LI LECCIÓN CONMEMORATIVA JIMÉNEZ DÍAZ

Madrid, 16 mayo 2019

"Genetic engineering: towards molecular medicine in cardiology"

Silvia G Priori, MD,PhD
Full Professor of Cardiology University of Pavia
Director of Molecular Cardiology, ICS Maugeri,
Centro de Investigaciones Cardiovasculares Carlos III, Madrid

The attempt to exploit the advancements in the field of molecular biology to develop "curative" approaches for genetic diseases dates back 20 years [1] [2, 3] however, it is only in the last decade that such a dream has turned into a safe and clinically used revolutionary approach for the management of inherited life-threatening diseases. In the field of cardiology, the application of gene therapies for the treatment of hereditary and acquired conditions has suffered a major delay as compared to other disciplines such as neurology, hematology and oncology. The reason for such a slower development is rooted in some characteristics of the heart that make it a more complex target than other organs: the heart is made of terminally differentiated cells, i.e. cells that cannot replicate thus preventing the use of the leading methods for gene editing. In addition, cardiac cells seem to be harder to infect with viral vector used to deliver gene therapy than other tissues and finally the heart is less accessible than other organs given its location inside the chest.

For this reasons, at a time in which hundreds of clinical trials are ongoing, no gene therapy for the heart is currently in clinical development.

Gene therapy: what is it?

According to the definition of the European Medicine Agency gene therapy is considered an Advanced Therapy and it consists in the administration of *" genes that lead to a therapeutic, prophylactic or diagnostic effect. They work by inserting 'recombinant' genes into the body, usually to treat a variety of diseases, including genetic disorders, cancer or long-term diseases. A recombinant gene is a stretch of DNA that is created in the laboratory, bringing together DNA from different sources*" (https://www.ema.europa.eu/en/human-regulatory/overview/advanced-therapy-medicinal-products-overview)

The design of a gene therapy strategy is a complex effort that requires a pondered selection of different elements. In its most schematic description, gene-therapy is composed of a ***vector*** and a ***construct***. The *vector* is the "carrier of the therapy": most often it is a modified virus that is used to shuttle the construct inside the target cell. The *construct* is the therapeutic component of the gene therapy and, in its basic essence, it includes a promoter and a nucleic acid that either is cDNA, siRNA, miRNA or shRNA.

The most difficult part of designing a gene therapy for a disease, resides in the identification of an approach that will target the mechanisms that are disrupted by the disease-causing mutations. Obviously, it is possible to design a gene therapy only when there is an extensive knowledge of the pathophysiological mechanisms of the

disease of interest and when an animal model that manifests the pivotal characteristics of the disease is available.

Despite the identification of the molecular strategy is the most critical step in development of a novel gene therapy, the selection of the other components of the construct is also critical to minimize the occurrence of side effects. For example, in the engineering of molecular strategies for cardiac diseases, it is important to ensure that the therapy is predominantly delivered to the heart and not to other organs. This goal can be achieved by selecting viruses that preferentially infect the heart (cardiac tropism) to deliver the therapeutic construct and by the use of cardiac specific promoters.

The design of gene therapy is based on the selection of one of three fundamental strategies: gene transfer, gene silencing and gene editing.

The choice of the best strategy is determined by the pathophysiological mechanisms of the disease for which the therapy is developed. For example, a disease-causing mutation may produce a dysfunctional protein that shows a loss of its function. Less commonly it is possible that a disease-causing mutation leads to a gain of function that causes a disease because the resulting protein is over-active. It is intuitive that in a disease caused by a protein that has lost its function, a promising therapeutic strategy may be represented by creating a construct that leads to the production of a normal protein to compensate the deficit induced by the mutation: this approach is called “gene transfer”. Unfortunately, there are limitations in the size of the gene that may be inserted in a virus and therefore, the gene transfer approach is not applicable to large genes. In the cardiac field, loss of function mutations in large genes such as titin that causes a form of inherited dilated cardiomyopathy could not be treated by administering the normal gene using a viral vector as the gene is too big to be positioned inside an adeno associated virus.

