Scientists at the Molecular Oncology Programme (MOP) aim to discover new molecular mechanisms that drive cancer onset, mediate its progression or influence the response to therapy. The ultimate goal is to generate knowledge that can be translated into applications that are beneficial to cancer patients and that can help in the diagnosis and treatment of their disease. To do so, we integrate leading groups that cover a wide range of complimentary expertise relevant in oncology, including DNA and chromosome stability (Maria A. Blasco, Óscar Fernández-Capetillo, Massimo Squatrito, Felipe Cortés Ledesma, and Ana Losada), oncogenes and cell cycle kinases (Mariano Barbacid), DNA replication (Juan Méndez), mitosis (Marcos Malumbres), melanoma (María S. Soengas), molecular pathophysiology of epithelial tumours (Francisco X. Real), metabolism and cell signalling (Nabil Djouder and Alejo Efeyan), and metastasis (Manuel Valiente, Eva González Suárez and Héctor Peinado). Our Programme will also soon incorporate a group working in cancer immunotherapy, which will certainly be of help as many of our existing groups have projects related to cancer immunity.

It goes without saying that 2020 has not been the easiest of times in any respect, including for scientific research. Yet, despite the limitations, scientists at the MOP continued to make significant contributions. Thanks to their work, we now have a better understanding of the mechanisms of resistance to cancer therapies and better ideas as to how to optimise treatments to overcome this resistance. We also made interesting discoveries related to how chromosome topology and structure influence cancer onset and overall adult health, and revealed key insights that could help to optimise the efficacy of cancer immunotherapies in tumours such as melanoma or breast cancer. While most of our scientific projects are strictly related to oncology, scientists at the MOP have also made very significant findings related to other age-related diseases, such as the discovery of a new mechanism linking viral infections to diabetes or advances in gene therapy for pulmonary fibrosis. Significantly, this last approach has led to the establishment of a new spin-off company that will try to advance the clinical development of telomerase-based gene therapy. Congratulations to Maria Blasco and her team for this important milestone. Finally, I want to note that while virology is not our area of expertise, scientists at the MOP have also tried to be of help and use their knowhow in the battle against covid-19. This exemplifies a common value among scientists: we are here to help.

My last sentence from the 2019 annual report was: “Let 2020 be our year”. Well, I was certainly not thinking of this. Yet, in some respects, it was our year. It was the year that we had to reinvent ourselves and overcome many limitations to show that we can still be productive even in the most challenging times. I am always proud to be the Director of the MOP, but even more so this year. Thank you all for making it possible.

“2020 has shown that MOP scientists are still productive in very challenging days. I sincerely wish that 2021 brings us all back the opportunity to enjoy the experience of being a scientist in its full.”
We study the mechanisms by which tumour cells are immortal and normal cells are mortal. Immortality is one of the most universal characteristics of cancer cells. The enzyme telomerase is present in more than 95% of all types of human cancers and absent in normal cells in the body. Telomeres are nucleoprotein complexes located at the ends of chromosomes, essential for chromosome protection and genomic stability. Progressive shortening of telomeres associated with organism ageing leads to ageing. When telomeres are altered, adult stem cells have a maimed regenerative capacity.

Our research focuses on:

- Generating 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 or TERRA.
- Testing telomerase gene therapy in “telomere syndromes” and age-related diseases.
- Role of telomerase and telomeres in adult stem cell biology and in nuclear reprogramming of differentiated cells to iPS cells.

“We have shown that the targeting of telomere maintenance mediated through the microRNA miR-490 could be therapeutically important in the treatment of glioblastoma.”
Short telomeres mice need active mTOR pathway for survival

The mechanistic target of rapamycin (mTOR) pathway is a central regulator of cell growth and metabolism. A variety of signals, including growth factors and nutrients, regulate mTOR activity. Inhibition of this nutrient-sensing pathway is considered a therapeutic target to delay ageing and age-related pathologies. mTOR exists in two distinct complexes, mTORC1 and mTORC2, each with different substrates and activities. Of the two, mTORC1 is the only sensitive to acute rapamycin treatment.

Genetic or pharmacological inhibition of mTORC1 with rapamycin, or with rapamycin-derived compounds, delays ageing and increases the lifespan of mice. There is evidence suggesting that lifespan extension by dietary restriction may partly arise from mTORC1 inhibition. Rapamycin significantly decreases cancer incidence in wild-type mice and it also has immunosuppressant properties.

**FIGURE 1** Opposite outcomes of rapamycin treatment depending on telomere length.

<table>
<thead>
<tr>
<th>Long telomeres</th>
<th>Short telomeres</th>
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<td>Decreased cancer and ageing</td>
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<td>Increased survival</td>
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Since mTOR inhibitors could represent potential treatments for human patients afflicted with telomere syndromes, we addressed whether rapamycin treatment could ameliorate the premature ageing phenotypes and the decreased longevity of telomerase-deficient mice with short telomeres. We found that while chronic rapamycin treatment in mice with telomeres of normal length inhibits mTOR activity and leads to a decrease of cancer and ageing and to increased survival (FIGURE 1), in telomerase-deficient mice with short telomeres it leads to the upregulation of the mTOR pathway and, quite unexpectedly, to the decreased longevity of these mice, a stark contrast to the lifespan extension observed in similarly treated wild-type mice (FIGURE 1). Altogether, our findings demonstrate that hyperactivation of the mTOR pathway as the consequence of short telomeres constitutes a compensatory survival mechanism. In agreement with this, inhibition of this pathway has deleterious effects in telomerase-deficient mice.

Telomerase treatment prevents lung fibrosis associated with physiological aging

Idiopathic pulmonary fibrosis (IPF) is a potentially lethal disease associated with certain mutations or advanced age, currently lacking a cure. We had shown that specific induction of telomere dysfunction aldehyde rodent type II (ATII) cells sufficed to induce progressive and lethal pulmonary fibrosis in mice, demonstrating that dysfunctional telomeres ATII cells are at the origin of IPF. We had also demonstrated that the presence of short telomeres in lung cells triggered IPF in telomerase-deficient mice upon treatment with a low dose of the lung-damaging agent bleomycin. We had also shown that treatment with a telomerase gene therapy that activated telomerase in the lungs stopped lung fibrosis progression in these mice. Evidence from human patients and mouse models with short telomeres indicates that short/dysfunctional telomeres are at the origin of IPF.

It remained unknown whether physiological ageing leads to short telomeres in the lung, and whether this increases the risk of IPF with ageing. We have now found that physiological ageing in wild-type mice leads to telomere shortening and a reduced proliferative potential of ATII cells and club cells, increased cellular senescence and DNA damage, increased fibroblast activation and collagen deposits, and impaired lung biophysics, suggestive of a fibrosis-like pathology. Treatment of ageing wild-type (FIGURE 2) and telomerase-deficient mice with telomerase gene therapy prevented the onset of lung profibrotic pathologies. Short telomeres associated with physiological ageing are at the origin of IPF, a potential treatment for IPF based on telomerase activation would be of interest both for patients with telomerase mutations and sporadic cases of IPF associated with physiological ageing.

**AWARDS AND RECOGNITION**

- Chair of SIREM (“Seven Others” Centres) and “Mara de Maeztu” Units of Excellence Alliance in Spain
- ERC-Advanced Grant SHELTERINS, European Research Council
- Chair of the Scientific Advisory Board of the Institute of Genetics and Molecular and Cellular Biology (IGBMC), Strasbourg, France.
- Chair of the Scientific Advisory Board of the Barcelona Cancer Research Centre – CIEMAT, Madrid, Spain.
- Editorial Board Member, International Journal of Cancer.
**Molecular Oncology Programme**

**Experimental Oncology Group**

**Overview**

KRAS oncogenes have been identified in one-fifth of all human cancers. In 2020, selective inhibitors against one of the KRAS oncogenic isoforms, KRAS<sub>G12C</sub>, have been developed. Yet all the other isoforms remain undruggable. Moreover, selective inhibitors of KRAS signalling pathways have failed in the clinic due to unacceptable toxicities. Previous work in our laboratory, allowed us to identify RAF1 as the only effector within the MAPK pathway whose elimination induced significant tumour regressions without causing major toxicities. In 2020, we have identified CDK4 as a second potential therapeutic target. Combined RAF1 ablation and expression of a CDK4 kinase dead isoform completely prevented tumour progression of KRAS/TRP53 driven lung adenocarcinomas and led to complete regression of a quarter of these tumours.

“Combined RAF1 ablation and expression of a CDK4 kinase dead isoform completely prevented tumour progression of KRAS/TRP53 driven lung adenocarcinomas and led to complete regression of a quarter of these tumours.”

