found modest evidence of increased inflammatory pathway activity. We interpret these findings as an adaptation of acinar cells to stress, aimed at increasing cell viability. These mice are currently undergoing extensive phenotyping and we are assessing their ability to respond to a variety of insults including pancreatitis and high fat diet, among others.

UROTHELIAL BLADDER CARCINOMA GENETICS, BIOLOGY, AND CLINICAL TRANSITION

We focus on understanding 2 new tumour suppressor genes that are mutated in bladder cancer that we identified through exome sequencing: STAG2 and RBM10. STAG2 codes for a cohesin subunit and RBM10 codes for a splicing regulator. We have generated conditional mouse models for these 2 genes and are exploring their role in development and urothelial biology as well as their cooperation with other cancer genes.

Bladder cancers that do not invade muscle largely maintain a luminal/urothelial phenotype characterised by the expression of transcription factors involved in the activation of this programme, such as GATA3 and FOXA1. However, upon invading muscle, a fraction of tumours loses this identity and displays a basal/squamous phenotype that is reminiscent of that of basal cells in the urothelium. This is generally associated with the loss of normal tumour-suppressor function. To acquire a more detailed understanding of the molecular mechanisms involved in this process, which is associated with more aggressive tumours, we set out to systematically identify novel transcriptional regulators that might participate in this process. Using bulk and single cell transcriptomic data from bladder cancer organoids and tumours classified as luminal or basal/squamous, we identified differentially expressed factors and searched for the enrichment of their binding motifs in tumours from basal cells and normal urothelium. This integrative work has unveiled several new transcription factors that are candidates to participate in urothelial transformation, including FOXG1, MRKCM, TRB3, API, proteins and TFP2, among others.

In addition, we continue to collaborate with our clinical colleagues from the GUARD consortium in the conduct of clinical studies with a strong translational component.
OVERVIEW

Over the last 2 decades, research has primarily focused on understanding the functions of mutated genes in cancer, neglecting the roles of environmental factors that can induce the expression of harmful proteins and tissue damage. These factors pose ongoing challenges, and their mechanisms in causing cancer-related pathologies are largely unknown. Identifying links between environmental stress and cancer progression is crucial for uncovering disease mechanisms and therapeutic targets.

Our laboratory employs genetically engineered mouse models and advanced technologies to investigate mechanisms of diseases associated with environmental stressors. We specifically study conditions related to toxic diets, nutrient imbalances, and sedentary lifestyles, which can lead to obesity and associated disorders, such as diseases from the digestive tract.

Our particular focus lies in diseases affecting the liver (non-alcoholic steatohepatitis, cirrhosis, and hepatocellular carcinoma), intestine (colitis and colorectal cancer), and pancreas (diabetes, pancreatitis, and pancreatic cancer). These organs are primarily affected by environmental stressors, including nutrient overload and lack of physical activity, that can cause severe inflammatory conditions. In addition, their functions are interconnected and potentially regulated by the nervous system, through unknown mechanisms. Accordingly, we recently started to explore the intricate relationship between diet and the nervous and immune systems in aggressive cancers, including metastasis, a perspective we plan to emphasise further in the future, within the emerging field of cancer neuroscience and neuroimmunomodulation.

Furthermore, our research encompasses tissue regeneration (intestine and liver), the dysregulation of metabolic pathways in cancer initiation, inflammatory processes, and the initial stages of embryonic development, shedding light on fundamental mechanisms applicable to various diseases. Our goal is to guide the development of novel medicines, with a special focus on potential immunomodulatory therapies for these disorders.

