REPORT
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STUDY OF THE ROLE OF TRANSCRIPTION FACTORS AS CHROMATIN MODULATORS AND CONDUCTORS OF LYMPHOID NEOPLASIAS

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1. Summary of the project

Alterations in the abundance and distribution of epigenetic marks throughout the genome are a hallmark of cancer. Although the downstream consequences of epigenetic deregulation in cancer are relatively well described, the causes leading to epigenomic changes are poorly described. In previous works, the teams of the applicants identified that epigenetic modifications in chromatin regions in lymphoid tumors seem to be mediated by the aberrant binding to the DNA of specific families of transcription factors (TF). Thus, the main hypothesis underlying this project was that such TFs may represent an initial molecular lesion (complementing genetics), which induces de novo enhancer/promoter activation leading to the generation of an oncogenic transcriptional program.

In this project, we have studied the role of TFs in chronic lymphocytic leukemia (CLL) and multiple myeloma (MM). These two neoplasms are among the most frequent lymphoid malignancies, and both exhibit substantial genetic heterogeneity and clinical variability. However, in spite of this heterogeneity, both display homogeneous disease-specific chromatin signatures enriched in binding sites of few TFs. Therefore, to obtain further insights into CLL and MM pathogenesis, we proposed three ambitious objectives: First, to identify the key TFs responsible for the CLL or MM's unique epigenomic signatures using a custom CRISPR/Cas9 TF screening. Second, to functionally define the causal role of the identified TFs as epigenetic drivers of CLL or MM, through the characterization of their binding sites and direct role in transcription and epigenetic modification. And third, to characterize the TFs' co-factors and study their potential as novel therapeutic strategies in CLL and MM, by identifying TFs' interaction proteins and assessing the impact of their inhibition.

To reach these goals, we used similar methodologies and experimental designs to study CLL and MM, although we adapted some of them to the available resources and experimental models available. During the course of the project, both teams worked very closely together, sharing tools, know-how, and resources, as well as reviewing and interpreting their respective results in regular videoconferences and in-person meetings.

In the next few pages, we will summarize the results obtained in CLL and MM separately, to later discuss the overall implications of our work and list the generated literature.

2. Results obtained

During the funding period, we have been able to fulfil all the proposed objectives, both in CLL and MM. A succinct summary of the main results obtained for each of the two diseases is shown below.

Chronic lymphocytic leukemia (project ID 201924-30)

In this project, we have performed an exhaustive analysis of the role of TFs in CLL. As available cell-line models of CLL poorly recapitulate some of the key molecular features of the disease, we initially characterized an in vitro system to culture primary CLL cells, and further developed a new strategy to perform mechanistic research using cells from primary CLL patients. Next, based on previous publications and data from our own research group, we selected TFs that are associated with the development and clinical aggressiveness of the disease to perform downstream mechanistic studies. In addition to other findings that have been summarized in the annual reports, in this project we evaluated in great detail the role of the LEF1 transcription factor in CLL. Due to the relevance of these results, they will be summarized in greater detail in this six-page summary of the project.

LEF1 is a key transcription factor involved in proliferation and differentiation of multiple normal cell types, but it is absent in mature B cells. In CLL, LEF1 expression seems to be an early oncogenic event, as it is already present in pre-malignant monoclonal B cell lymphocytosis (MBL). Additionally, several LEF1 isoforms are known to have different roles in normal and cancer cells. Although LEF1 expression is a hallmark of CLL and a differential diagnostic marker, its contribution to CLL pathogenesis remains elusive. Therefore, identifying the precise oncogenic role of LEF1 in CLL was a major focus of our project. In particular, we determined the functional role of LEF1 in primary samples from CLL patients and MBL donors using intracellular flow cytometry, CUT&RUN, LEF1 CRISPR-Cas9 KO and nuclear IP-mass spectrometry analysis. Additionally, LEF1 isoforms were overexpressed in CLL cell lines (MEC1 and HG3).