In the presence of a gain of function molecular defect that causes a dominant disease, the preferred approach may consist in a gene silencing strategy that aims at reducing the production of the mutant protein while preserving the expression of the wild type protein. To achieve this goal, it is possible to design targeted short RNA molecules able to inhibit gene expression or translation, by neutralizing the mRNA-transcript of the mutant allele. This approach may be used to design treatment for inherited diseases caused by either "gain of function" mutations or “loss of function” mutations with a dominant negative effect and in the presence of the detrimental increase of a protein in the setting of an acquired disease. To mitigate the risk of haploinsufficiency when silencing a dominant negative mutation, it is possible to

combine silencing of the mutant allele with expression of the wild type gene (hybrid therapy).

The most exciting gene therapy approach is called “gene editing” and it consists in the possibility of repairing a disrupted coding sequence in a mutant gene to restore the physiologic DNA sequence.

In other word this approach is able to “cancel” the mutation to create a “normal gene” in its physiological location. The method called CRISPR-CAS9 has been the first gene editing strategy and it has represented a true revolution in gene therapy: unfortunately, the method works when cells are replicating therefore in the post-natal setting, when cardiac myocytes lose their replicating ability, it is not possible to use gene-editing to treat inherited diseases of the heart.

A pivotal challenge that applies to all these strategies is represented by the need to strictly and adequately control the effect of the introduced molecule, in order to prevent the development of adverse side effects like toxic or inappropriate overexpression of the exogenous protein for gene transfer, the excessive and detrimental reduction of the target molecule for gene silencing and the off target cleavage for gene editing. Finally, despite gene therapy provides interesting approaches to counteract a variety of abnormalities, it should be considered that gene transfer may be ineffective even if cells are correctly transduced but there is failure of downstream steps such as a failure in proteins folding, co-assembly or localization.

Gene therapy for heart diseases: where do we stand?

The choice of gene therapy for treating heart rhythm disorders appears reasonable given both the evident hardiness to pharmacologically manipulate electrical properties of cardiomyocytes and the increasing understanding of the molecular mechanisms involved in arrhythmic diseases. Evidences to make real this new frontier of molecular medicine derive from a large series of preclinical data that clearly confirm the actual effectiveness of the system in the heart environment, in small and large animals. Most importantly, the Calcium Up-regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) study, that is the first human gene therapy trial for cardiac gene transfer, showed the efficacy and safety of cardiac gene therapy using AAV vectors**.** In this study an AAV-1 vector, was used to deliver sarcoplasmic reticulum Ca2+-ATPase (SERCA2) cDNA to advanced heart failure patients via percutaneous intracoronary delivery.

SERCA2 is pivotal to controlling the flow of calcium ions between the sarcoplasmic reticulum and cytoplasm and so plays a key role in regulating myocardial

contractility. Deficiency of the enzyme often occurs in advanced heart failure and is considered a mechanism of progressive systolic and diastolic dysfunction. The choice of Ca2+ cycling targets is supported by several preclinical data showing that the overexpression of SERCA2a can improve cardiac function in various small- and large-animal heart failure models [4]. Furthermore, it has been demonstrated that SERCA2 plays a central role in the molecular mechanism of cardiac alternans in heart failure and speculated that its restoration would exert an antiarrhythmic effect [5]. The group of Roger Hajjar was the first to bring this assumption forward in the translational setting [6]. In the open-label phase 1 study, nine patients received AAV-1 SERCA2a gene therapy at low (1.4×10^{11} DNase resistant particles, DRP), middle (6×10^{11} DRP) and high (3×10^{12} DRP) doses, with three patients in each group. One patient in the medium dose group died for reasons unrelated to gene therapy and no adverse effects were observed in the remaining eight patients. Six patients showed some level of improvement while two patients (one in the low-dose group and another in the high-dose group) had high pre-existing anti-AAV1 neutralizing antibodies and they did not respond to therapy as expected. Recently, a double-blind placebo-controlled phase 2 trial was conducted[7]. Thirty-nine patients were recruited in the study and AAV1-SERCA2a was delivered at the dose of 6 × 1011 DRP (low-dose), 3 × 1012 DRP (middle dose) and 1 × 1013 DRP (high-dose) through the coronary circulation. The study met its primary efficacy endpoint, including the simultaneous assessment of patients' clinical outcomes, exercise tolerance, heart failure symptoms, biomarkers, and cardiac function. At three years post-administration, there were 13 deaths: six in the placebo group, three in the low-dose group, three in the mid-dose group and one in the high-dose group. Despite the initial promising results, the most important trial, i.e. the phase IIb CUPID 2 trial, failed to document efficacy of the therapy [8]. High-risk ambulatory patients with New York Heart Association class II-IV symptoms of heart failure and a left ventricular ejection fraction of 35% or less due to an ischemic or non-ischemic cause were randomly assigned to receive a single intracoronary infusion of 1 × 10(13) DNase-resistant particles of AAV1/SERCA2a or placebo. The primary efficacy endpoint was time to recurrent events (hospital admission for heart failure or ambulatory treatment for worsening heart failure). Patients were followed up for at least 12 months but unexpectedly AAV1/SERCA2a gene therapy did not improve time to recurrent events compared with placebo (hazard ratio 0·93, 95% CI 0·53-1·65; p=0·81). Interestingly, no safety signals were noted. The authors of the trial concluded that "despite promising results from previous studies, AAV1/SERCA2a at the dose tested did not improve the clinical course of patients with heart failure and reduced ejection fraction" and they could not indicate any likely reason for the observed failure.