“We continuously strive to generate new and unique preclinical mouse models to elucidate the mechanisms of diseases and capture the complexity of human disorders, with a particular focus on diseases associated with obesity and the digestive tract.”
RESEARCH HIGHLIGHTS

Using genetically engineered mouse models, along with other model systems and cutting-edge technologies (including cell biology with organoid culture and quantitative imaging, biochemistry, and functional genomics), and human data, our laboratory has dedicated significant effort over the past years to comprehend the molecular, cellular, and pathophysiological mechanisms that connect environmental stresses to disease pathogenesis. In particular, we have studied the mechanisms of diseases associated with obesity and the digestive system, with a focus on liver and intestinal disorders. These conditions often stem from unhealthy diets, nutrient imbalances, and sedentary lifestyles, all of which can contribute to severe inflammatory conditions (see Figure 1). Organs of the digestive system are indeed primarily impacted by environmental stressors but are also physiologically interconnected and influenced through their euscline and/or endocrine functions. Significant discoveries have been made, and several future research projects are planned as follows:

**Mechanisms of obesity**

Our groundbreaking work has uncovered the mechanisms behind the inflammatory properties of nutrients and their connection to various disorders. Our recent research has linked inflammation, particularly IL-17A, to obesity and autoimmune disorders, connecting them to hepatitis and liver disease-induced hepatocellular carcinoma. Our findings have gained significant attention from pharmaceutical companies exploring IL-17A blockers as potential treatments for these disorders. Our ongoing research aims to further understand how nutrients can be inflammatory by themselves leading to obesity. Additionally, we will dedicate special efforts to identify the specific inflammatory cells responsible for obesity and its associated metabolic disorders.

**Diet, nutrients and cancer**

We discussed in an extensive review how various diets could impact cancer development. This suggests that nutritional interventions could be beneficial for both the prevention and treatment of cancer.

**Mechanisms of liver cancer progression**

Environmental stress, nutrient overload and toxic diet can lead to chronic liver diseases, including cirrhosis, which may progress to hepatocellular carcinoma (HCC). To comprehend the influence of cirrhosis on HCC development, our work focuses on studying the mechanobiology of liver tissue at the molecular, cellular, and tissue levels. This involves investigating and genetically manipulating in mice the mechanical forces within and between various liver cells, as well as their interactions with microenvironments. Mathematical models and bioinformatics analyses will be used to complement our studies, with the ultimate goal of understanding the progression from an injured and diseased liver to a cancerous tissue.

**Cell dormancy in HCC relapse**

Despite numerous therapeutic strategies, cancer relapse is common, occurring months to years after treatment. Tumour relapse is thought to be driven by dormant, non- or slow-cycling resistant cells, yet conclusive proof-of-concept studies are lacking. Our laboratory aims to address this gap by utilising a genetically modified mouse model. We plan to label and track dormant cells to understand their role in HCC recurrence.

**Mechanisms of intestinal diseases and colorectal cancer**

Colorectal cancer (CRC) is a multi-hit neoplasia originating from APC mutation-induced adenomatous polyps, which progress to malignancy through the acquisition of p53 loss. Our research is focused on comprehending the mechanisms underlying CRC initiation and the transitional mutations leading to the transformation of polyps into malignant carcinomas. Additionally, we are prioritising the investigation of why the majority of colorectal cancers exhibit resistance to immune checkpoint inhibitors, which have proven ineffective in patient treatment.

**Mechanisms of totipotency-to pluripotency transition**

We are currently elucidating the mechanisms that govern the smooth and precise transition from totipotency to pluripotency, which is a crucial process in embryonic development. This transition generates pluripotent stem cells with the capability to form all cell types.

**Structure of the URI prefoldin-like complex**

One of our future goals is to determine the functions of the URI prefoldin-like complex by unravelling its structural organisation through advanced techniques such as electron microscopy.
Tumours exploit and manipulate for their benefit the same mechanisms that regulate homeostasis in healthy tissue. In the Transformation and Metastasis Group, we aim to understand normal mammary gland development and the key events that lead to tumour initiation, progression, and metastasis, and to identify novel therapeutic targets to combat breast cancer. We use complementary tools, including primary cell cultures and organoids, lineage tracing mouse models, and clinical samples with the goal of translating basic knowledge into clinically relevant findings.

“Luminal Rank loss impairs lactation, awakening basal bipotency to restore functional milk production in parous glands.”