**Experimental Oncology Group**

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<th>Graduate Students</th>
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<tr>
<td>Oksana Brehey (since November), Laura de Esteban (until August), Fernando Fernández, Jing Li, Vasiliki Lialis, Lucia Moralés, Marina Salmón, Manuel Sanclemente</td>
<td>Irene de Diego, M. Carmen González (TS), Silvia Jiménez, Marla San Román, Raquel Villar</td>
<td>Yaiza Arranz (February-June) (BS Thesis, Universidad Autónoma de Madrid, Spain), Lidia Atencia (until July) (Master’s Thesis, Universidad Complutense de Madrid, Spain), Marina Matamoros (until May) (Master in Molecular Oncology, CIB, Madrid, Spain), Noelia Santander (until June) (Master’s Thesis, Universidad Complutense de Madrid, Spain)</td>
<td>Alfredo Carrato (Hospital Ramón y Cajal, Madrid, Spain), Monica Musteanu (Universidad Complutense de Madrid, Spain), Bruno Sainz (Universidad Autónoma de Madrid, Spain), Juan Velasco (Eli Lilly, Alcobendas, Spain)</td>
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<td>Anna Kick (February-November) (FH Campus Wien, Vienna, Austria)</td>
<td>Patricia F. Guerra</td>
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<td>Sara García-Alonso, Carolina Navas (until July), Guillem Paniagua</td>
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<td>Matthias Drosten, Raquel García-Medina (on medical leave), Carmen Guerra, Monica A. Musteanu (until September)</td>
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**Staff Scientists**

Mariano Barbacid

**Group Leader**

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Most KRAS mutant lung adenocarcinomas remain intractable for targeted therapies. During 2020, 2 companies, Amgen and Mirati, developed selective inhibitors against one of the KRAS oncogenic isoforms, KRAS\(^{G12C}\), based on the unique properties of the cysteine residue to form covalent bonds. Yet, all other KRAS isoforms remain undruggable. Likewise, no selective inhibitor against KRAS downstream effectors has been approved by the FDA due to their unacceptable toxicities. Genetic interrogation of members of the MAPK pathway along with the interphase CDKs identified CDK4 and RAF1 as the only targets capable of inducing therapeutic responses without causing significant toxicities. We have interrogated the therapeutic consequences of expressing a kinase dead isoform of CDK4 in combination with RAF1 ablation in advanced \(Kras/Trp53^{+/FSFG12V+}/F\) mice for 4 weeks with a combination of 5-azacytidine (5-AZA) and a PI3K inhibitor (right panel) or vehicle (open circles) or 5-AZA (left panel) (solid circles). Student’s T test. ****p < 0.0001. (D) Representative tumour clones from a hypermethylated resistant clone treated with vehicle or with 5-AZA (left panel) or PI3K inhibitor (right panel). Scale bar: 1 cm.

**Figure 1** Pharmacological validation of the hypermethylated and PI3K activated resistance mechanisms. (A) Heatmap representing colour-coded expression levels of differentially expressed genes among the indicated CDK4/RAFI resistant clones. (B) Colony formation assay of parental cell lines and CDK4/RAFI resistant clones treated with 10\(^{-6}\)M DMFSO (upper panel) or with 2\(\mu\)M of 5-aza-dc (5-AZA) (left panel) or with 1\(\mu\)M of PI3K inhibitor (right panel) for 9 days. (C) Tumour growth of CDK4/RAFI resistant clones treated with vehicle (open circles) or with 5-AZA (left panel) or with PI3K inhibitor (right panel) (solid circles). Student’s T test. ****p < 0.0001. (D) Representative tumour clones derived from a hypermethylated resistant clone treated with vehicle or with 5-AZA (left panel) or PI3K inhibitor (right panel). Scale bar: 1 cm.

**Characterization of CDK4/RAF1 resistant tumour cells**

In spite of the significant therapeutic response observed upon CDK4 and RAF1 inactivation, most tumours (66%) only underwent partial responses (PRs), indicating the presence of resistant cells. To interrogate those mechanisms implicated in the lack of response to CDK4/RAF1 inactivation, we selected cells able to proliferate in the absence of wild type CDK4 and RAF1. Comparison of the transcriptional profiles of these resistant clones with that of 2 independent resistant mechanisms. They included a ‘hypermethylated’ phenotype leading to a significant decrease in the expression of a series of tumour suppressor genes and a transcriptional profile suggestive of a PI3K activated phenotype. To pharmacologically validate this bioinformatic analysis, we exposed the CDK4/RAF1 resistant clones with the hypermethylated phenotype to the demethylation agent 5-azacytidine (5-AZA). These resistant clones, but not their parental cells, were exquisitely sensitive to this drug. Likewise, exposure of resistant clones displaying a transcriptional profile consistent with a PI3K activated phenotype were effectively inhibited by PI3K inhibitors. Interestingly, \(Kras/Trp53\) mutant lung tumour cells were not sensitive to either 5-AZA or PI3K inhibitors unless CDK4 and RAF1 were previously inactivated, thus demonstrating that these pharmacological vulnerabilities to methylation or PI3K inhibitors represent bona fide resistance mechanisms. Nevertheless, the high toxicities displayed by 5-AZA and PI3K inhibitors in the clinic underscores the need for better compounds to combat the resistance to CDK4/RAF1 inhibition. Hopefully, the design of more potent and selective inhibitors against these targets should allow the translation of these results to the clinic in a not-too-distant future.

**RESEARCH HIGHLIGHTS**

CDK4 and RAF1 are essential for progression of \(Kras/Trp53\) driven lung adenocarcinomas

- Inhibitors of CDK4 and RAF1 are particularly active against KRAS downstream effectors. This combination induced complete regression in 25% of the tumours. To pharmacologically validate our genetic studies, we compared the therapeutic effect of expressing CDK4\(^{KD}\) with that of 2 independent CDK4/6 inhibitors, palbociclib and abemaciclib, in the context of RAF1 ablation. Unfortunately, both inhibitors failed to phenocopy the cooperative effect observed upon genetic inactivation of CDK4. Likewise, we also attempted to pharmacologically validate the therapeutic effect of RAF1 ablation by inhibiting its kinase activity with 4 independent RAF inhibitor kinase inhibitors, including MLN2480 (Millennium), GSK1836785 (GSK), PLX8094 (Plexinon), and LSN3074753 (Eli Lilly). Only the latter displayed a sub-micromolar IC50 against cell lines derived from 2 independent KRAS mutant lung PDX models. Treatment of tumour-bearing mice with LSN3074753 mice for 4 weeks with a combination of abemaciclib and LSN3074753 reduced, but did not prevent tumour growth and only induced partial regressions in 10% of the tumours. No complete regressions were observed. Therefore, pharmacological validation of our genetic results will require the generation of more potent and selective inhibitors.

**Publications**


**Awards and Recognition**

- Anaida de Honor, Spanish Ministry of Defense, Madrid, Spain.
- 15th International AISECA Congress, Santiago de Compostela, Spain.
- III Keynote Lecture, III CONCURSO DE JÓVENES INVESTIGADORES, Madrid, Spain.
- Spanish Academy of Sciences, Madrid, Spain.
- Closing Lecture, 11th International AISECA Congress, Santiago de Compostela, Spain.
- Keynote Lecture, 3rd EBHERIDC Young Researchers Meeting, Madrid, Spain.

**ANNUAL REPORT 2020**

**SPAINISH NATIONAL CANCER RESEARCH CENTRE, CNIO**

**MOLECULAR ONCOLOGY PROGRAMME | EXPERIMENTAL ONCOLOGY GROUP**
The Cell Division and Cancer Group is interested in deciphering the mechanisms by which cell division and cell proliferation are regulated in mammalian cells. Our scientific interests are:

- to understand the basic control mechanisms that regulate the cell division cycle.
- to characterise the physiological and therapeutic consequences of cell cycle deregulation.
- understanding self-renewal and pluripotency in stem cell biology and tumour development.
- improving the use of old and new targets for cancer therapy.

As a final goal, we aim to generate information that will be useful for understanding basic mechanisms of cell function and to improve therapeutic strategies against cancer cell proliferation.

