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# TARGETING THE GENETIC CAUSES OF DIABETES THROUGH GENE THERAPY: THERAPEUTIC APPROACHES FOR MODY

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## 1. Summary

Maturity-onset diabetes of the young (MODY) comprises a heterogeneous group of monogenic disorders characterized by onset of hyperglycemia at an early adult age, generally before 25 years. MODYs are a rare cause of diabetes, although collectively they may represent 1-2% of all cases. MODY patients are generally misdiagnosed as type 1 or type 2 diabetic patients and treated with lifestyle interventions or drugs that do not address the underlying genetic defect of their disease. The genetic cure of MODYs has never been attempted before. Thus, the main objective of this proposal was to develop gene therapy approaches for the most prevalent forms of MODY (MODY1 and MODY3) based on the use of adeno-associated viral (AAV) vectors. The gene transfer of the therapeutic genes to beta-cells would be *per se* curative, and significant benefit over existing therapeutic strategies could reasonably be expected. To this end, AAV vectors encoding the genes of interest under the control of the most appropriate regulatory sequences have been designed and produced. The therapeutic efficacy following intraductal delivery was demonstrated in animal models of the disease through a battery of biochemical, functional and histopathological tests. Thus, the successful accomplishment of the objectives of this proposal would not only pave the way to the treatment of MODY patients but also to the development of gene therapy approaches for other monogenic forms of diabetes or for type 2 diabetes associated with hypofunctionality of beta-cells.

The ***specific objectives*** of this project were:

**1)** To produce a collection of disease-specific expression cassettes carrying the predictive curative coding sequence for each MODY.
**2)** To manufacture AAV vector batches carrying these therapeutic cassettes.
**3)** For those forms of MODY for which a model is not available, to generate and/or phenotype the mouse model.
**4)** To provide proof of concept of the therapeutic efficacy of the generated AAV vectors in the corresponding mouse models.
**5)** To generate intellectual property (IP) for those approaches for which therapeutic efficacy is clearly demonstrated.

The main achievements of this collaborative project have been the generation and characterization of new MODY1 and MODY3 mouse models and proof of concept data for the efficacy of an *in vivo* gene transfer with AAV vectors for a MODY-specific gene.

## 2. Results obtained

During this project we have been able to obtain and characterize 2 new MODY1 and MODY3 mouse models. Most of the existing mouse models of MODY1 and MODY3 do not properly reproduce the phenotype of the human disease. Although two different lines of transgenic mice with beta-cell-specific overexpression of dominant negative mutants of HNF1A closely recapitulates the beta-cell dysfunction and diabetes observed in MODY3, without extra-pancreatic phenotype these lines cannot be used to assess the therapeutic potential of the AAV-mediated gene therapy for MODY3 proposed in this project. Indeed, in the engineered beta-cells the dominant negative mutants would sequester the wild-type form of the HNF1A protein derived from the AAV. Thus, to circumvent this limitation a beta-cell-specific HNF1A knockout mouse model of MODY 3 has been generated by means of a unique and novel strategy based on the CRISPR/Cas9 technology. To this end we designed an RNA guide that recognizes a specific sequence in the mouse HNF1A gene and a DNA donor comprising two copies of the target sequence of a pancreas-specific miRNA. miRNAs are small non-coding RNAs that bind specifically to certain mRNAs preventing their translation. Incorporation of target sequences of a pancreas specific miRNA in the locus of HNF1A gene precluded the production of HNF1A specifically in this tissue, generating a new mouse model of MODY3. First, we microinjected the RNA guide, Cas9 and the DNA donor comprising two copies of miRNA in the pronucleus of one-cell C57Bl/6 mouse embryos. Once we obtained the mice from the microinjection they were genotyped by PCR as well as by Sanger sequencing of the PCR product. Based on the results of the genotyping, knock-in (KI) MODY3 mice were selected as founders and were crossed with control (C57BL6) mice to segregate possible CRISPR/Cas9 off-target mutations.

Similarly, a beta-cell-specific HNF4A knock-in mouse model of MODY 1 that prevents HNF4A protein translation has been generated. To strongly reduce the expression of the HNF4A protein in β cells, we generated a MODY1 knock-in mouse line using a similar strategy to that for MODY3, by means of the CRISPR/Cas9 technology. A total

of 54 mice were born after pronucleus injection of the target DNA and transfer into foster mothers. All mice were genotyped by PCR and subsequent enzymatic digestion as well as Sanger sequencing of the PCR product. Based on these results, three founder animals were selected and crossed to C56BL/6N wild-type mice to receive the F1 generation. Again, all mice were genotyped by PCR and subsequent enzymatic digestion as well as Sanger sequencing of the PCR product. Five heterozygous F2 breeding pairs have been bred to set up the final F3 cohort.