“RANK pathway inhibitors can restore sensitivity to CDK4/6i and prevent acquired resistance in breast cancer.”
**RESEARCH HIGHLIGHTS**

**RANK is a poor prognosis marker and a therapeutic target in ER-negative postmenopausal breast cancer**

Analyses of RANK and RANKL expression in more than 2000 breast tumours revealed that tumour RANK expression associated with poor prognosis in ER-negative breast cancer and in postmenopausal breast cancer patients. Gene set enrichment analysis (GSEA) showed that RANK protein expression in tumour cells in postmenopausal ER-negative breast tumours was associated with multiple immune and metabolic pathways, suggesting that RANK signalling increases after menopause. Our results demonstrate that RANK expression is an independent biomarker of poor prognosis in postmenopausal patients with ER-negative breast cancer and support the therapeutic benefit of RANK pathway inhibitors in breast cancer patients with RANK-positive, ER-negative tumours after menopause. (Ciscar M et al., EMBO Mol Med 2023).

**Luminal Rank loss decreases cell fitness leading to basal cell hypopotency in parous mammary glands**

The Rank signalling pathway regulates mammary gland homeostasis and epithelial cell differentiation. By combining temporal/lineage-specific Rank genetic deletion with lineage tracing techniques, we found that loss of luminal Rank reduces the luminal progenitor pool and leads to aberrant alveolar-like differentiation with high protein translation capacity in virgin mammary glands. These Rank-deleted luminal cells are unable to expand during the first pregnancy, leading to lactation failure and impairment of protein synthesis potential in the parous stage. The unfit parous Rank-deleted luminal cells in the alveoli are progressively replaced by Rank-proficient cells early during the second pregnancy, thereby restoring lactation. Transcriptomic analysis and functional assays point to the awakening of basal bipotency after pregnancy through the induction of Rank/NF-κB signalling in basal parous cells to restore lactation and tissue homeostasis. (Rocha AS, Collado-Sole A et al., Nat Commun 2023).

**Microglia Rank signalling regulates GnRH function and the hypothalamic-pituitary-gonadal axis**

We have demonstrated a novel role of hypothalamic microglia in controlling reproductive hormones through Rank signalling. Congenital and microglia Rank deletion leads to severe hypogonadotropic hypogonadism (CHH) in males and females, resulting from a direct alteration in gonadotropin-releasing hormone (GnRH) regulation. In addition, we identified rare sequence mutations of RANK in patients with congenital hypogonadotropic hypogonadism (CHH). Moreover, inducible Rank deletion during puberty and adulthood also leads to HH. Transcriptional profiling at single-cell level of hypothalamic microglia revealed the importance of Rank signalling in the maintenance of a functionally active homeostatic microglia. Our data have revealed the crucial role of microglia, mediated by Rank signalling, in regulating GnRH function and the hypothalamic-pituitary-gonadal (HPG) axis, which is essential for reproductive maturation and fertility. (Collado et al., under review in Nature).

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**FIGURE 1** Mammary gland at G9.5 showing in pink luminal cells and in cyan IR expression.

**FIGURE 2** Defective HPG axis in microglia Rank null models.
Brain metastasis is the most common neurological complication of cancer and, in spite of the progress made with local (i.e., surgery and radiation) and systemic (i.e., targeted therapy, immunotherapy) therapies, prognosis remains poor. Indeed, the increased incidence of brain metastases is partially due to systemic therapies that work extra-cranially but do not provide the same therapeutic benefit in the brain. We study why and how cells from different cancer types (breast cancer, lung cancer, and melanoma) are able to access the brain, survive, and colonise this vital organ. We dissect the biology of these processes in vivo using experimental models and patient-derived material to challenge this unmet clinical need. Our research has identified novel brain metastasis mediators, characterised the metastasis-associated microenvironment, designed better experimental models, and explored novel methods to target brain metastasis as well as to prevent or revert the frequent impact of metastasis on brain function.

“We challenged mass effect as the only source of neurocognitive impairment in brain metastases and instead suggest that a molecular programme present in cancer cells could underlie such a process.”
How brain metastases impair neural communication

We published a manuscript that suggests an alternative way to look at the impact brain metastasis can cause on brain function. Rather than the compression and destruction of neurons in the peritumoral area derived from the mass effect as the tumour grows, we showed that the molecular profile of cancer cells might generate aberrant ways in which they influence surrounding intact neural circuits. By exploiting different brain metastasis models, we showed that they recapitulate the heterogeneous impact observed in patients with respect to the negative influence on neuronal communication. As such, we detected differences in the peritumoral electrophysiology from different models that correlated with the decrease in both inhibitory synapses as well as calcium activity. Furthermore, simply analysing brain activity associated with the presence of different brain metastasis models depicted a novel biomarker. In brief, computational analysis of brain activity using artificial intelligence demonstrates the possibility to predict the presence and the subtype of metastasis in the brain. Both aspects (the use of a novel biomarker as well as the molecular mediators of the impact on brain activity) are currently being followed-up to be exploited clinically through the National Network of Brain Metastasis, RENACER.