Overall the CUPID experience over the 3 trial represents a valuable contribution to the field despite the failure of the CUPID 2. The trials represent the “first in man” experience of percutaneous delivery of an therapeutic AAV vector to the heart of patients demonstrating both feasibility and safety of the approach. The studies proved that the choice of AAV vector has been successful both for the documented efficiency in transducing cardiomyocytes after a single intracoronary administration of the vector and for the long term expression of the transgene in the few patients in which it was assessed. Most importantly, no safety concerns were noted during the three-year follow-up period.

In 2010 we started working on the idea to develop a gene therapy for inherited arrhythmogenic disorders. Under this name is currently grouped a set of disorders caused by mutations in genes that encode proteins that are pivotal players in the regulation of cardiac rhythms. Most of them are ion channels, i.e proteins that regulate inflow and outflow of ions such as sodium, potassium and calcium ions that regulated cardiac excitability. The major conceptual obstacle to the development of a gene therapy strategy for the heart resides in the view that cardiac cells need to have a uniform electrical behavior as any voltage discrepancy across cells is likely to predispose to arrhythmias. Despite being aware of this challenge, we decided to pursue our project supported by the desire to address the unmet clinical and therapeutic needs of our young patients.

Based on our large population of patients affected by a disease called Catecholaminergic Polymorphic Ventricular Tachycardia or CPVT, we were able discover in 2001 that the disease, that causes exercise and emotion driven arrhythmias in children and adolescent, is related to mutations in the cardiac ryanodine receptor [9] and we decided to devote to this disease our efforts. Of relevance, molecular mechanisms underpinning CPVT has been characterized in animal models that we [10,11,12] and others [13,14] developed and therefore we were confident to have a mechanistically-driven insight in the selection of the gene therapy strategy as well as clearly defined readouts to assess its efficacy.

As a first line of work, we attempted to correct *CASQ2* molecular defects present in our two mouse models of recessive CPVT (rCPVT): knock-in mice carriers of the R33Q mutation [10] and CASQ2 Knock-out mice (CASQ2 null) [11]. CASQ2 mutations lead to a variety of functional abnormalities that ultimately reduce CASQ2 protein levels. The reduction of CASQ2 is accompanied by the decrease in the "CASQ2 sister

proteins" Triadin and Junctin, possibly by altering the stoichiometry of the three proteins and disrupting their localization promoting proteins' degradation. [10]

At the ultrastructural level, electron microscopy studies in heart tissue from CASQ2 mutant mice have highlighted unique abnormalities [10,11] occurring at the junctional SR (JSR), that becomes enlarged, fragmented and in its vesicles it is no longer possible to visualize the CASQ2 polymer.

