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INVESTIGATING GENETIC AND MECHANISTIC INTERACTORS IN FAMILIAL CARDIOMYOPATHY THROUGH ADVANCED DISEASE MODELING

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1. Summary of the project

Hypertrophic cardiomyopathy (HCM) is the most prevalent hereditary cardiac disease, affecting 0.2% of the population. HCM is a disease marked by phenotypic and genotypic heterogeneity. Left ventricular non-compaction cardiomyopathy (LVNC) is frequently associated with HCM, but whether HCM and LVNC are genetically and/or mechanistically connected disease entities is unknown. Our main objective is to identify genetic and mechanistic interactors linking HCM and LVNC, which could account for the co-morbidity present in a subset of HCM patients, as well as illuminating the currently poorly understood genotype-phenotype relationship in these hereditary cardiomyopathies. For this purpose, we propose to generate: 1) Clinical and imaging data from a large cohort of HCM patients (1,090), which will be analyzed to establish the prevalence of LVNC. 2) Next-generation sequencing (exome) in a selected group of 50 families with HCM and LVNC, followed by analysis of pathogenicity and co-segregation and by genotype-phenotype correlation. 3) Advanced disease models using lines of induced pluripotent stem cells (iPSC) from HCM and LVNC patients with known mutations, and in vitro generated myocardium-endocardium organoids. 4) Putative interactors linking HCM and LVNC, found in genetic studies of patients and mechanistic analyses in advanced disease models, will be functionally validated using patient-derived cell lines and zebrafish. Our results should 1) identify genetic variants that predispose HCM patients to undergo LVNC; 2) identify pathogenic mechanisms that cooperate in patient-derived cardiomyocytes to develop HCM and/or LVNC phenotypes; and 3) provide a clearer understanding of genotype-phenotype relationship in hereditary cardiomyopathies

2. Results obtained

Specific Aim 1: Identification of genetic variants predisposing HCM patients to manifest LVNC.

Specific Task 1.1: Patient selection for imaging analysis

Partner 3 produced and reviewed a list of patients with HCM with high-quality echo images (Group1-Echo, n= 800), of which 29% of cases met LVNC criteria. A list of patients with CMR images available for traditional and automatic evaluation (dedicated software) (Group 1-CMR, n= 299) was also produced. All cases in this group were

reviewed and classified as meeting (27%) or not meeting (73%) current LVNC criteria. An additional list of silent carriers (no LVH) with CMR available was also produced (Group 2, n= 35), thus completing patient selection.

Specific Task 1.2: Cardiac imaging.

Partner 3 completed the review of stored images from echocardiography (n=1,090) (Xcelera) and cardiac magnetic resonance (n=424) (PACS and Xcelera) from HCM patients. The lists of patients of the 3 groups was produced and their clinical information collected in a dedicated database. 100% of echocardiographic images were reviewed and individuals classified into LVNC and non-LVNC criteria. 100% of CMR images were reviewed and patients similarly classified as meeting or not meeting LVNC CMR criteria. CMR images were also collected and converted to the file format appropriate for automated measurement of trabeculations. Analysis of these images was completed by the contracted engineer.

Specific Task 1.3: Patient selection for genetic studies

Based on preliminary results from cardiac imaging analysis, Partner 3 produced a list of 50 candidate families with HCM and LVNC. The final list was fully annotated with the trabeculation results from automated CMR imaging analysis with a dedicated software. Families with a larger number of affected individuals were prioritized for exome analysis, their samples collected, stored in a biobank (IMIB), and DNA extracted. In coordination with Partner 2, other 50 unrelated families with LVNC were selected for deep sequencing, their samples collected, stored in a biobank (IMIB), and DNA extracted.

Specific Task 1.4: Next-generation sequencing

Exome sequencing was completed in a coordinated effort by Partners 1, 2, and 3 on samples from index cases and evaluated relatives from the 50 families initially selected with HCM-LVNC, and from index cases and evaluated relatives from the additional 50 families with LVNC. Detected variants were confirmed by Sanger sequencing. The studies of pathogenicity of the variants, cosegregation and genotype/phenotype correlations were performed by Partners 2 and 3. These activities led to the identification of candidate genes and to the generation of the following mouse models (see more details below in Specific Task 3.1): Bcl7a$^{AG,GA/+}$; Mib1$^{V943F/+}$, Cep192$^{T1522M/+}$, and Tmx3$^{F191X/+}$.

Specific Aim 2: Generation of advanced disease models to investigate mechanistic interactions between HCM and LVNC.