Progress on the co-evolution of the metastasis-associated microenvironment

Our programme exploring the evolution of the microenvironment into a pro-tumoral niche to be exploited for novel therapies against brain metastases has been significantly expanded. We have deepened our knowledge on metastasis-associated astrocyte heterogeneity as well as on the processes by which they are reprogrammed to acquire new roles as a strong local immunomodulatory cell type. Indeed, we have interrogated strategies to manipulate CD8+ T cells and evaluated their ability to target brain metastases using adoptive T cell transfer experiments. This strategy is giving us the opportunity to develop immunotherapies optimised to work on clinically relevant (symptomatic) brain metastases. The study of the plasticity of the microenvironment has been complemented by analysing a novel subpopulation of macrophages that emerges in the metastasis-associated microenvironment.

Development of preventive strategies against brain metastasis

Our systematic dissection of metastatic colonisation of the brain has made it possible to define different steps. By focusing on the early moments after extravasation, we are studying the process by which virtually all potentially metastasis-initiating cells rely on pre-existing vessels to survive, using the process termed vascular co-option. Deconstructing the crosstalk between co-opting metastatic cells and co-opted endothelial cells is giving us new molecular insights to develop preventive strategies to stop the development of micrometastases.

Consolidating RENACER as a pioneering strategy for a more efficient translation

The National Network of Brain Metastasis (RENACER) initiated in 2021 has expanded to 18 hospitals throughout the country, attracted competitive grants, and initiated clinical studies and trials (NCT06353734, NCT06896191) based on the use of Patient-Derived Organotypic Cultures (PDOC). Furthermore, the unique resource of patient-derived material generated over these years is allowing us to initiate research projects starting from findings in human data.

PATENT


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PATENT

ORGAN CROSSTALK IN METABOLIC DISEASES GROUP

The Organ Crosstalk in Metabolic Diseases Group is dedicated to understanding how metabolic alterations and obesity trigger other secondary diseases such as cancer, diabetes, and cardiovascular diseases. Our research takes a holistic approach, aiming to comprehend how these alterations occurring in obesity disrupt the communication between organs. In this context, we have found that during obesity several stress kinases are activated in different tissues, and that this activation can affect the development of a tumour.

OVERVIEW

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RESEARCH HIGHLIGHTS

The Group focuses on 3 main aspects induced by obesity:

1. The alteration of adipose tissue and, consequently, the secretion of adipokines. We have observed that in adipose tissue, during obesity, stress kinases are activated, and the circadian clock is altered. Our aim is to delve into whether these factors could serve as drivers of adipose tissue dysfunction during obesity and the associated comorbidities. The primary focus of our investigation lies in unraveling the endocrine function of adipose tissue, with particular attention to the distinctive role of brown adipose tissue in thermogenesis.

2. The onset of chronic inflammation, which is associated with an increased risk of cancer. We want to comprehend how stress kinases and alterations in metabolism within inflammatory cells impact the development of the disease.

3. Cell metabolism alteration as a driver of disease. Metabolism controls the functionality of cells in our body, as it is their means of obtaining ATP to carry out their functions or, in the case of tumours, to proliferate. Therefore, disruptions in cellular metabolism can serve as drivers of diseases, but the modulation of metabolism can also offer new therapies for cardiovascular diseases and cancer. By utilising animal models to manipulate metabolism, we aim to understand how metabolism is implicated in the development of diseases.

Our main research areas are:

→ Organ Interaction and Health-Related Disorders.
→ Adipose Tissue Dysfunction in Pathological Progression.
→ Chronic Inflammation and Cancer Association.
→ Cell Metabolism as a Driver of disease.