The functional consequence of these alterations is the occurrence of abnormal diastolic calcium release following adrenergic stimulation. CASQ2 knock-in mice develop Ca^{2+}sparks with shortened coupling intervals and generate spontaneous Ca^{2+} waves unable to propagate as cell-wide waves[11]. Isolated myocytes from the heart of KI and KO recessive CPVT mice develop adrenergically induced delayed afterdepolarizations (DADs) that may trigger action potentials [10,11]. In vivo ECG recording revealed that KI and KO mice develop bidirectional and polymorphic ventricular tachycardia upon adrenergic stimulation.

In this fully characterized animal model of recessive CPVT, we tested the hypothesis that delivery of a construct that would transduce cells expressing wild type calsequestrin could restore normal calcium handling physiology. We used an AAV9 vector to deliver a construct that consisted of a cytomegalovirus promoter, the cDNA of cardiac calsequestrin and GFP as a reporter gene. Thanks to the well characterized disease-models used, it was rather straightforward to select a composite panel of "clinical" end-points to define the efficacy of our gene-therapy. The success of our gene therapy was therefore defined as the ability to abolish all the molecular, ultrastructural and electrophysiological disease-induced abnormalities.

In our initial set of experiments, we infected homozygous CASQ2 KO mice at birth by intraperitoneal injection of AAV9-CASQ2 and studied them at 20 weeks of age. Through a series of in vitro and in vivo experiments, we were able to demonstrate that the AVV-CASQ2 gene transfer is able to revert all the pathologic abnormalities of KO mice [12.]. In the subsequent studies we documented the efficacy of AAV9-CASQ2 gene replacement therapy in R33Q knock in mice[13]. Specifically, we were able to demonstrate that mice infected at birth as well as mice infected in the adult age, show a complete prevention of all the phenotypical manifestations of CPVT. Interestingly, 12 months after the administration of a single dose of the viral construct, the animals were still showing a full therapeutic effect [12,13].

Encouraged by these preliminary results, we moved on to develop a molecular strategy for the dominant form of CPVT in which gain of function mutations lead to an abnormal release of calcium from the sarcoplasmic reticulum into the cytosol of cardiac

myocytes inducing spontaneous diastolic calcium waves and delayed afterdepolarizations that precipitate life threatening ventricular arrhythmias. In order to reduce the transcription of the RyR2 mutant R4496C allele present in our knock in RyR2 mice [14], we developed an in vitro mRNA and protein-based assays to screen multiple siRNAs for their ability to selectively target the mutant mRNA over the corresponding wild-type allele [15]. Once identified the most performant of these siRNAs, we inserted it in a construct that was delivered by intraperitoneal injection to mice heterozygous for the R4496C mutation. After 8 weeks, protein quantification showed that the ratio between the mutant and the wild type protein has doubled in favor of the normal protein. This reduction of the mutant protein was paralleled by a reduction of the dysfunctional channels leading to a dramatic decrease (>90%) of arrhythmias.

A very interesting aspect of the success of the two gene therapies delivered to our CPVT mice models, has been the impact that our studies have had in the field. As of today, another therapeutic gene therapy approach has been developed in CPVT mice models. This study [16] results from a collaboration with the group of Sandor Gyorke in which our knock in mice with recessive CPVT were treated to receive a construct containing an engineered Calcium Calmodulin variant able to prolong refractoriness of mutant RyR2 thus reducing the diastolic release of calcium from the sarcoplasmic reticulum and preventing adrenergically mediated arrhythmias. Interestingly, also this innovative approach was able to obtain a significant reduction of arrhythmias in vivo.

Ethical Aspects of gene therapy and genetic engineering

The research that we have presented provides a solid hope for “cure” for both dominant and recessive CPVT. Interestingly the data also show how important is the development of animal models of human diseases: the field of gene therapy for inherited arrhythmias and cardiomyopathies is in fact slowed in its advancement by the lack of proper animal models on which therapy can be thoroughly tested. At a time in which the hope for use of induced-pluripotent stem cells derived cardiac myocytes or in silico models of electrical diseases, the community working in gene therapy for inherited diseases strongly supports the importance of animal models that can provide an in vivo answer to the efficacy and tolerability of advanced treatment that may profoundly impact survival of patients. Clearly, transition to a “first in man study” cannot be advocated based on cellular data or compute based simulations so animal research should be regulated but should not be limited when models are needed to test potentially lifesaving new therapies for diseases with major unmet clinical needs.

