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iPSC-technology to model human cardiac diseases

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Abstract

The discovery that somatic cells can be reprogrammed to pluripotency as induced pluripotent stem cells (iPSC) has generated much interest since it presents an opportunity to generate patient- and disease-specific cell lines from which normal and diseased human cardiomyocytes (CMs) can be obtained. In most cases, the iPSC-derived CMs have been show to be able to recapitulate the disease phenotype and have provided opportunities for gaining novel insights into heart pathophysiology. These genetically diverse human model systems can be used *in vitro* to decipher mechanisms of diseases and to develop innovative strategies and reagents for personalized medicine approaches (Maaike H. et al., 2012): iPSC-based models can be indeed employed as a platform for screening the efficacy of new therapeutic molecules, as well as their toxicity and side effects in a human context. Preclinical data supporting the safety and efficacy of iPSC are also accumulating. However, some recent reports also indicate risk factors for the use of iPSC, such as genetic and epigenetic abnormalities that could take place during reprogramming or maintenance in subsequent cell culture (Hideyuki O. et al., 2013) and mainly due to retroviral transgene activation and/or retroviral insertion mutagenesis into iPSC-derived cells. For this reason, increasing efforts have been recently directed toward the generation of insertion-less or insertion-free iPSC using chemical compounds, adenoviral vectors, transposons, modified mRNA, plasmids, recombinant proteins, episomal vectors, Sendai vectors (Hideyuki O. et al., 2013).

In our experiment we used CytoTune™-iPS Reprogramming, a system based on replication incompetent Sendai virus (SeV) expressing the four “pluripotency” Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc). These reprogramming vectors have been engineered to increase biological and environmental safety. In fact these viruses are confined into the cytoplasm and do not integrate into the host cell genome. Furthermore, due to their temperature sensitivity, they can be cleared from the host cells by incubation at 38°C for few hours. After cell reprogramming occurs, iPSC have been characterized by checking the expression of markers typical of pluripotency (Oct-4, SSEA-1, TRA1-60) using several methodologies, such as RT-

PCR, immunofluorescence, Western Blot, FACS, and alkaline phosphatase activity detection.

The ability to differentiate into cells that derive from all the three germ layers is another key feature of a pluripotent cell: when iPSC are removed from differentiation suppression conditions and grown in suspension aggregates [called embryoid bodies (EBs)] in presence of serum, differentiation into cells from the three germ layers occurs, including spontaneously beating CMs. In the protocol we are using in the laboratory, ascorbic acid is added to increase the efficiency of induction toward the mesoderm lineage and CMs (Di Pasquale E. et al., 2013).

The projects I have been involved in the laboratory have been mainly focused on the use of iPSC technology to model and treat cardiac diseases.

Cardiac arrhythmias and heart failure are major causes in the Western Countries. In younger patients, the majority of sudden cardiac deaths underlies Mendelian genetic causes. iPSC models have been described for some cardiac arrhythmia syndromes, including Brugada Syndrome, catecholaminergic polymorphic ventricular tachycardia (CPVT) and other cardiac diseases, such as Timothy syndromes and cardiomyopathies (CMPs – hypertrophic and dilated). In my project, I focused my interest on developing iPSC-based system to investigate recessive CPVT and familial dilated CMPs (DCM)

Recessive CPVT is associated with mutations in the *CASQ2* gene and is characterized by the absence or the drastic reduction of cardiac calsequestrin, a protein critical for the regulation of cytoplasmic calcium homeostasis in cardiac myocytes.

In our study, we exploited a human knock-out model of CPVT caused by the homozygous mutation D307H calsequestrin protein. Our final propose was to explore whether viral gene transfer using AAV9, engineered to carry the wild type CASQ2 gene, was able to revert the complex set of abnormalities due to the lack calsequestrin. The distinguishing features of recessive CPVT include: severe reduction of the sarcoplasmic reticular (SR) proteins TrD (Triadin) and JnC (Junctin) that, together with calsequestrin, regulate the release of calcium from the SR mediated by ryanodine receptor; ultra-structural changes of the SR; development of adrenergically mediated diastolic delayed after-depolarizations and triggered

activity and occurrence of sustained polymorphic or bidirectional ventricular tachycardia elicited by stress or emotion.

On the other, familial DCM is a progressive disease of the heart muscle, clinically characterized by presence of left ventricular enlargement and reduced systolic dysfunction, myocyte death and myocardial fibrosis leading to severe heart failure and increased risk of sudden death due to arrhythmia. In many cases the disease is inherited (about 20-30% of cases) and associated to mutations in genes encoding components of a wide variety of cellular compartment and pathways, and comprising the nuclear envelope (i.e. lamin A/C), the contractile apparatus and the sarcomere (i.e. titin, alpha and beta myosin), gene transcription and splicing machinery and calcium handling (Watkins H et al., 2011).