At the gene expression level, LEF1 expression was similar in MBLs and CLLs regardless of their IGHV mutational status. Remarkably, at the protein level clinically-aggressive IGHV unmutated cases (U-CLL) showed significantly higher LEF1 levels than the more indolent IGHV mutated (M-CLL) or MBL cases. This discordance between mRNA and protein levels was explained by increased protein stability in U-CLL. After 8 hours of protein synthesis inhibition with cycloheximide, LEF1 protein levels were reduced by 20% in U-CLL and 60% M-CLL cells. We then analyzed the genome-wide distribution of LEF1 DNA binding sites by CUT&RUN and identified 2.7-fold more peaks in U-CLL than M-CLL. While most of the peaks identified in M-CLL were also found in U-CLL (n=5,777 peaks), 68% of the LEF1 binding sites identified in U-CLL cells were unique (n=12,059 peaks). The target genes of the common peaks were involved in functions such as BCR signaling, whereas the U-CLL specific target genes were linked to cell cycle progression. In line with this finding, we observed higher LEF1 protein levels in the proliferative (CXCR4 low CD5 high) as compared to the resting fraction (CXCR4 high CD5 low). LEF1 protein levels of primary CLL cells also increased in vitro upon receiving proliferative stimuli. We validated that LEF1 is involved in cell cycle regulation by inducing LEF1 CRISPR-Cas9 bulk KO in primary CLL samples. In this model, RNA-seq analysis showed a downregulation of cell cycle genes, and cells lacking LEF1 showed lower Ki67 levels than LEF1 expressing cells.

We also investigated the nuclear interactome of LEF1 in U-CLL and discovered the cell-cycle regulator CDC5L as LEF1 partner. Unexpectedly, CDC5L interacted with LEF1 shorter isoforms lacking exon 6 and not with the full length (FL) transcript.

Concordantly, proliferative stimuli decreased the LEF1-FL levels and increased the LEF1-Δexon 6 isoforms. Moreover, overexpression of LEF1-FL but not of LEF1-Δexon 6 decreased proliferation in CLL cell lines. RNA-seq analysis showed that LEF1-FL induces a quiescent transcriptional profile, that is recovered by the expression of LEF1-Δexon 6 isoforms.

Overall, we discovered that de novo expression of LEF1 has a dual role in CLL pathogenesis depending on protein levels and isoform expression. Lower LEF1 protein levels are related to initial CLL development, while higher LEF1 levels and increased relative abundance of the LEF1-Δexon 6 isoform drive proliferation in more aggressive forms of the disease.

Multiple myeloma

In this project we interrogated the essentiality of 54 TFs, which showed TF binding sites (TFBS) enriched in de novo active regions in MM, using a custom CRISPR-Cas9 library. The CRISPR-Cas9 library screening into two MM cell lines, MM.1R and KMS-11, showed the essentiality of 22 TFs. Intriguingly, among these TFs, we detected three members of the IRF family, IRF1, IRF2, and IRF5, along with the positive control IRF4, suggesting the importance of IRF TFs in MM. Single CRISPR-Cas9 knockdowns identified IRF2 and, as expected, IRF4, as the most essential IRF TFs in MM. To determine the signaling pathways regulated by IRF2 and IRF4, we analyzed the chromatin regions bound by these 2 TFs (and IRF1 as non-essential TF) by CUT&RUN in the same two MM cell lines. We identified 16,300 chromatin regions bound by IRF2 in MM cells, significantly enriched in active promoters, compared to IRF4 (7,512 detected peaks) and IRF1 (showing 5,891 peaks). 60% of the chromatin regions bound by IRF2 were exclusive to IRF2, associated with genes that participate mainly in the cell cycle, cell differentiation, and adhesion. The remaining 40% of IRF2 binding regions were shared with IRF4 and/or IRF1, related to genes implicated mostly in immune system, cell differentiation and adhesion. Furthermore, IRF2 was present in 32% (498/1,556) of de novo active chromatin regions where 201 peaks corresponded uniquely to IRF2 and regulate genes involved in cell migration, osteoblast differentiation, and kinase activation, while 297 peaks corresponded to the three IRFs and were related to oxidative stress genes. Interestingly, IRF2 regulating genes were mostly overexpressed in MM compared to healthy tonsillar and bone marrow plasma cells. Then, transcriptome analysis after IRF2 silencing revealed that more than 30% of the downregulated genes were IRF2 targets identified by CUT&RUN. Moreover, downregulated genes were associated with cell cycle, proliferation, cell differentiation, adhesion, and immune system. These results suggest that IRF2, besides pathways also regulated by IRF4, controls specific pathways involved in the biology of MM and not regulated by other members of the IRF family. Finally, we examined the prognostic impact of IRF2 expression on MM and observed that patients with increased expression of IRF2 presented a worse progression-free survival (PFS) and overall survival (OS) in a multivariate analysis including classical genetic alterations used for the stratification of MM patients. In conclusion, we have demonstrated that IRF2 plays a significant role as a biomarker and as an essential gene in the pathogenesis of MM by participating in the regulation of genes related to cell cycle, differentiation, and adhesion. These results establish IRF2 as a promising target for new therapeutic strategies in MM.