“Our group has proposed new therapeutic uses of CDK4/6 inhibitors in metastatic cancer, as well as new strategies to improve the function of pluripotent cells in regenerative medicine.”
Cell cycle inhibition in cancer in cancer therapy

Inhibition of the cell cycle kinases CDK4 and CDK6 is currently part of the standard-of-care for the treatment of hormone receptor-positive, metastatic breast cancer. Inhibiting CDK4/6 activity is also considered an attractive therapeutic intervention for multiple other malignancies. However, it is generally assumed that these inhibitors should not be used in combination with classical chemotherapy, given that CDK4/6 inhibition arrests cells in G1, thereby protecting tumour cells from the cytotoxic effect of classical chemotherapy acting either in S-phase or mitosis in proliferating cells. Unfortunately, classical chemotherapy (DNA damaging agents, topoisomerase inhibitors, taxanes etc.) remains the treatment of choice for most patients with advanced disease. Using pancreatic adenocarcinoma (PDAC) as a model, we recently generated data suggesting that, both in vitro and in vivo, applying CDK4/6 inhibitors right after taxanes strongly cooperates to prevent tumour cell proliferation (FIGURE 1). We also demonstrated that the mechanism behind these observations is different from the classical model in which CDK4/6 are required for S-phase entry. We described that CDK4/6 activity is required for homologous recombination and DNA repair, and the recovery from the chromosomal damage imposed by taxanes or DNA damaging agents. This mechanism immediately suggests that the mechanism behind these observations is different from the chromosomal damage imposed by taxanes or DNA damaging agents. Unfortunately, classical chemotherapy (DNA damaging agents, topoisomerase inhibitors, taxanes etc.) remains the treatment of choice for most patients with advanced disease.

Mechanistically, this effect is mediated through the repression of de novo DNA methyltransferases Dnmt3a and Dnmt3b and, globally, but transient, genome demethylation. Application of miR-203 to iPSCs or ESCs mediates the resetting of the epigenetic memory and improves the developmental potential of these cells in multiple assays, including generating or live differentiation of mesenchymal cells from pluripotent cells, or interspecies embryos in which human pluripotent cells are aggregated into mouse embryos. Exposure to miR-203 enhances the differentiation of mesenchymal cells from pluripotent cells in vitro, and improves the recovery from heart injuries in a model of cardiac infarctions in mice. These findings may have important potential implications in regenerative medicine that we plan to study in the upcoming years.

Finally, we are using a variety of genetically-modified mice and iPSC/ES cells with specific mutations in cell cycle regulators to understand the basic mechanisms of control of cell cycle progression and self-renewal in pluripotent cells. Our preliminary data suggest interesting connections between cell cycle kinases and phosphatases, the developmental potential of neural progenitors, and the generation of developmental syndromes with defects in the nervous system, including primary microcephaly, a developmental defect resulting in smaller brain at birth. The molecular connections between centrosome dynamics, cell cycle regulation, and cell fate in neural progenitors are currently under analysis in these models.

Improving the use of pluripotent cells in regenerative medicine

How pluripotent cells control their self-renewal and differentiation potential is becoming a major research topic in our laboratory. Our recent work suggests that a microRNA expressed in early development, miR-203, is able to induce naive pluripotency in both murine and human induced pluripotent cells (iPSC) and embryonic stem cells (ESCs), thereby enhancing the potential of these cells in vitro and in vivo. Mechanistically, this effect is mediated through the repression of de novo DNA methyltransferases Dnmt3a and Dnmt3b and, globally, but transient, genome demethylation. Application of miR-203 to iPSCs or ESCs mediates the resetting of the epigenetic memory and improves the developmental potential of these cells in multiple assays, including generating or live differentiation of mesenchymal cells from pluripotent cells, or interspecies embryos in which human pluripotent cells are aggregated into mouse embryos. Exposure to miR-203 enhances the differentiation of mesenchymal cells from pluripotent cells in vitro, and improves the recovery from heart injuries in a model of cardiac infarctions in mice. These findings may have important potential implications in regenerative medicine that we plan to study in the upcoming years.

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**REFERENCES**

In the Genomic Instability Group, our main goal is to understand the molecular mechanisms underlying cancer and other age-related diseases, and then use this knowledge for the development of new therapies. To this end, we combine molecular and cellular biology approaches with chemical and genetic screens that allow us to identify cancer cell vulnerabilities and new druggable targets. In parallel, we develop mouse models of disease, which we can later use as platforms for testing new treatments. With all these tools, in recent years, we have made exciting discoveries in several areas, from basic to translational research. We have contributed to the understanding of fundamental aspects of DNA replication, unveiled new mechanisms of resistance to cancer therapies, and developed anticancer drugs that are now in clinical development. Ultimately, our objective is to translate our findings into better treatments for human disease. During 2020 we made significant advances in several areas related to cancer ontogeny and therapy. For instance, we revealed a tumour suppressor role for the RNA-binding protein EWSR1 and made significant advances in the development of chemical inhibitors of the histone methyltransferase SETD8.
EWSR1 loss drives nuclear stress and cancer development

Metastatic Ewing Sarcoma (ES) is a paediatric bone tumour driven by translocations that frequently involve the RNA-binding protein EWSR1 (e.g., EWSR1-FLI1). We previously demonstrated that ES shows remarkable susceptibility to a treatment with ATR inhibitors that were developed by our Group and the CNIO Experimental Therapeutics Programme. To address this possibility, we developed constitutive and conditional knockout mouse models of EWSR1. Constitutive EWSR1 nullzygosity leads to anaemia and embryonic lethality, indicating a particular impact on the immune system. Consistently, ubiquitous deletion of EWSR1 in adult animals leads to a fully penetrant early onset of thymomas to which mice succumb within the first 6 months of life (FIGURE 1). These results are consistent with recent large-scale cancer genomic studies that have identified recurrent EWSR1 gene deletions in human thymomas. EWSR1-deficient MEF or thymoma cell lines presented an accumulation of nuclear stress, which increased their sensitivity to RNA Polymerase I inhibitors. Altogether, this work identified EWSR1 as a tumour suppressor and revealed vulnerabilities in EWSR1-deficient cells that could be exploited for potential treatments.

Developing new SETD8 chemical inhibitors

Drugs targeting the epigenetic machinery are a promising avenue for cancer therapy, particularly in the context of paediatric tumours where the mutational load is low and they are frequently associated with stem-cell differentiation anomalies. In this context, recent evidence has indicated that targeting the histone methyltransferase SETD8 might have antitumour effects in certain paediatric tumours of poor prognosis, such as neuroblastoma or medulloblastoma. However, the few currently available SETD8 inhibitors show poor potency and pharmacological properties, and none has reached clinical trials. Our group, through a previous collaboration with the laboratory of Dr Modesto Orozco at the IRB (Barcelona), identified a new class of chemical SETD8 inhibitors, with the main compound (SETD8iCNIO) significantly reducing histone 4 monomethylation (H4K20me1) levels in cellular assays. The compounds also trigger the known cellular effects associated with SETD8 depletion by siRNA, such as increasing the expression of P53 or inhibiting the function of DNA repair factors like 53BP1 (FIGURE 2). Hence, we currently have a new class of SETD8 inhibitors with cellular activity in the mid-nanomolar range. Regarding their mechanism of action, our current data already reveal that, contrary to current proposals, and despite the increase in p53 levels observed when cells are exposed to SETD8 inhibitors, the cell death induced by these compounds is not related to P53 and instead related to other aspects of cellular metabolism such as nuclear integrity. We did in the past for ATR inhibitors, we are currently exploring other essential aspects to optimise the clinical application of these new agents as anticancer drugs, such as to define the type of tumours that are more likely to respond to the therapy (biomarkers), and the mechanisms of resistance that might emerge upon treatment.

**PUBLICATIONS**


**AWARDS AND RECOGNITION**

- ERC Proof of Concept (PoC) Grant to develop SETD8 inhibitors, European Research Council.

- Section Editor, Molecular Oncology.
DNA topoisomerases have a dual relationship with the genome. They are essential to solve the topological problems inherent to all DNA transactions, but their intrinsic mechanism of action can result in the formation of DNA breaks, either accidentally during normal cellular metabolism or upon chemotherapy treatment with the so-called topoisomerase poisons. Imbalances in DNA topoisomerase activity can therefore compromise cell survival and genome integrity, entailing serious consequences for human health, such as developmental and degenerative problems and, very importantly, neoplastic transformation processes and their subsequent response to treatment.

We are interested 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.

“We have proven a causal link between spontaneous DNA breaks induced by topoisomerase II and tumorigenesis in mouse models of lymphoid and prostate cancer.”
RESEARCH HIGHLIGHTS

In 2020, our work mostly focused on understanding DNA topoisomerase II (TOP2) function and how double-strand breaks (DSBs) derived from its aberrant action can compromise genome integrity and drive tumorigenesis.

Machine learning to predict topoisomerase II function genome wide

We have performed an unbiased analysis of available chromatin and DNA sequence features in order to establish which of them determine TOP2 binding genome wide. We achieved highly accurate predictions, with accessible chromatin and architectural factors being the most informative features. Strikingly, we found that TOP2 is sufficiently explained by only 3 features: DNase I hypersensitivity, CTCF and cohesin binding, for which genome-wide data are widely available. Based on this, we developed a predictive model for TOP2 genome-wide binding that can be used across cell lines and species, and generate virtual probability tracks that accurately mirror experimental ChIP-seq data. These results deepen our knowledge on how the accessibility and 3D chromatin organisation of TOP2 function and constitute a proof of principle regarding the in silico prediction of sequence-independent chromatin-binding factors. The methodology may now be used to predict TOP2 function in multiple cell-types, organisms and conditions, boosting our understanding of TOP2 biology and its implications in the origin of oncogenic translocations and other types of chromosomal rearrangements as potential cancer drivers.