The full characterization of these animal models showed that they closely recapitulate MODY 1 and MODY 3. Specifically, we have shown that both homozygous male and female mice from both KI MODY3 lines showed highly decreased HNF1A expression and protein levels specifically in islets. Monitoring of glycemia revealed that, similarly to patients, both male and female MODY3 homozygous mice were mildly hyperglycemic under fed and fasted conditions. Moreover, male and female MODY3 mice showed impaired glucose tolerance in comparison with WT mice at young and adult ages. Overall, diabetic phenotype was more exacerbated in male than female MODY3 mice. No striking differences in islet morphology were detected between MODY3 mice and WT mice. Nevertheless, MODY3 mice showed reduced mean islet area and β-cell mass in comparison to WT mice. In agreement, both male and female homozygous MODY3 mice presented reduced insulinemia. Moreover, MODY3 mice exhibited defects in insulin secretion both *in vivo* and *in vitro*. These results indicated that the pancreas phenotype of homozygous MODY3 mice resembled that of MODY3 patients, with defects in β-cell and insulinopenia. In pancreatic β-cells, HNF1A has been reported to regulate expression of the insulin and β-cell transcription factors genes as well as expression of proteins involved in glucose transport and metabolism and mitochondrial function, all of which are involved in insulin secretion. Both male and female MODY3 mice showed markedly reduced expression of all HNF1A target genes examined. Thus, a new β-cell specific MODY3 mouse model that faithfully mimics the clinical phenotype of MODY3 patients has been developed.

The second main achievement of this project is a preclinical study of the efficacy of a gene therapy product for MODY3. Our results clearly show that this developed gene therapy product can counteract diabetes in a MODY3 mouse model.
The development of the gene therapy products first requires the generation and characterization of AAV vectors. This process consists of the following steps:

*a) Generation of AAV vector expression cassettes containing HNF1A genes under control of beta-cell specific promoter*

To generate AAV8 vectors encoding HNF1A under the control of the most appropriate beta-cell specific promoter, three candidate β-cell specific promoters were selected (βP1, βP2 and βP3). Expression cassettes encoding the green fluorescent protein (GFP) under the control of either the βP1, the βP2 or the βP3 candidate promoter and flanked by the inverted terminal repeats (ITRs) of AAV2 were generated.

AAV8 vectors encoding GFP under the control of each candidate promoter (AAV8-βP1-GFP, AAV8-βP2-GFP or AAV8-βP3-GFP) were produced by triple transfection in HEK293 cells. To evaluate beta-cell specificity wild-type mice were administered intraductally with AAV8-βP1-GFP, AAV8-βP2-GFP or AAV8-βP3-GFP vectors. Measurement of GFP expression confirmed that βP1 mediated the highest expression and production of GFP in islets. Double immunostaining against GFP and insulin in pancreas sections confirmed that transgene expression mediated by the βP1 and βP2 candidate promoters in islets was restricted to beta cells, while βP3 presented GFP+ acinar cells. Therefore, βP3 was discarded for future experiments as transgene expression mediated by this candidate promoter was not beta-cell specific.

*b) Production, purification and characterization of AAV8 vector batches: AAV8 vectors encoding the HNF1A or GFP genes or null*

Once we selected the promoters, AAV vector expression cassettes coding for HNF1A cDNA under the control of the βP1 or the βP2 promoter were generated and cloned into single-stranded AAV backbone plasmids. AAV8 vectors were produced by triple transfection in HEK293 cells (AAV8-βP1-HNF1α, AAV8-βP2-HNF1α).

*c) In vivo evaluation of the biological activity of the different AAV vector batches after intraductal administration to healthy mice*

To evaluate whether βP1 and βP2 were able to mediate HNF1α expression in beta cells and to assess if this overexpression was safe, healthy mice were administered intraductally with AAV8-βP1-HNF1α, AAV8-βP2-HNF1α vectors or PBS. Measurement of HNF1α expression by qPCR confirmed overexpression of the transgene in islets of animals administered with AAV8-βP1-HNF1α and AAV8-βP2-HNF1α vectors in comparison to control mice.

Mice treated intraductally with AAV8-βP1-HNF1A vectors showed decreased number of islets and β-cell mass, while AAV8-βP2-HNF1A treated mice exhibited normal pancreas

morphology. Moreover, no differences in body weight, glycemia or glucose tolerance were observed between control and mice administered with AAV8-βP2-HNF1α vectors, further highlighting safety. Therefore, AAV8-βP2-HNF1A vectors were chosen for subsequent evaluation of therapeutic efficacy for MODY3.

Once the candidate AAV vectors have been obtained and characterized, their therapeutic efficacy has been evaluated in the MODY3 mouse model.