Specific Task 2.1: Genome editing of patient-specific iPSC

iPSC lines representing two HCM patients carrying the K600fs mutation in MYBPC3, available at the laboratory of Partner 1, were gene-corrected by CRISPR/Cas9-mediated gene edition to generate isogenic controls. Patient-specific iPSC harboring the MIB1^{R530X} mutation were similarly edited to produce isogenic controls. A wild type iPSC used as a control was also successfully gene-edited to incorporate the MIB1^{R530X} mutation. Gene correction of patient-specific iPSC lines carrying the MIB1^{V943F} mutation presented additional difficulties. To address this, Partner 1 tested and implemented a robust platform based on the nucleofection of Cas9 protein and chemically-modified sgRNAs for higher efficiency of genome editing. Moreover, the screening was optimized further for the selection of successfully edited iPSC clones. These modifications resulted in the successful generation of isogenic controls for iPSC lines carrying the MIB1^{V943F} mutation, the introduction of the MIB1^{V943F} mutation in control iPSC, and the introduction of the MYBPC3^{K600fs} mutation in heterozygosis in two different wild type control hiPSC. The optimization of genome edition containing large insertions was performed by comparing different sizes of homology arms and template formats. A fluorescent reporter hiPSC line for NKX2.5 (the main transcription factor expressed in cardiac progenitors) was developed for this purpose. Purification of early cardiac progenitor populations was instrumental for the development of cardiac organoids (see Specific Task 2.4 below).

Specific Task 2.2: Cardiac differentiation of patient-specific iPSC

Partner 1 achieved cardiac differentiation of control, MYBPC3^{K600fs}, and MIB1^{R530X} mutant iPSC using monolayer differentiation conditions and onto feeder layers of mouse embryonic ventricular endocardial cells (MEVEC, provided by Partner 2). The extent of differentiation was examined using qRT-PCR of several cardiac markers and considered to be too immature. To overcome this issue, we implemented several systems to increase cardiac maturation: 1) The use of procardiogenic factors and hormones, such as triiodothyronine (T3) in combination with insulin growth factor 1 (IGF-1) and dexamethasone, which resulted in cardiomyocytes showing increased hypertrophic response and higher contraction capacity; and 2) The use of a parallelized bioreactor for continuous perfusion and electrical stimulation and recording of cardiac macroscale constructs. Using a combination of these methods, Partner 1 set up the

conditions to detect HCM-related phenotypes in cardiomyocytes from iPSC carrying the MYBPC3^{K600fs} mutation, and obtained samples during cardiac differentiation of the patient specific iPSC lines and isogenic controls described in Specific Task 2.1 above.

Specific Task 2.3: Expression profiling during cardiac differentiation

hiPSC derived from patients with HCM and/or LVNC were differentiated into cardiomyocytes along with their isogenic CRISPR-corrected lines. Samples were taken at different time points for gene expression profiling. Preliminary analyses identified important hypertrophic biomarkers, such as NPPA, upregulated in cardiomyocytes derived from HCM iPSC, highlighting the potential of this system for recapitulating the patient's phenotypes. Moreover, total RNA extracted from the samples of cardiomyocytes at different times of the differentiation process were quality controlled and sent to Partner 2 for gene expression profiling by RNAseq. The results of these analyses are currently being compared with those of the mouse models (see Specific Task 3.1 below) and will be submitted for publication shortly.

Specific Task 2.4: Analysis of myocardium-endocardium organoids

Partner 1 has developed an in vitro 3D cardiac organoid system that resembles the early stages of cardiac development. It is based on the co-culture of early cardiac progenitors and ventricular endothelial cells. Using the fluorescence reporter described in Specific Task 2.1 we have been able to co-culture FACS-purified cardiac progenitors together with ventricular endothelial cells in 3D spheroids. The ventricular endothelial cells were able to integrate within the cardiomyocytes and promoted their growth and maturation. Generation of cardiac organoid cultures in 3D was tested with cardiomyocytes derived from control wild type hiPSC and compared with iPSC carrying the MIB1^{R530X} mutation for further validation. In this case, we found that secreted signals within the co-culture system were diminished in the mutants, highlighting the potential use of this 3D co-culture system for validation of further targets

Specific Aim 3: Functional validation of putative genetic and/or mechanistic interactors.

Specific Task 3.1: Functional validation of rare genetic variants

As described in Specific Task 1.4 above, our exome sequencing analyses identified two new LVNC-causing candidate genes in the extended family carrying the MIB1^{R530X} mutation: APCDD1 and ASXL3, for which missense mutations were found co-

segregating with LVNC and with the mutation in MIB1. The functional validation of these results was carried out by generating new genetically modified mouse lines using CRISPR/Cas9-based gene edition. Specifically, Apcdd1 and Asxl3 mutant alleles were generated by microinjection of sgRNAs, Cas9 protein, and ssODNs in the pronuclei of one-cell stage mouse embryos carrying the $Mib1^{R530X}$ allele. The analysis of these triple mutant mice is currently being finalized, but our results thus far indicate that triple heterozygous animals show a LVNC phenotype. In the family carrying the $MIB1^{V943F}$ mutation we identified three co-segregating mutations in the CEP192, BCL7A, and TMX3 genes. Functional validation was carried out by generating mouse lines carrying the Cep192, Tmx3 and/or Bcl7A mutations independently or in combination with the $Mib1^{V943F}$ allele. While the detailed characterization of these mouse models will extend beyond the timeline of this project, our preliminary data indicate that triple heterozygous mice mutant for $Mib1^{V943F}$, Cep192, and Tmx3 develop cardiac valve abnormalities with high penetrance. Altogether, our results support the hypothesis that LVNC (at least that involving NOTCH signaling alterations) has an oligogenic, rather than monogenic, autosomal dominant inheritance.