Topoisomerase II-induced DNA breaks and prostate cancer

TOP2 has been previously linked to the regulation of hormone-induced transcription, and in particular, to the activation of androgen-responsive genes. The mechanism by which this occurs, and whether the induction and repair of DSBs is involved, remain, however, poorly understood. In collaboration with the group of Hiroyuki Sasanuma and Shunichi Takada (University of Kyoto) we have addressed the involvement of TOP2 and TDP2 in the response to androgen. We found that physiological concentrations of androgens induce TOP2-mediated DSBs that are repaired by TDP2 in human prostate cancer cells and prostate epithelium in mouse models. Furthermore, we found that TDP2-deficient mice spontaneously develop higher levels of prostate hyperplasia when compared to wild-type animals. These results suggest that endogenous TOP2-mediated DSBs resulting from androgen signalling can drive prostate hyperplasia and influence the development of prostate cancer.

Atm" mouse models are particularly predisposed to develop lymphoid cancers derived from deficient repair of RAG-induced DSBs during V(D)J recombination. We have unexpectedly found that specifically disturbing the repair of TOP2-induced DSBs by genetically removing the highly specialised repair enzyme TDP2 strongly increases the incidence of thymic tumours in Atm" mice, but without changing their molecular characteristics or underlying genomic rearrangements, including a significant association with Tcr loci. Furthermore, we found that TOP2 strongly colocalises with RAG, both on a genome-wide scale and specifically at sites undergoing V(D)J recombination, in a manner that is consistent with its involvement in solving topological problems associated to 3D genome organisation, and that results in an increased chromosomal fragility of these regions. Thus, our findings demonstrate a strong causal relationship between spontaneous TOP2-induced DSBs and cancer development, confirming these lesions as major drivers of ATM-deficient/lymphoid malignancies, and potentially other conditions and cancer types.

**PUBLICATIONS**


**FIGURE 1** Comparison between experimentally determined (ChIPseq) and machine-learning-predicted TOP2 binding genome wide. (A) Representative USCC genome browser views of experimental and predicted TOP2 binding in a representative genome region in the indicated mouse tissues or cell types. (B) Representative USCC genome browser views of experimental (top independent ChIP-seq experiments: R1 and R2) and predicted TOP2 binding in the human MCF7 breast cancer cell line. Annotated genes in each region are indicated.

**FIGURE 2** Model to explain aberrant TOP2 activity as a driver of ATM-deficient thymus tumours. TOP2 activity accidentally results in DSBs throughout the genome (top). Additionally, TOP2-DSBs are associated to V(D)J genome reorganisations (bottom), concurring with RAG-mediated DSBs. TOP2 and ATM limit the oncogenic potential of these lesions.
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. Mutations in cohesin have been found in several tumour types, most prominently in bladder cancer, Ewing sarcoma and acute myeloid leukaemia. Germline mutations in cohesin and its regulatory factors are also at the origin of human developmental syndromes collectively known as cohesinopathies.

Our goal is to understand how cohesin works, how it is regulated, and how its dysfunction contributes to cancer and other human diseases. In particular, we are intrigued by the existence of different versions of the cohesin complex. We use human cells and mouse models carrying knock out alleles of genes encoding variant cohesin subunits to investigate their functional specificity.

“We have found that cohesin STAG2 is essential during embryonic development, while its loss in adult mice reduces fitness without increasing tumour incidence.”
RESEARCH HIGHLIGHTS

Cohesin STAG2 is essential for mouse embryonic development

Cohesin consists of 4 core subunits, SMC1, SMC3, RAD21 and STAG1/2. There are two versions of the STAG subunit in vertebrate somatic cells, STAG1 and STAG2, giving rise to two distinct complexes. These are present in all tissues and cell types, but their functional specificity is not well established. STAG2 is commonly mutated in cancer, and germline mutations in both STAG1 and STAG2 have been recently identified in cohesinopathy patients. To identify specific functions of STAG2 at the cellular and organisational levels and to better understand the pathological consequences of its loss, we generated and characterised STAG2 conditional knock out mouse strain in collaboration with the groups of F. X. Real (CNIO) and M. Manzanares (CNIC, CBMSo).

We found that embryos lacking cohesin-STAG2 die by mid-gestation, showing global developmental delay and a selective defect in the developing heart, most prominently in structures derived from secondary heart field progenitors. Both decreased proliferation and altered transcription of tissue-specific genes likely contribute to these defects. In contrast to the embryonic lethality, STAG2 is largely dispensable in adults, and its tissue-wide inactivation does not lead to tumours but reduces fitness and affects both haematopoiesis and intestinal homeostasis.

We also analysed the consequences of Stag2 deletion in mouse embryonic fibroblasts (MEFs). Stag2-null MEFs show mild centromeric cohesin defects and proliferate more slowly than wild type MEFs, but they are viable. Likewise, we had previously reported that Stag2-null MEFs display telomere cohesin defects that impair chromosome segregation, but they are also viable. Thus, cells growing in culture can survive with a single cohesin complex carrying either STAG1 or STAG2, while the two complexes are required to fulfil embryonic development (FIGURE 1).

Different dynamics of the two cohesin variants underlie their differential contribution to 3D genome organisation

Cohesin and the proteins that regulate its association to chromatin (NIPBL, PDS5A/B, WAPL, ESCO1/2, CTCF) are key for shaping genome architecture. Our previous studies in human epithelial cells and mouse embryonic stem cells knocked down for STAG1 or STAG2 identified differential contributions of the two complexes. Cohesin-STAG2, together with the architectural protein CTCF, plays a more important role in the demarcation of topological associated domains (TADs) while cohesin-STAG1 promotes local chromatin contacts that are relevant for tissue-specific transcription independent of CTCF. Analysis of the distribution of cohesin and its regulators in MEFs confirmed that the two variants occupy CTCF-bound positions while STAG2 can be additionally found at non-CTCF sites (FIGURE 2). PDS5A and PDS5B are located at the former positions while NIPBL is enriched preferentially at the latter. Unlike previous observations in Stag2 deficient MEFs, in which the distribution of cohesin changed to include new non-CTCF positions, the number of cohesin binding sites detected in Stag2 deficient MEFs is restricted to those overlapping with CTCF. This situation, which occurs in several tumours with loss of function mutations in STAG2, alters gene expression. Salt extraction of chromatin fractions and fluorescence recovery after photobleaching (FRAP) experiments in MEFs show that cohesin-STAG2 binding to chromatin is more dynamic than binding of cohesin-STAG1. In addition, we have observed a preferential association of STAG2 with the chromatin releasing factor WAPL, while others have reported a stronger interaction between STAG1 and CTCF. We are currently exploring the molecular determinants of these preferences and how they contribute to shape chromatin architecture. 

Figure 1 Scheme that summarises the characterisation of Stag2 KO and Stag1 KO cells (MEF, mouse embryonic fibroblasts, top) and embryos (bottom). Both Stag1 KO and Stag2 KO cohesins are required for embryonic development, while either one is sufficient for cell viability.

Figure 2 Heatmaps showing the distribution of CTCF and cohesin subunits in wild type (WT) MEFs. Two types of positions are found, with or without CTCF. STAG2 is the preferred variant in non-CTCF cohesin positions, whereas STAG1 occupies by cohesin STAG1, even in the absence of STAG2.
The process of DNA replication is responsible for many of the genomic alterations underlying the activation of oncogenes or the inactivation of tumour suppressor genes. While some of these alterations are inherent to life, e.g. the introduction of mutations due to DNA polymerase errors, others are caused by environmental agents — UV light, ionising radiation, toxic chemicals in tobacco smoke, and other pollutants — that induce chemical modifications in the DNA and complicate its replication. The capacity to generate difficult-to-replicate DNA modifications, e.g. covalent links between the two strands of the double helix, is the basis for the cytotoxic effect of cisplatin and other drugs used in cancer therapy. Our laboratory studies how the “replisome” machinery is capable of operating through these lesions, a step that normally leads to the activation of specific DNA repair pathways. In 2020 we focused on the study of the PrimPol enzyme that mediates the replicative tolerance of DNA crosslinks generated by common chemotherapy agents.