In the laboratory a familial case of DCM due to mutations in Lamin A/C and titin has been recently described: in this work the authors found two mutations in the genes encoding Lamin A/C and Titin (LMNA and TTN) were segregating with the DCM phenotype. In particular, they observed that all the affected family members were carrying the c.656A>C variant in LMNA gene (already associated to DCM), whereas and the four most severely affected family members were found to have a previously not described variant in the TTN gene (c.14563C>T), that have been proposed to act as a "modifier" gene. However, further genotype/phenotype correlation studies are required to determine how commonly rare variants in multiple genes associated with DCM are associated with a particularly severe clinical course. (Roncarati et al, 2013).

In order to prove causal-link between the disease and the identified mutations we developed a gene correction strategy to revert both Lamin A/C and Titin mutations: such method is based on helper-dependent adenoviral vectors (HDAdVs), shown to be a highly efficient and safe for correcting mutations in large genomic regions in human iPSC.

In conclusion, the development of targeted genome-editing strategies to be applied to iPSC-based model systems will have enormous rebounds for future cell therapy approaches to human genetic diseases.

INTRODUCTION

Induced Pluripotent Stem Cells technology to study and cure of human diseases

"**Induced Pluripotent Stem Cells**" (iPSC) are adult somatic cells that have been that have been genetically reprogrammed to an embryonic stem cell-like state by being forced to express genes and factors important for maintaining the defining properties of embryonic stem cells (ESCs).

IPSC are indistinguishable from ESCs for morphology and are pluripotent; iPSC are able to grow indefinitely and posses the potential to give rise derivatives of all the three germ layers, i.e. ectoderm, mesoderm and endoderm.

The discovery of iPSC dates back to 2006 when Shinya Yamanaka showed that a differentiated cell was able to go back to an embryonic state through the forced expression of transcription factors typical of the state of pluripotency .This discovery was first demonstrated in the mouse and, the following year, iPSC were generated also from human fibroblasts in the laboratories of S. Yamanaka and J. Thomson, through the forced expression of a different set of transcription factors typical of the state of pluripotency, that are Oct4, Sox2 and either Klf4 and c-Myc or Nanog and Lin-28[42].Oct4, Sox2 and Nanog are transcription factors that are essential for maintenance of the state of pluripotency; Klf4 and c-Myc, are oncogenes and act by inducing proliferation of reprogrammed cells, while Lin-28 is a micro-RNA binding protein, thought to regulate the self-renewal of stem cells.

The reprogramming process lasts about 20-30 days *in vitro* reprogrammed cells are first detectable by morphology typical of ESC. these cells grow aggregated in colonies, possess prominent nuclei and a high ratio nucleus/cytoplasm due to a high transcription in the nucleus.

Major applications of iPSC are: i) disease modeling, they could be a valuable approach for studying the pathophysiology of genetic human diseases *in vitro* in order to design strategies and reagents for new therapies[20,21] ;ii) drug discovery, the ability to get in culture patient-specific disease cells could be useful for the testing of new drugs and to test their toxic effects; iii)regenerative medicine, being autologous cells may be used for transplantation in order to regenerate the tissues, damaged cells, without triggering the immune system.