PUBLICATIONS AT OTHER INSTITUTIONS


Hepatology 78, 874-887.

• Ferreira V (2023). The outcome of boosting BAT thermogenesis through IL-12-FGF21 axis. AJP Cell Physiol 77, 874-887.


• Zamir S, Ferreira V, Mora A.,... Sabio G (2023). The role of the p38δ MAPK pathway in regulating the metabolic rewiring that prevents hepatic metabolic rewiring. Hepatology 78, 874-887.


AWARDS AND RECOGNITION

• Carmen y Severo Ochoa Award in Molecular Biology, Fundación Carmen y Severo Ochoa, Spain.

• Premio Extremadura en Getafe Science Award, La Casa Regional de Extremadura en Getafe, Spain.

• “Hombres que Inspirar”, Women Space Extremadura, Spain.

• Juan González Molina Foundation Award to the best scientific trajectory, cátedra extramuros de la Fundación FJAPERVA, Spain.
Our Laboratory focuses on elucidating the crosstalk between tumours and their microenvironment throughout metastatic progression. Currently, we are examining the relationship between extracellular vesicles (EVs) and DNA damage responses (DDR). This project aims to uncover the potential role of EVs in controlling intrinsic/extrinsic DDR and their impact on tumour evolution and metastasis. Additionally, we are exploring the investigation of body mass index, coagulation parameters, and disease outcomes in TNBC patients.

Deciphering the role of NGFR in metastasis and resistance to therapy. The nerve growth factor receptor (NGFR) has been involved in therapy resistance and metastasis in melanoma. We postulate that therapies against NGFR will not only enhance therapeutic responses but also reduce metastasis. We are currently investigating the use of NGFR inhibitors as anti-metastatic drugs and the combination with immunotherapy in melanoma. Moreover, we are studying the significance of NGFR in tumour development and metastasis in head and neck cancer, aiming to define its relevance in chemotherapy resistance.

**OVERVIEW**

*We are investigating the dynamic interplay between systemic factors and the tumour microenvironment in metastatic progression and therapy resistance.*

**TECHNIQUES**

- Jan Hochstadt (until August) (Master’s Thesis, Univ. Autónoma de Madrid, Spain), Noelia Lara (March-June) (IES Jaime Ferrán Clúa, San Fernando de Henares, Spain)

**RESEARCH HIGHLIGHTS**

Exploring the link between EVs and DNA damage responses (DDR). EVs secreted by tumour cells contain a broad variety of biomolecules including DNA. Notably, we have identified molecules related to DDR within tumour-secreted EVs. Intriguingly, tumour-secreted EVs extrinsically impact DDR, leading to an increase in surrounding cells. Our project is dedicated to analysing the potential role of EVs in controlling DDR and their influence on tumour evolution and metastasis.

Impact of obesity in breast cancer pre-metastatic niche formation. Obesity, particularly in postmenopausal women and patients with triple-negative breast cancer (TNBC), is linked to unfavourable outcomes. Beyond its role in primary tumour initiation and growth, we found that obesity significantly influences metastasis. Our research is investigating the correlation between obesity, coagulation, and the formation of pre-metastatic niches in TNBC. Notably, a high-fat diet activates platelets and induces vascular leakiness, promoting tumour cell homing and metastasis (FIGURE 1). Additionally, we are exploring the correlation of body mass index, coagulation parameters, and disease outcomes in TNBC patients.