“We have identified that PrimPol facilitates the tolerance and repair of DNA inter-strand crosslinks, making it a suitable molecular target to enhance the efficacy of chemotherapy.”
RESEARCH HIGHLIGHTS

The slowdown of DNA synthesis caused by DNA lesions, hard-to-repair special structures or collisions with transcription proteins is referred to as “replicative stress” (RS). In previous years we reported that human PrimPol protein mediates the bypass of UV-generated DNA lesions by synthesising primers that allow re-initiation of DNA synthesis from a downstream point, leaving behind short non-replicated gaps. In 2020 we found that PrimPol-mediated repriming is involved in the tolerance and repair of inter-strand crosslinks (ICLs), one of the most cytotoxic DNA lesions. We also participated in collaborative studies that underscore the importance of repriming in cancer cells deficient in BRCA proteins, and in the response to DNA lesions induced by benzo[a]pyrene, an ubiquitous environmental carcinogenic.

Replicative tolerance mediated by PrimPol

PrimPol is the only enzyme with primase activity identified in mammalian cells besides the Polα/primase that initiates replication through UV-induced CPD and 6,4pp DNA adducts. During the last year we found that PrimPol is required to elicit the repair of DNA ICLs caused by endogenous aldehydes, chemotherapy agents (e.g. cisplatin), and chemicals used to treat certain skin conditions (e.g. trimethyl psoralen). ICL recognition and repair requires DNA replication and a combination of homologous recombination, transcription syntheses and nucleotide excision repair. Mutations in ICL repair genes cause Fanconi Anaemia, a rare but severe disease associated to congenital abnormalities, bone marrow failure, and predisposition to leukaemia and solid tumours.

Our recent research indicates that PrimPol interacts with proteins that recognise ICLs such as the BTR complex (Bloom’s-Top2A-RM1-RM2) and the FANC translocase complex (FANC-MHF1-MHF2-FAPA-F24), and plays an important role in the progression of the replisome through ICLs, also called “ICL traverse”. Using an assay to monitor DNA replication in stretched DNA fibres in the presence of ICLs, we have found that the catalytic activity of PrimPol is required for efficient ICL traverse (FIGURE 1). Genetic ablation of PRIMPOL in human cells and mice leads to hypersensitivity to ICL-inducing agents, as indicated by the higher incidence of chromosomal lesions (FIGURE 2), and delays ICL repair. Of note, the FA pathway can be activated without PrimPol by an alternative way that requires the convergence of two replication forks at each ICL lesion. The role of PrimPol in ICL traverse reveals a new molecular element in the complex pathways leading to ICL repair (González-Acosta et al., 2020).

In collaboration with A. Vindigni (Washington University, St Louis, USA), we learnt that PrimPol mediates an adaptive response to cisplatin in BRCA-deficient cells that have lost the ability to stabilise stalled forks in situations of RS (Quinet et al., 2020). In addition, we participated in a study led by E. Petermann (University of Birmingham, UK), showing that PrimPol acts on bulky DNA adducts caused by benzo[a]pyrene-diol-epoxide (BPDE). In this case, Rad51 protein that PrimPol acts on bulky DNA adducts.

Because PrimPol counteracts the cytotoxic effect of DNA crosslinks, we hypothesise that it could be targeted to increase the efficacy of chemotherapy based on crosslinking agents. A screening for PrimPol small molecule inhibitors is underway, supported by the CNIO Experimental Therapeutics Programme.

DNA replication in Pds5-deficient cells

We are also interested in the molecular connections between DNA replication and the cohesin complex involved in sister chromatid cohesion, chromatin organisation, transcriptional regulation, and DNA repair. In collaboration with Ana Losada’s Group (CNIO), we learnt that the cohesin-associated factors PDS5A and PDS5B are required for proper replication fork progression and BRCA2-mediated protection of stalled forks (Morales et al., 2020).

FIGURE 1 PrimPol mediates ICL traverse. (A) Left, experimental design. Right, individual DNA fibres with different patterns of DNA synthesis around an ICL lesion. (B) Percentage of each pattern in the experimental conditions tested. A, Aα, PrimPol catalytic mutant; O, wild-type, primase-/primase that initiates replication fork reversal in BRCA-deficient cells. (C) Examples of chromosome breaks and fusions are highlighted. (D) Quantification of chromosome aberrations per metaphase (cavase and SEM) in each condition. Statistical analysis: one-way ANOVA and Bonferroni post-test. *, p<0.05; **, p<0.01. Adapted from González-Acosta et al. (2020).

FIGURE 2 PrimPol loss sensitises cells to ICL-inducing agents. (A) Experimental design and metaphase spreads from control or TMP-UVA-treated WT and PrimPol KO cells. (B) Percentage of each pattern in the experimental conditions tested. A, Aα, PrimPol catalytic mutant; O, wild-type, primase-/primase that initiates replication fork reversal in BRCA-deficient cells. (C) Examples of chromosome breaks and fusions are highlighted. (D) Quantification of chromosome aberrations per metaphase (cavase and SEM) in each condition. Statistical analysis: one-way ANOVA and Bonferroni post-test. *, p<0.05; **, p<0.01. Adapted from González-Acosta et al. (2020).

• PUBLICATIONS


Melanomas are the only tumours where lesions barely over one millimetre in depth can be at risk for metastasis. An increasing number of (epi)genetic alterations and mechanisms of immune evasion have been identified in this disease. Nevertheless, no molecular biomarker has been approved as a bona fide prognostic indicator. The field is also in need of improved treatments, as a significant fraction of patients is resistant to targeted and immune-based therapies. The long-term goal of our Group is to identify new progression biomarkers and anticancer agents. We are particularly interested in defining lineage-specific vulnerabilities that distinguish melanomas from other tumours with lower metastatic potential (publications in Nature, Cancer Cell, Nature Cell Biology, Nature Communications, among others). Our laboratory has also generated first-in-class lymphoreporter mice for non-invasive imaging of pre-metastatic niches in melanoma (Nature) and has identified actionable immune suppressive mechanisms with implications for patient treatment (Nature Medicine). Our ultimate objective is to improve the management of patients with otherwise refractory metastatic melanomas.

“We have visualised and targeted (pre)metastatic niches in melanoma and defined mechanisms of immune suppression with clinical implications for cancer patients.”
ANNUAL REPORT 2020

CNIO Melanoma Group: objectives and model systems

Melanomas are aggressive solid tumours and a paradigm of how basic and clinical research have significantly improved patient prognosis. Nevertheless, despite great success with targeted and immune-based therapies, sustained clinical responses are still limited. Moreover, the field lacks molecular markers of diagnosis, and the knowledge about how melanomas progress is largely incomplete. One of the main objectives of our Group is to define modulators of this aggressive behaviour. In particular, we are interested in identifying mechanisms that drive (pre)metastatic niche formation in vivo, specifically those acting in a systemic manner already from early stages of melanoma development, creating “permissive” microenvironment(s) for tumour progression.

Our Group’s main aims are to:

→ define when and how melanomas act “at a distance” (on stromal and immune compartments) before tumour cell dissemination.
→ determine how melanoma cells evade the immune system, and whether distinct mechanisms may be activated at different anatomical sites.
→ develop anticancer agents to prevent and eliminate metastatic sites.

New immune suppressors that favour melanoma progression

One of the long-term objectives of the Melanoma Group is to discover new melanoma drivers. We previously identified a cluster of endosulfonyl-associated genes that distinguish melanomas from over 35 additional malignancies (Alonso-Curbelo et al., Cancer Cell 2014). Further analyses of lysosomal-dependent pathways also revealed distinctive features of autophagy genes (ATG5) and RNA binding proteins (CELF1 and IGF2BP1) with selective roles in melanoma (García-Curbelo et al., 2016; Perez-Guijarro et al., Cancer Discovery 2019). All these proteins had potent autocrine effects on the tumour cells where they were expressed. However, we were also interested in melanoma-secreted factors that could exert long-range activities at visceral organs, particularly in the generation of premetastatic niches.

Our Group pioneered the analysis of such systemic effects in vivo by exploiting melanoma “MetAlert” mice, which have the unique feature of visualising tumour-activated lymphatic vasculature (Olmeda et al., Nature 2017). “MetAlert” animals, in combination with human tissue specimens, revealed the growth factor MDKine (MDK) as a new driver of melanoma metastasis. We have now performed loss- and gain-of-function studies of downstream effectors of MDK in vitro and in vivo (mouse xenograft models), combined with expression studies in large patient cohorts. These studies have revealed yet a new function of MDK in immune suppression. Specifically, we identified a MDK-associated gene set that was able to separate melanoma patients with a differing transcriptional phenotype, involving in particular a variety of immunomodulators (Cerezo-Wallis et al., Nature Medicine 2020). Curiously, although MDK promoted an inflammatory secretome (driven in part by NF-kB), the ultimate outcome was an immunotolerant microenvironment whereby macrophages are recruited to tumours, but instead of attacking the cancer cells, promote a dysfunctional state in CD8+ T cells, ultimately favouring immune escape (FIGURE 1, left part). This “Jekyll and Hyde syndrome” described above, whereby the immune system shift from an anti-tumoural to a pro-tumoural phenotype depending on MDK expression, was recently published in Nature Medicine (Cerezo-Wallis et al., 2020) and featured on the cover of the journal.