To evaluate the therapeutic efficacy of AAV8-βP2-HNF1A, this vector was intraductally administered in homozygous MODY3 mice. Wild-type mice injected with PBS were used as healthy controls, and homozygous MODY3 mice administered with PBS served as disease controls. MODY3 mice treated with AAV8-HNF1α exhibited an important overexpression of HNF1A that increased the expression of some HNF1A gene targets. Moreover, AAV8-HNF1α treated mice showed counteraction of the mild-hyperglycemia under fed and fasted conditions and improvement of glucose tolerance. Analysis of circulating insulin levels revealed that MODY3 mice treated with AAV8-HNF1α vectors presented an increase in fed insulinemia.

Currently, an in-depth study is being performed to further understand the mechanisms underlying the therapeutic efficacy of AAV8-HNF1A vectors.
A similar approach has been used to develop a gene therapy product for MODY 1 and to evaluate its therapeutic efficacy following intraductal delivery to MODY1 mice.

## 3. Relevance to possible future implications

The development of animal disease models that closely reproduce the human disease is crucial to enable pre-clinical assessment of therapeutic strategies. In this project we have generated two new knock-in mouse models by means of a unique and novel strategy based on the incorporation of tissue-specific miRNA target sequences in the HNF4a or HNF1 locus using the CRISPR/Cas9 technology. Noticeably, these two new mouse models mimic the human clinical phenotype of MODY1 and MODY3 patients, which allowed testing of the therapeutic efficacy of the novel AAV-based gene therapy strategies also developed in the project. These mouse models will also be very useful to develop and test other therapeutic interventions (including antidiabetic drugs,

protein replacement therapy, and gene therapy approaches) for MODY in the future. It is worth mentioning that the novel technology developed to generate the new MODY1 and MODY mouse models can be applied to reduce expression of any endogenous gene and obtain novel genetically modified organisms (animals as well as plants) not only for monogenic but also for complex/polygenic diseases (*patent application EP21382080.6*). The novel technology is associated with fewer side effects compared to conventional global and unconditional knock-out and knockdown models.

In this project, we have also developed AAV-based gene therapy strategies to successfully treat the diabetic phenotype of the newly generated MODY mouse models. The genetic cure of MODY had never been attempted before. Our results constitute the first demonstration for the genetic counteraction of a monogenic diabetes and pave the way to the treatment of MODY patients in the future (*patent application EP21382079.8*). Moreover, our results will also contribute to the future development of novel gene therapy approaches for other monogenic forms of diabetes or even for type 2 diabetes. We have demonstrated that gene transfer of the correct coding sequence of the affected protein to the afflicted tissues is curative. Hence, significant benefit over existing therapeutic strategies or others under development is reasonably expected. *In vivo* gene therapy, in particular, offers the possibility of a one-time treatment, with the prospect of lifelong beneficial effects, such as the production of the therapeutic protein for extended periods of time after a single administration of the gene therapy product has been repeatedly demonstrated in several animal models and humans. Presently, seven gene-based therapies have already received marketing authorization from the regulatory bodies in Europe and the United States. Moreover, the important progress in the clinical arena will likely keep feeding the pipeline of approved advance therapeutic medicinal products (ATMP) in the near future. The ultimate goal of our project is transferring the AAV-mediated therapeutic strategies for MODY developed in this project to a biotech or pharmaceutical company to accelerate their clinical translation.

## 4. Scientific outcomes of the Project

The results obtained in this project have enabled the filing of two patent applications. The first patent application claims the new method of generation of tissue-specific knock-down mouse models by means of the incorporation of microRNA target

sequences in the desired gene, with the examples of MODY1 and MODY3 mice (**EP21382080.6)**. The second one claims the development of AAV-based gene therapy approach for MODY3 (**EP21382079.8)**. Additional details corresponding to these two patent applications are specified below:

TITLE: ***Down-regulation of endogenous genes***
OWNER: Universitat Autonòma de Barcelona (Spain) and Helmholtz Zentrum Munich (Germany)
INVENTORS: Fatima Bosch, Veronica Jimenez, Miquel Garcia, Estefania Casaña, Martin Matthias Hrabě de Angelis, Gerhard Kurt Herbert Przemeck, Anna-Lena Amend
APPLICATION NUMBER: **EP21382080.6**
PRIORITY COUNTRY: Europe
PRIORITY DATE: 30-1-2021

TITLE: ***Gene therapy for monogenic diabetes***
OWNER: Universitat Autonòma de Barcelona
INVENTORS: Fatima Bosch, Veronica Jimenez, Miquel Garcia, Estefania Casaña
APPLICATION NUMBER: **EP21382079.8**
PRIORITY COUNTRY: Europe
PRIORITY DATE: 30-1-2021

Currently, two research papers including the results derived from the research project are being prepared to be submitted to high impact scientific journals in the near future. Estefania Casana, Veronica Jimenez, Miquel Garcia, Alba Casellas, Tura Ferré, Meritxell Morró, Victor Sacristan, Claudia Jambrina, Xavier Leon, Roger Cox, Martin Hrabé de Angelis, Steve Brown and Fatima Bosch. ***Treatment of MODY3 disease by AAV-mediated gene therapy*** (*in preparation*).