### Publications


In the Metabolism & Cell Signalling Lab we study the links between nutrients, cancer and ageing. All our cells integrate signals emanating from the abundance of intracellular nutrients and from the nutritional state of the entire organism. Integration of these signals is key for adjusting metabolic functions, as well as for energy storage and expenditure. Importantly, the components of these signalling cascades are generally corrupted in cancer and are drivers of the metabolic complications of chronic nutrient overload. Conversely, dietary restriction regimes are extremely efficacious interventions against tumorigenesis and to delay the process of ageing, albeit we still ignore the fundamental molecular underpinnings of these protective effects. We are combining mouse genetics with cell biological tools to gain insight into the genetic and molecular mechanisms that drive these responses. We are focusing on key nutrient sensing pathways, such as the AMP-activated protein kinase (AMPK). Interestingly, we have found that the inability of liver cells to respond to fluctuations in nutrients and insulin is neither required nor established before birth. With the start of oral feeding, these fluctuations in nutrient and hormonal signalling are a trigger for neonates to segregate liver functions only when needed: after birth and with the start of oral feeding cycles. At the molecular level, constitutive mTOR signalling impairs the action of the Wnt/β-catenin pathway. We have generated mice with liver cells incapable of detecting drops in nutrient and insulin levels, hence with mTOR always active in the liver, regardless of mice being fed or fasted. We found that the mTOR instructs the maturation of liver zonation and the spatial segregation of metabolic functions in a Wnt/β-catenin-dependent manner. Importantly, the lack of postnatal establishment of metabolic zonation in the liver was recapitulated in a model of constant feeding intermittency, fluctuations that occur with feeding cycles after birth operate as a GPS for the mammalian liver, and this spatial information is blurred in liver disease.

"We have found that the fluctuations in nutrient levels that occur with feeding cycles after birth operate as a GPS for the mammalian liver, and this spatial information is blurred in liver disease."
Myeloid cells are abundant in solid tumours. While their heterogeneity has been widely described, efficient ways of manipulating these cells are scarce. My laboratory focuses on the identification and therapeutic targeting of myeloid checkpoint programmes in cancer. By studying the microenvironment in which lung, ovarian, and breast cancer emerge, we examine how macrophages crosstalk with the stroma and how they modulate their malignant conversion into cancer-associated fibroblasts. As metastasis is the major cause of death in breast cancer, we explore the mechanisms by which neutrophils exacerbate metastasis malignancy. Lastly, understanding how tissue physiology is perturbed in cancer is key to investigating novel ways to modulate anti-tumour immunity: we do this by exploring the circadian biology of immune responses, interrogating how our diet modifies the lipid metabolism of macrophages, and mining the dysfunctional properties of bone marrow-derived haematopoietic cells.

During 2023, my Group successfully incorporated 3 new Ph.D. students: Mariola Munárriz and Eduardo Garvín, funded by “Severo Ochoa” Excellence and Retos del Conocimiento programmes, respectively, and Jan Hochstad, funded by “La Caixa” Foundation Health Research Grant. Their projects are starting to bloom, and their work has already been presented at national symposia (ASEICA 40th Anniversary Meeting and the Annual Sociedad Española de Inmunología Congress). In addition, a new lab manager (Mónica Gómez) and 2 postdoctoral fellows joined the team: Alba de Juan (funded as a Ramón y Cajal Junior Fellow) and Sarai Martínez Pacheco (funded by the H2020 Transcan Network).

Ovarian tumours are massively infiltrated by macrophages, yet their origin and expansion remain unexplored. Clonal haematopoiesis is known to confer the advantageous expansion of certain haematopoietic stem or progenitor clones, and its presence correlates with increased inflammatory output from mutated cells. Interestingly, tumours in which WT and Tet2-deficient clones cohabit display increased macrophage numbers, as opposed to macrophages derived from a fully WT haematopoietic environment (FIGURE 1A–B). These macrophages full of lipids (or lipid-loaded macrophages (LLM)) expressed higher levels of immunosuppressive molecules (PDL1, Arg1, among others) and a significant expansion at the tumour site. Given these observations, we are currently modulating an LLM immunosuppressive phenotype using different diets and combining this with immune checkpoint blockade (ICB). Interestingly, a high-fat diet turns ovarian Tet2-ICB-resistant tumours into ICB-sensitive. We are intrigued by this result and are currently exploring how this occurs mechanistically.

During 2023, we also obtained funding from the European Research Council (ERC StG2021). In this project, we are uncovering the mechanisms by which circadian rhythms of the immune system become dysfunctional in tumours. Our goal is to understand ways to design time-based therapies to harness anti-tumour immunity and block time-dependent immune suppression. Interestingly, mice lacking circadian modulation in neutrophils and macrophages showed opposing trends when controlling tumour growth (FIGURE 1C), suggesting that BMAL1 controls specific programmes in particular subsets of myeloid cells.