Gene signatures that define response to immune checkpoint blockade in melanoma patients

Having found that MDK promoted immune suppression, our next approach was to block its function genetically or pharmacologically. Using various murine systems, we found that MDK inhibition favoured the response to vaccination treatments, and importantly, promoted an interferon (IFN)-driven secretome that enhanced the effect of immune checkpoint blockers (ICB) (summarised in FIGURE 1, right part). This IFN-signalling resulting from MDK blockade was enriched in 6 independent clinical cohorts of melanoma patients treated with ICB (see examples in FIGURE 2).

Therefore, these results provided proof of principle for MDK inhibition as a strategy to prime immunologically unresponsive tumours into “hot” lesions with an improved response to ICB. The novelty and physiological relevance of these data received considerable attention in the media (TV, press, radio) and were echoed in independent News & Views in Nature Reviews Cancer, Cancer Discovery and in Pigment Cell and Melanoma Research.
We focus on the molecular pathophysiology of pancreatic ductal adenocarcinoma (PDAC) and urothelial bladder carcinoma (UBC) with a disease-oriented approach. We use patient samples, cultured cells, and genetically modified mice, giving a similar weight to the 3 model systems. Observations made at either of these levels are then extended through additional work. To translate the findings, we bring this knowledge to a “population” level – leveraging on information and samples from large patient cohorts – in close collaboration with Núria Malats’ Group (CNIO).

In PDAC, a main hypothesis is that cell differentiation is a potent tumour suppressor mechanism acting early in carcinogenesis. We use the excellent genetic mouse models available because these processes cannot be readily studied in humans. In mice, PDAC can originate in pancreatic progenitors and in adult acinar and ductal cells. Understanding the contribution of early molecular events is crucial to design better strategies for prevention and early tumour detection.

In UBC, we focus on identifying new genes, using them for improved tumour taxonomy, characterising the mechanisms of action, and applying this knowledge for improved prediction of outcome and therapy.

**RESEARCH HIGHLIGHTS**

**Pancreatic cancer molecular pathophysiology**

The genetic/genomic changes associated with PDAC have been extensively described by the genome consortia, and there is increasing interest in defining the molecular changes that precede tumour development. Our lab has pioneered the notion that cell differentiation is the first tumour suppressor mechanism in the pancreas. Focusing on acinar cells, we have identified several novel transcriptional regulators involved – including GATA6, GATA4, NR5A2, HNF1A, and NFIC. Dysregulation of these genes is associated with a scenario of pre-inflammation or inflammation, dependent on a functional interaction with the microbiome: antibiotic administration to deplete gut bacteria enhances the activity of the acinar programme and rescues the inflammatory predisposition of Nrsa2 heterozygous mice, with lesser effects on wild type mice. The relevance of these findings to PDAC development are being analysed. These studies provide the basis for the pharmacological and genetic manipulation of acinar differentiation as a tumour preventative strategy.

GATA6 is a master regulator of the “classical” PDAC transcriptional programme and its loss is associated with poor patient outcome. In mice, GATA6 loss promotes metastasis and immune evasion (with P. Martinelli). GATA4 loss also favours PDAC development/progression in mice. However, these proteins play opposite roles in inflammation and they contribute differently to tumour initiation. In collaboration with an international consortium, we have shown that tumours that lose both GATA6 and GATA4 have the worst outcome and we are assessing the hypothesis that GATA6 amplifications are associated with long-term survivorship, possibly by locking cells in a differentiated state. We are focusing on deciphering their overlapping and unique transcriptional programmes using a combination of mouse models and genomic approaches (i.e., RNA-Seq and ChIP-Seq).

New conditional knockout mouse models of Hnf1a, developed with J. Ferrer (CRG, Barcelona) and Sagrario Ortega (CNIO), show that HNF1A can act as a tumour suppressor in PDAC initiation. Using a dual recombinase system, we are assessing the role of HNF1A and its partner NR5A2 in tumour maintenance.
The activity of these transcription factors is intertwined, and our overarching goal is to establish the rules and hierarchies governing the control of acinar differentiation and their contribution to pancreas and cancer.

Urothelial bladder cancer (UBC) genetics, biology, and clinical translation

We focus on understanding 2 new UBC tumour suppressor genes that we identified through exome sequencing. STAG2 and RBM10. STAG2 codes for a cohesion 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 and their cooperation with other cancer genes.

Increasing evidence shows that STAG2 acts as a tumour suppressor through rather unique mechanisms, largely unrelated to the canonical role of cohesion in chromosome segregation. In normal urothelial cells, the genomic effects of STAG2 loss are dependent on the differentiation state and clinical translation. In normal urothelial cells, the genomic effects of STAG2 loss are dependent on the differentiation state and their segregation. In normal urothelial cells, the genomic effects of STAG2 loss are dependent on the differentiation state and their segregation.

Our translational studies focus on the prediction of response to cisplatin-based chemotherapy and to immune checkpoint blockade (ICB). In collaboration with Núria Malats and Spanish u-oncologists, we are assessing the value of our immune signatures to stratify patients to receive neoadjuvant therapy (cisplatin-based chemotherapy vs. ICB) in a randomised clinical trial.
Our laboratory focuses on understanding mechanisms of diseases associated to the digestive system, including liver, intestine and pancreas. Our work aims to integrate mouse models mimicking human disease with state-of-the-art genomics, proteomics, bioinformatics, metabolic pathways and gut microbiome analyses, and therapeutic technologies combined with human data, to: find out what goes wrong in diseased and cancerous tissues; understand how organs can regenerate; and, if regeneration goes awry, to determine how it contributes to cancer.

We put a special emphasis on studying the mechanobiology of tissue development in a health and disease context, from the physical and mechanical perspective at the molecular, cellular, and tissue levels, with the eventual goal to understand how an injured liver progresses to a cancerous tissue, in order to find new therapeutic targets. Additionally, the application of mathematical models to quantitatively study and analyse mechanical forces and cellular plasticity is an important focus in collaboration with other research groups. Finally, the use of nanotechnology combined with in vivo disease models generated in our laboratory might provide additional opportunities to complement our work and impact the field of medicine in diagnosis and treatment.

“We put much effort into understanding the mechanisms of diseases by generating and using genetically engineered mouse models that recapitulate the pathological features of human syndromes in order to guide the design of novel medicines.”
RESEARCH HIGHLIGHTS

Our interest is mainly driven by the discovery of two components initially identified in our laboratory to be downstream targets of the growth factor and nutrient signalling cascades: the URI (Unconventional prefoldin RPBS Interactor) and MCRS1 (Microspherule protein 1) proteins. URI and MCRS1 expression turned out to be regulated in response to various environmental factors (radiation, nutrients, bacteria, viruses, etc.), compromising their functions and activating pleiotropic circuits to support complex cell signaling networks with non-oncogene addiction functions and dependence, provoking severe outcomes. Importantly, URI and MCRS1 are respectively part of two independent protein complexes: the URI prefoldin-like and the non-specific lethal (NSL) complexes. While the URI prefoldin-like complex has some co-chaperone activities, both complexes seem to be critical for chromatin dynamics and remodelling, and are most likely involved in cellular plasticity and tissue regeneration.

Using genetically engineered mouse models (GEMMs) generated in our laboratory for URI and MCRS1 gain- and loss-of-function, combined with other model systems and cutting-edge technologies – including cell biology with organoid culture and quantitative imaging, biochemistry and functional genomic methodologies – and human data, our laboratory has devoted substantial effort over the last 5 years to understanding the molecular, cellular, and pathophysiological mechanisms linking environmental stresses to disease pathogenesis affecting organs of the digestive system. We put emphasis on studying diseases associated to the liver, intestine, and pancreas, as these organs are physiologically interconnected and influenced through their exocrine and/or endocrine functions (FIGURE).

Research in the last decade has focused mainly on understanding the functions and roles of newly discovered mutated genes in the development of cancer and associated diseases. With this focus, less attention has been paid to environmental factors leading to the expression of virulent factors or tissue damage that also present a permanent challenge for an organism. How environmental and temporal and spatial kinetics of signaling pathways and cell types involved, as well as the chronological evolution of the regenerative response during disease progression, will help us to understand what controls liver regeneration in chronic injury and HCC.