Another important ethical aspect that should be discussed is whether as we are approaching the clinical testing of CPVT gene therapies, the issue of using gene

therapies in the uterus, after prenatal diagnosis has assessed the presence of the disease in the fetus. Albeit, testing of therapies in human-embryos has been banned in several countries, a first experiment of gene therapy in a human embryo carrier of a mutation in the Myosin binding protein C 3 (MYBCP3) gene that causes hypertrophic cardiomyopathy has been carried on. The article was published in Nature in 2017 [17] and demonstrated that feasibility of using a CRISPR-Cas9 method to correct the MYBC3 mutation in human embryos before implantation with a high efficiency of repair. This study provides not only an important proof of concept but indirectly it also brings to the forefront the issue on how to regulate research and investigations on human embryos.

To this end an indirect response to the question raised by the study of Ma and collaborators has recently come from an article published in Nature [18] in 2018 in which a group of scientists involved in gene therapy calls for a regulation for germline genome editing experiments. The statement of the authors reads as follows:

- *We call for a global moratorium on all clinical uses of human germline editing — that is, changing heritable DNA (in sperm, eggs or embryos) to make genetically modified children.*
- *By 'global moratorium', we do not mean a permanent ban. Rather, we call for the establishment of an international framework in which nations, while retaining the right to make their own decisions, voluntarily commit to not approve any use of clinical germline editing unless certain conditions are met.*

This initiative seems very appropriate and timely as it calls for considerations on the future use of genetic engineering at the time in which not only gene therapy is used for treating malignant diseases that create major disabilities and death among affected patients, but at time in which we can see appearing on the horizon of knowledge the proposal of using genetic engineering for increasing performance of human beings. The idea that genetic manipulation might be used to potentiate performance in sport, to select color of eyes and stature or to make individuals with more powerful minds is raising safety issues and ethical concerns especially if this molecular techniques are applied to germline cells that will transmit the induced changes to the offspring.

All scientists working at the frontier of science who are using tools that allow to modify somatic and germline cells need to avoid breeching ethical boundaries and at the same time they should protect the independence of science as in virtue of this freedom biomedical research has eradicated diseases that used to cause devastating pandemics and it has greatly contributed to the improvement of the quality of human life on the planet.

In conclusion, I would like to make a personal statement: working on gene therapy is a great commitment and a major responsibility toward patients and their families who are following the work of scientists hoping that a breakthrough will come soon to free them from the spell of the burden of a disease that will accompany them for the entire life. Furthermore, an inherited condition will continue to remain in the family as it will be transmitted to children.

My teams in Madrid and Pavia and I will do our best to continue to keep the ethical bar high but nonetheless to work hard to shorten the time needed to make gene therapies available to patients around the world.

REFERENCES

1. Donahue, J.K. et al. Focal modification of electrical conduction in the heart by viral gene transfer. *Nat Med* 6, 1395-8 (2000)

2. Priori, S.G. & Napolitano, C. From catheters to vectors: the dawn of molecular electrophysiology. *Nat Med* 6, 1316-8 (2000)

3. Edelberg, J.M., Aird, W.C. & Rosenberg, R.D. Enhancement of murine cardiac chronotropy by the molecular transfer of the human beta2 adrenergic receptor cDNA. *J Clin Invest* 101, 337-43 (1998).

4. Lompré, A.M. et al. Ca2+ cycling and new therapeutic approaches for heart failure. *Circulation* 121, 822-30 (2010)

5. Cutler, M.J., Wan, X., Laurita, K.R., Hajjar, R.J. & Rosenbaum, D.S. Targeted SERCA2a gene expression identifies molecular mechanism and therapeutic target for arrhythmogenic cardiac alternans. *Circ Arrhythm Electrophysiol* 2, 686-94 (2009)

6. Jaski, B.E. et al. Calcium upregulation by percutaneous administration of gene therapy in cardiac disease (CUPID Trial), a first-in-human phase 1/2 clinical trial. *J Card Fail* 15, 171-81 (2009)