Specific Task 3.2: Functional validation of signaling pathways

We have generated mice with targeted mutations in MIB1 identical to those alleles identified in LVNC patients (Val943Phe and Arg530X), using the CRISPR/Cas9 technology. We have established inbred lines carrying both mutations and bred them with the Mib1flox allele. Interestingly, the R530X mutation combined in trans with the floxed allele inactivated in the myocardium ($Mib1^{R530X}/Mib1^{flox}$;cTnT-Cre/+) causes LVNC, while the V943F mutation does not cause LVNC in any condition (V943F/+, V943F/V943F or $Mib1^{V943F/flox}$;cTnT-Cre/+). We have already bred both genotypes with the null alleles for Notch1 and RBPJ, the main receptor and the unique effector of the Notch pathway, in an attempt to examine whether these Mib1 alleles compromise Notch signaling function by reducing the amount of wild type Notch function, in a typical genetic sensitization experiment. We plan to finish this part of the project in the spring of 2020 and then submit the results for publication.

3. Relevance and potential future implications

Relevance

This project has advanced our knowledge on the molecular mechanisms underlying LVNC and related cardiomyopathies, which is expected to aid the design of preventive or therapeutic strategies for these prevalent diseases. In particular, the successful development of this project has generated new knowledge about the molecular and cellular bases of HCM and LVNC: There was very little knowledge about the process of trabecular compaction and the onset of cardiac hypertrophy and how its alteration may cause cardiomyopathy. Our results have contributed to improve our understanding of these two diseases and thus to the design of new diagnostic and therapeutic approaches.

Potential future implications

-Our results can be applied to improve treatments and clinical decision: Our work has contributed to the establishment of genetic and imaging criteria for HCM and LVNC diagnosis and improved patient stratification.

-This project has generated information valuable for making administrative decisions or policies and accurate genetic counselling for LVNC, resulting in improved patient management that helps rationalizing and reducing cost to our National Health System.

-The implementation of this project should help improving the quality of life of LVNC patients and overall patient satisfaction. Our research has contributed to improve the genetic diagnosis of both HCM and LVNC and to better manage the patients, based on the novel genetic and image information made available.

4. Scientific literature generated

Peer-reviewed publications

Casanova JD, González-Carrillo J, Martín-Jiménez J, Cuenca-Muñoz J, Burillo E, de la Pompa JL, Raya A, Gimeno-Blanes JR, Sabater-Molina M, Bernabé-García G. Trabeculated myocardium in Hypertrophic Cardiomyopathy. Clinical implications. *Under revision*

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polyploidy in the migrating zebrafish epicardium. Nat Mat, doi: 10.1038/s41563-019-0381-9. IF ISI: 38.887. PMID: 31160803

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PhD Theses

"Development of a biomimetic mechanical stimulation system to improve the maturation of human iPS-derived myocardial grafts", by Juan Crespo Santiago, Universitat de Barcelona, 21/07/2016; Excellent 'cum laude'

"Biotechnological approaches to cardiac differentiation of human induced pluripotent stem cells", by Claudia Di Guglielmo, Universitat de Barcelona, 09/02/2016; Excellent 'cum laude'

"Bioengineering approach to study the role of cell migration during zebrafish heart regeneration", by Isil Tekeli, Universitat de Barcelona, 03/02/2016; Excellent 'cum laude'

"Development of an advanced 3D culture system for human cardiac tissue engineering", by Maria Valls Margarit, Universitat de Barcelona, 07/07/2017; Excellent 'cum laude'

"Coronary vessel development in the mouse: Role of Notch signaling", by Stanislao Travisano, Universidad Autónoma de Madrid, 15/10/2018; Excellent 'cum laude'
"Study of extracellular matrix remodeling and the role of periostin b during zebrafish heart regeneration", by Anna Garcia-Puig, Universitat de Barcelona, 22/03/2019; Excellent 'cum laude'

"Role of Nrg1 in mouse heart development", by Paula Gómez Apiñániz, Universidad Autónoma de Madrid; 28/06/2019; Excellent 'cum laude'

"Role of caveolin-1 and midkine-a in zebrafish heart regeneration", by Dimitrios Grivas; Universidad Autónoma de Madrid, 19/12/2019; Excellent 'cum laude'

"Modelling the heterogeneity and complex inheritance of Left Ventricular Non-Compaction", by Marcos Siguero-Alvarez; Universidad Autónoma de Madrid, 17/01/2020 2020; Excellent 'cum laude'