This work will be facilitated by our long-standing research interest in liver diseases and tissue regeneration, and impacted by the generation of sophisticated mouse models, recapitulating clinical features of the disease. Moreover, new approaches for the quantitative assessment, mathematical modelling, and bioinformatics analysis of single cell RNA sequencing will be specifically developed. Special effort will also be made to elucidate mechanisms of liver diseases from a patho-physiological standpoint which implicates organ crosstalk via metabolic pathways, gut microbiome, and cross-immune reactions. Recently, we have also developed an interest in mechanobiology and nanotechnology-based theranostics combined with the latest imaging technologies. This will allow us to associate conceptual advances arising in our laboratory, together with the GEMMs we generate, with these new technologies to guide the design of new therapeutic approaches to prevent and treat liver diseases and cancer. The final goal will be to promote healthy liver regeneration and to identify and functionally validate targets with potential preventive and therapeutic values. Doing so will enable us to treat frequent human lethal disorders with increased worldwide incidence and unmet medical needs.

Recently, we made the key discovery that URI marks the slow-cycling, label-retaining (LR) cells in the intestinal crypt, which are essential for organ regeneration following ionising radiation. Reduced URI levels render LR cells highly proliferative by activating the β-catenin/c-MYC axis. Consequently, LR cells become radiosensitive, thereby increasing gastrointestinal syndrome severity. We conclude that: (1) URI protects LR cells to promote intestinal tissue regeneration in response to high-dose irradiation; and (2) LR cells represent the facultative stem cell pool essential for organ regeneration following ionising radiation. This work was published in Science (Chaves et al., 2019).

We intend in the near future to continue deconstructing the mechanisms of pathologies associated to the digestive system in response to environmental stressors. We will focus on understanding the mechanisms of liver diseases to find out what goes wrong in diseased and cancerous tissues, and to understand how the organ can regenerate; we will also investigate how regeneration in chronic injury can impact hepatocellular carcinoma (HCC) development, the most common and one of the most lethal and aggressive human liver cancers. A complete understanding of the mechanisms and temporal and spatial kinetics of signalling pathways and cell types involved, as well as the chronological evolution of the regenerative response during disease progression, will help us to understand what controls liver regeneration in chronic injury and HCC.

This work will be facilitated by our long-standing research interest in liver diseases and tissue regeneration, and impacted by the generation of sophisticated mouse models, recapitulating clinical features of the disease. Moreover, new approaches for the quantitative assessment, mathematical modelling, and bioinformatics analysis of single cell RNA sequencing will be specifically developed. Special effort will also be made to elucidate mechanisms of liver diseases from a patho-physiological standpoint which implicates organ crosstalk via metabolic pathways, gut microbiome, and cross-immune reactions. Recently, we have also developed an interest in mechanobiology and nanotechnology-based theranostics combined with the latest imaging technologies. This will allow us to associate conceptual advances arising in our laboratory, together with the GEMMs we generate, with these new technologies to guide the design of new therapeutic approaches to prevent and treat liver diseases and cancer. The final goal will be to promote healthy liver regeneration and to identify and functionally validate targets with potential preventive and therapeutic values. Doing so will enable us to treat frequent human lethal disorders with increased worldwide incidence and unmet medical needs.
Research in the Transformation and Metastasis Group aims to identify novel therapeutic targets for epithelial cancer treatment and to elucidate resistance mechanisms to drugs currently available. Tumours exploit and manipulate for their benefit the same mechanisms that work correctly in the healthy tissue. Thus, we first aim to understand normal development, and then to identify the key events that lead to tumour initiation, progression and metastasis in order to avoid and combat them. Complementary tools including primary cell cultures and organoids, mouse models and clinical samples are used with the final goal of translating basic knowledge into clinically relevant findings.

One of our research lines aims to characterise the role of the TNF family member RANK in mammary gland development and breast cancer and to elucidate its therapeutic potential.

“Clinical and preclinical findings support that activation of RANK signalling in breast cancer cells induces immunosuppression and that its blockage leads to a T cell dependent anti-tumour response.”
Therapeutic impact of targeting RANK or RANKL in breast cancer and the tumour-immune crosstalk

Most breast cancers exhibit low immune infiltration and are unresponsive to immunotherapy. We hypothesised that inhibition of the RANK signalling pathway may enhance anti-tumour immune response. Using preclinical mouse models, we found that loss of RANK signalling in tumour cells increases infiltration by leukocytes, lymphocytes, and CD8+ T cells, and reduces macrophage and neutrophil infiltration. CD8+ T cells mediate the attenuated tumour phenotype observed upon RANK loss, whereas neutrophils, supported by RANK-expressing tumour cells, induce immunosuppression. Moreover, RANKL inhibition increases the anti-tumour effect of immunotherapies in mouse mammary tumours through a tumour cell mediated effect. Comparably, pre-operative single-agent denosumab in premenopausal early-stage breast cancer patients from the Phase-III B-DEYOND clinical trial (NCT01864798) was well tolerated, inhibited RANK pathway, and increased tumour infiltrating lymphocytes and CD8+ T cells. Higher RANK signalling activation in tumours and serum RANKL levels at baseline predict the immune-modulatory effects driven by denosumab.

Altogether, our preclinical and clinical findings reveal that tumour cells exploit the RANK pathway as a mechanism to evade immune surveillance and support the use of RANK pathway inhibitors to prime luminal breast cancer for immunotherapy (Gomez-Aleza et al., Nat Commun, 2020).

RANK signalling increases after anti-HER2 therapy contributing to the emergence of resistance in HER2-positive breast cancer

Around 15-20% of primary breast cancers are characterised by HER2 protein overexpression and/or HER2 gene amplification. Despite the successful development of anti-HER2 drugs, intrinsic or acquired resistance represents a major hurdle. RANK and RANKL proteins are more frequently detected in HER2-positive tumours that have acquired resistance to anti-HER2 therapies than in treatment-naive ones. RANK (but not RANKL) gene expression increased after dual anti-HER2 neoadjuvant therapy in the cohort from the SOL-T1-114 PAMELA trial. Results in HER2-positive breast cancer cell lines recapitulate the clinical observations, with increased RANK expression after short-term treatment with anti-HER2 therapies and enhanced NF-κB activation in lapatinib resistant HER2+ breast cancer cells. Moreover, we found that overactivation of the RANK signalling pathway enhances ERK and NF-κB signalling and increases lapatinib resistance in different HER2-positive breast cancer cell lines. Our results indicate that ErbB signalling is required for RANK/RANKL-driven activation of ERK in several HER2-positive breast cancer cell lines. In contrast, lapatinib is not able to counteract the NF-κB activation elicited after RANKL treatment in HER2-overexpressing cells. Finally, we showed that enhanced RANK pathway activation alters HER2 phosphorylation status and RANK binding to HER2 in breast cancer cells. Altogether, our data support a physical and functional link between RANK and HER2 signalling in breast cancer and demonstrate that increased RANK signalling may contribute to the development of lapatinib resistance through NF-κB activation (Sanz-Moreno et al., Breast Cancer Research, 2020).

Figure 1: The immunomodulatory role of anti-RANKL in breast cancer. (a) Representative micrographs of multiple IHC of pre- and post-treatment tumour sections. (b) Bar-plots showing the change from baseline (A) post-metastasis treatment values of TILs, CD8+ and CD4+ T cells. For each measured parameter, the corresponding boxplot is displayed on the right-hand side. (c) RANK expression in breast cancer cells. Favourable recruitment of TAMs and TILs in immunosuppressive populations which interferes with lymphocyte T cell recruitment and/or activity. Denosumab (anti-RANKL) or RANKL signalling inhibition results in increased TILs, lymphocytes and CD8+ T cell infiltration, transforming immune “cold” tumours into “hot” ones and attenuating tumour growth.

Figure 2: (a) RANK but not RANKL expression increased after dual anti-HER2 therapy in patient samples (n = 150) from the PAMELA Trial. Ladder plots showing gene expression before (baseline) and after (Surgery) dual anti-HER2 treatment. (b) Representative images of RANK and RANKL in treatment-naive and anti-HER2 resistant HER2 breast cancer tumour samples. (c) Overactivation of RANK signalling in HER2-positive cell lines increases NF-κB activation and lapatinib resistance. Relative survival in lapatinib of HER2+ breast cancer cells overexpressing RANK (c) and downstream pathways (d).
Our Group aims to understand the crosstalk between tumour cells and their microenvironment during metastatic progression. Microenvironmental cues are important at all steps of the metastatic process, for which the recruitment of a variety of stromal cells is crucial. Secreted factors play an essential role in this mechanism including soluble factors and extracellular vesicles. These mechanisms of cell-cell communication have become as a novel language of cancer that we aim to decode. We are interested in: 1) understanding how tumour cells crosstalk with stromal cells involved in lymph node and distal metastasis in melanoma, lymphoma, prostate cancer and malignant peripheral nerve sheath tumours; 2) the influence of obesity in melanoma and breast cancer metastasis. We found that nerve growth factor receptor (NGFR) is overexpressed in metastatic melanoma cells, secreted in EVs, and that it is shuttled to lymphatic endothelial cells inducing lymphangiogenesis and metastasis. We are also studying the use of NGFR inhibitors as a new strategy to block melanoma metastasis. Finally, we are defining the role of secreted EVs in prostate cancer lymph node metastasis.