7. Jessup, M. et al. Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID): a phase 2 trial of intracoronary gene therapy of sarcoplasmic reticulum Ca2+-ATPase in patients with advanced heart failure. *Circulation* 124, 304-13 (2011)

8. Greenberg B, Butler J, Felker GM, Ponikowski P, Voors AA, Desai AS, Barnard D, Bouchard A, Jaski B, Lyon AR; Pogoda JM, Rudy JJ, Zsebo KM. Calcium upregulation by percutaneous administration of gene therapy in patients with cardiac disease (CUPID 2): a randomised, multinational, double-blind, placebo-controlled, phase 2b trial.Lancet 387:1178-86 (2016)

9. Priori SG, Napolitano C, Tiso N, Memmi M, Vignati G, Bloise R, Sorrentino V, Danieli GA.Mutations in the cardiac ryanodine receptor gene (hRyR2) underlie catecholaminergicpolymorphic ventricular tachycardia. Circulation;103:196-200 (2001)

10. Rizzi, N. et al. Unexpected structural and functional consequences of the R33Q homozygous mutation in cardiac calsequestrin: a complex arrhythmogenic cascade in a knock in mouse model. *Circulation Research* **103**, 298-306 (2008)

11. Liu, N. et al. Abnormal propagation of calcium waves and ultrastructural remodeling in recessive catecholaminergic polymorphic ventricular tachycardia. *Circ Res* **113**, 142-52 (2013)

12. Denegri, M. et al. Viral gene transfer rescues arrhythmogenic phenotype and ultrastructural abnormalities in adult calsequestrin-null mice with inherited arrhythmias. *Circulation Research* 110, 663-8 (2012)

13. Denegri, M. et al. Single delivery of an adeno-associated viral construct to transfer the CASQ2 gene to knock-in mice affected by catecholaminergic polymorphic ventricular tachycardia is able to cure the disease from birth to advanced age. *Circulation* 129, 2673- 81 (2014)

14. Cerrone M, Colombi B, Santoro M, di Barletta MR, Scelsi M, Villani L, Napolitano C, Priori SG. Bidirectional ventricular tachycardia and fibrillation elicited in a knock-in mouse model carrier of a mutation in the cardiac ryanodine receptor. Circulation Research; 96:e77-82 (2005)

15. Bongianino R, Denegri M, Mazzanti A, Lodola F, Vollero A, Boncompagni S, Fasciano S, Rizzo G, Mangione D, Barbaro S, Di Fonso A, Napolitano C, Auricchio A, Protasi F, Priori SG.Allele-Specific Silencing of Mutant mRNA Rescues Ultrastructural and Arrhythmic Phenotype in Mice Carriers of the R4496C Mutation in the Ryanodine Receptor Gene (*RYR2*). Circulation Research 121:525-536 (2017)

16. Liu B, Walton SD, Ho HT, Belevych AE, Tikunova SB, Bonilla I, Shettigar V, Knollmann BC, Priori SG, Volpe P, Radwański PB, Davis JP, Györke S. Gene Transfer of Engineered Calmodulin Alleviates Ventricular Arrhythmias in a Calsequestrin-Associated Mouse Model of Catecholaminergic Polymorphic Ventricular Tachycardia. J Am Heart Assoc 7e008155 (2018)

17. Ma H, Marti-Gutierrez N, Park SW, Wu J, Lee Y, Suzuki K, Koski A, Ji D, Hayama T, Ahmed R, Darby H, Van Dyken C, Li Y, Kang E, Park AR, Kim D, Kim ST, Gong J, Gu Y, Xu X, Battaglia D, Krieg SA, Lee DM, Wu DH, Wolf DP, Heitner SB, Belmonte JCI, Amato P, Kim JS, Kaul S, Mitalipov S. Correction of a pathogenic gene mutation in human embryos. Nature. 548:413-419; (2017)

18 Lander ES, Baylis F, Zhang F, Charpentier E, Berg P, Bourgain C, Friedrich B, Joung JK, Li J, Liu D, Naldini L, Nie JB, Qiu R, Schoene-Seifert B, Shao F, Terry S, Wei W, Winnacker EL. Adopt a moratorium on heritable genome editing Nature 567:165-168 (2018)