Furthermore, in this project, we studied the possible pharmacological modulation of de novo active regions in MM (Ordoñez R, et al. Genome Research 2019) and its relationship with the functionality of TFs. We treated three MM cell lines (KMS-11, RPMI8226, and KMS-12) with 1μM of JQ1 bromodomain and extraterminal domain (BET) proteins inhibitor (BETi) during 72h. After treatment with JQ1, we detected a significant downregulation of more than 10% of genes regulated by de novo chromatin activation in MM. Interestingly, SMILO, an essential lncRNA in MM (Carrasco-León A, et al. Leukemia 2021), presented one of the greatest inhibitions, even in comparison with well-described genes regulated by BETi. Through ChIP-seq and ATAC-seq analysis, we verified that SMILO decrease was not due to changes in chromatin activation and accessibility produced by JQ1 in MM cells, suggesting that JQ1 may displace the transcriptional machinery, such as TFs, from de novo active regions related to SMILO. The Reverse-ChIP study revealed the binding of 16 proteins in the chromatin region related to SMILO. Among these, we selected as our candidate the TF FLI1 for several reasons: 1) FLI1 was overexpressed in MM in comparison to healthy B cells; 2) it presented a positive correlation of expression with SMILO; 3) MM patients with higher expression of FLI1 presented worse PFS and OS both in univariate and multivariate analyses considering classical genetic alterations of MM and 4) FLI1 showed a significant decrease in H3K27ac at the promoter region after JQ1 treatment, which correlated with a decrease in FLI1 protein levels. To ascertain that FLI1 is causally involved in the expression of SMILO, FLI1 was silenced using CRISPR-Cas9 technology, which substantially depleted FLI1 protein levels giving rise to a significant reduction of the expression of SMILO. These results explain at least one of the mechanisms by which JQ1 downregulates SMILO via downregulation of the TF FLI1. In conclusion, our study highlights the potential of BETi to modulate the expression of de novo active regions in MM and specifically, the expression of the lncRNA SMILO in MM. This opens the door to the development of new therapeutic strategies directed against RNA molecules for the treatment of MM and other types of human tumors.

3. Relevance and potential future implications

The relevance and impact of the results, and the future implications of the data generated in the course of the project are related to:

1. Knowledge generation: we have provided fresh insights into the mechanisms underlying epigenetic deregulation in CLL and MM. We identified several TFs playing a key role in these diseases, most remarkably LEF1 in CLL and IRF2 in MM. These results will foster additional research in the field. Furthermore, the applied experimental approach can be extrapolated to other cancers to generate a catalogue of specific TFs involved in different forms of cancer.

2. Data resources: we have generated data from CRISPR-Cas9 screenings in MM, and well as multiomic data in both CLL and MM, which upon publication we will make all the data available through international data repositories (such as EGA or GEO). These data will become a resource for the community of researchers exploring these diseases.

3. New tools and techniques: we have generated a new CRISPR/Cas9-based method to allow gene editing in B cell malignancies such as CLL.

4. Several of the TFs identified in this study are indeed excellent therapeutic targets, which will constitute the basis to develop specific inhibitors or degraders as potential new therapies.

4. Generated scientific bibliography

ARTICLES PUBLISHED OR UNDER REVIEW

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In addition to these articles, we are currently drafting two additional manuscripts derived from the project that will be submitted in Q3-4 2024.

ABSTRACTS IN CONGRESSES

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