Impact of high fat diet in metastasis

We are currently analysing how obesity impacts breast cancer lung metastasis by reinforcing pro-coagulant activities. We are testing novel approaches to reduce tumour-platelet interactions and develop anti-metastatic therapies.

Novel approaches in liquid biopsies

We are developing state-of-the-art technologies to implement EV-based liquid biopsies in the diagnosis and prognosis of patients with melanoma. We have found that the detection of BRAFV600E mutation in circulating EVs from the lymphatic exudate obtained post-lymphadenectomy can be used to identify melanoma patients at risk of relapse (FIGURE).

Novel mechanisms driving in local and distal metastasis

We are investigating the mechanisms involved in melanoma and prostate cancer metastasis. We found that nerve growth factor receptor (NGFR) is overexpressed in metastatic melanoma cells, secreted in EVs, and that it is shuttled to lymphatic endothelial cells inducing lymphangiogenesis and metastasis. We are also studying the use of NGFR inhibitors as a new strategy to block melanoma metastasis. Finally, we are defining the role of secreted EVs in prostate cancer lymph node metastasis.

We are also analysing how adipose tissue renews melanoma metastasis by promoting tumour cell homing and metastatic behaviour.

Tumour-stroma interactions in metastasis

We are studying how alterations in the lymph node microenvironment influence lymphoma progression. We are analysing the role of NGFR in lymph node stromal cells and its influence in follicular lymphoma. We are also exploring novel therapeutic strategies against malignant peripheral nerve sheath tumours (MPNSTs). We are currently testing a combination therapy with MEK inhibitors and anti-angiogenic antibodies as a novel treatment for MPNSTs.

We are interested in understanding how tumour cells corrupt the tumour microenvironment along metastatic progression and the main mechanisms involved, with the aim to develop novel anti-metastatic therapies.34

**References**


**PUBLICATIONS**

- Martinez-Lage M, Torras-Ros R, Puig-San
Brain metastasis is the most common neurological complication of cancer. When metastatic cells reach the brain, prognosis is poor given that local therapies (i.e., surgery and radiation) have limited benefits for patients and the disease inevitably progresses. The rise in the number of patients with brain metastasis is partially due to the increasing number of systemic therapies that work extra-cranially but are unable to provide real benefits for patients with brain metastases.

During 2020, the Group participated in an international effort to investigate the growth of aggressive tumours with mathematical models that have helped to obtain key principles that govern cancer cell colonisation in experimental models and in patients.

**Research Highlights**

A “white book” for brain metastasis research

Together with 19 laboratories worldwide, we have built a public resource for organotropic cancer cell lines that are metastatic to the brain (The Brain Metastasis Cell Lines Panel: https://apps.cnio.es/app/BrainMetastasis/CellLines). This is the most valuable research tool available to interrogate brain metastasis. In addition, we jointly describe the main strategies to study brain metastasis, their current problems, and the open key questions.

Modelling the aggressive growth of brain metastasis

During 2020, the Group participated in an international effort to investigate the growth of aggressive tumours with mathematical models that have helped to obtain key principles that govern cancer cell colonisation in experimental models and in patients.

**Publications**

- Vlor et al. Brain Metastasis Cell Lines Panel is the first effort to collect existing information on existing brain tumor cell lines, their behaviour in vivo, and potential use for research on metastasis. A publicly available webpage describes this open resource.

**Awards and Recognition**

- Manuel Valente: ERC Consolidator Grant, European Research Council (ERC).
- Laura Adriana Álvaro: Fundación Alfonso de Borja-Ingelheim (FDAI), Spain.
- ESMO Faculty Member, CNS tumours faculty group (2020-2024). European Society for Medical Oncology (ESMO).
- Appointed Next Chair of the EANO Scientific Committee, European Association of Neuro-Oncology (EANO; expected mandate 2022-2024).
- Laura Adriana Álvaro was recipient of a Boehringer Ingelheim Fonds MD Fellowship.
To understand the negative impact of chronic nutrient overload in systemic metabolism, and because the liver has a key role in metabolic homeostasis, we generated mice that have chronically high nutrient signalling only in hepatocytes (by liver-specific expression of an active RagA allele: RagA\textsuperscript{Q119L}). RagA\textsuperscript{Q119L} livers exhibited high phosphorylation of mTOR targets (S6K1 and 4EBP1, FIGURE A), and, importantly, the sole activation of RagA in the liver, without altering nutrient intake, impaired glucose homeostasis, as revealed by loss of glucose tolerance (FIGURE B). This result highlights the relevance of a chronic nutrient surplus – liver RagGTPase signalling axis in metabolic complications of the obesity state.

Studying the connections of nutrients and cancer, we previously found that activating mutations in the gene called RagC (key player, together with RagA, in the signal transduction of cellular nutrient levels) result in a subtype of B cell lymphoma, follicular lymphoma. Thus, a lot of interest to develop pharmacological inhibitors of this nutrient signalling pathway has recently spurred, but these drugs are still to be developed. Thus, to determine both the efficacy and safety of inhibition of nutrient signalling against follicular lymphomas, we undertook a genetic approach; we now generated mice expressing a hypomorphic allele of RagC, and asked whether 1) decreased nutrient signalling could suppress the development of follicular lymphoma; and 2) unanticipated side effects could preclude the use of such inhibitors. Hypomorphic RagC mutant mice (RagC\textsuperscript{Q119L}) showed a significant extension of survival when follicular lymphomas were induced (FIGURE C), and an exhaustive analysis of potential side effects revealed that B cells were selectively affected (FIGURE D), importantly, without detectable undesirable trade-offs in other organs (not shown). These results support both the efficacy and safety of nutrient signalling inhibitors in the treatment of B cell neoplasms.
Malignant brain tumours represent about 3% of all cancers, and annually about 100,000 new cases are diagnosed worldwide. In Spain, there are about 4,000 new cases a year. Gliomas are a large collection of brain tumours of which Glioblastoma Multiforme (GBM) is the most frequent and aggressive primary central nervous system (CNS) tumour in adults. Regardless of the recent advances in treatment modalities, GBM patients usually respond weakly to all therapeutic approaches, and prognosis remains dismal (approximately 15 months).

In our laboratory, we use a combination of genomic analysis, mouse models and primary tumour cell cultures, with the main goal of identifying the molecular mechanisms that could provide the basis for novel therapeutic modalities for GBM patients.

"The central focus of our Group is to uncover the genetic alterations present in GBM patients that are responsible for the aggressiveness of this tumour type, with particular interest in the identification of the signalling pathways that lead to poor treatment response."

The molecular basis underlying Glioblastoma (GBM) heterogeneity and plasticity are not fully understood. GBM is a very heterogeneous disease for which multiple transcriptional subtypes have been described. Among these subtypes, the Mesenchymal (MES) GBMs tend to have the worst prognosis. The most frequent genetic alterations — Neurofibromatosis type 1 gene (IDH) copy number loss or mutation — and important regulators of the MES subtype, such as STAT3, CEBPB and TAZ, have been identified. Nevertheless, the mechanisms regulating MES GBMs are still not fully understood. Even though each subtype is associated with specific genetic alterations, there is also considerable plasticity among different subtypes co-exist in the same tumours, and shifts in subtypes can occur over time. This plasticity may be explained by the acquisition of new genetic and epigenetic abnormalities, by stem-like reprogramming or by clonal variation. Using transcriptomic data of patient-derived brain tumour stem cell lines (BTSCs), classified according to GBM-intrinsic signatures, we identified the AP-1 transcription factor FOSL1 as a key regulator of the mesenchymal subtype. We provided a mechanistic basis for the role of NF1, a negative regulator of the RAS/MAPK pathway, in GBM mesenchymal transformation through the modulation of FOSL1 expression. Depletion of FOSL1 in NF1-mutant human BTSCs and Kras-mutant mouse neural stem cells results in loss of the mesenchymal gene signature, reduction in stem cell properties and in vivo tumorigenic potential. Our data demonstrated that FOSL1 controls GBM plasticity and aggressiveness in response to NF1 alterations.

**FIGURE 1** NF1 regulates mesenchymal glioblastoma plasticity and aggressiveness through the AP-1 transcription factor FOSL1. (A–B) FOSL1 is upregulated in GBM with worse prognosis and is associated with poor treatment response. (C) NF1-MAPK-FOSL1 signalling modulates MES GBM plasticity.