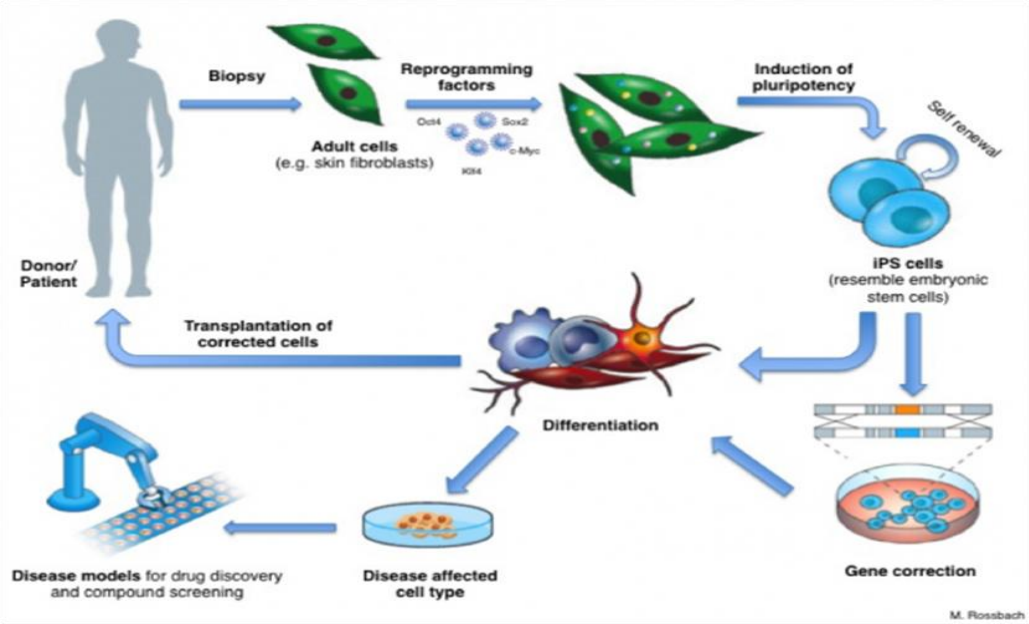


Fig.1: *iPSC-approach to human diseases*
There are multiple potential uses of the human iPSC, including: study of the mechanisms of disease, drug screening, human genetics, developmental biology, gene therapy and autologous cell therapy

However, recent studies have showed iPSC may carry genetic and epigenetic abnormalities that may compromise their use for preclinical and therapeutic purposes. Among the multiple methods described so far to generate iPSC, cell transduction with retroviral/lentiviral vectors is still one of the most popular method to induce reprogramming for the high efficiency and moderated cost; however vectors integration into host chromosomes is required to express reprogramming genes and may retroviral transgene activation and/or insertional mutagenesis into iPSC-derived cells.

For this reason, increasing efforts have been recently directed toward the generation of insertion-less or insertion-free iPSC using chemical compounds,

adenoviral vectors, transposons, modified mRNA, plasmids, recombinant proteins, episomal vectors and, Sendai vectors 30.

DNA-based vectors such as adenovirus, adeno-associated virus, and plasmid vectors exist episomally and do not require integration. However, their reprogramming efficiency is lower and they may still be integrated into host chromosomes at certain frequencies. Modified-RNA, DNA or proteins have been also proposed as alternative methods for iPSC generation, but their use is either technically complex or seem to give extremely low efficiency of reprogramming (0,001%).

Use of vectors based on Sendai virus expressing the four "pluripotency" factors has been recently demonstrated to efficiently generate transgene-free iPSC. Based on these premises, we decided to employ such methodology for our reprogramming experiments.)

Sendai virus (SeV) and reprogramming

Sendai virus is a respiratory virus of mouse and rat, classified as mouse parainfluenza virus type-I belonging to the *Paramyxoviridae* family. SeV was first isolated in Japan in the early 1950s and is also called Hemagglutinating Virus of Japan (HVJ). SeV is an enveloped virus of 150–250 nm in diameter whose genome is a single chain RNA (15,384 bases) in the minus sense. Six genes coding for viral proteins are situated sequentially on the genome of the wild-type SeV in the following order (starting from the 3' end):

- ✓ *Nucelocapsid protein (NP)* forms the core nucleocapsid complex with the genome RNA.
- ✓ *Phosphoprotein (P)* is the small subunit of the RNA polymerase.
- ✓ *Matrix protein (M)* supports the envelope structure from the inside.
- ✓ *Fusion protein (F)* fuses the viral envelope with cell membrane when the virus enters the cell.
- ✓ *Hemagglutinin-Neuraminidase (HN)* recognizes the cell surface receptor, sialic acid.
- ✓ *Large protein (L)* is the large subunit of RNA polymerase.

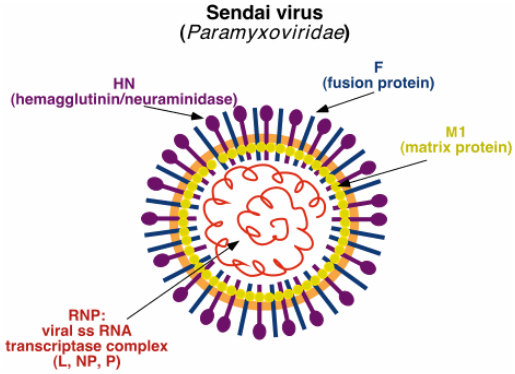


Fig.2: *Structure of the Sendai Virus*

The Sendai-based viral vectors have been designed to safely and efficiently express the four "pluripotency" Yamanaka factors (Oct4, Sox2, Klf4 and c-Myc) in a variety of different cell types. These reprogramming vectors have been engineered to increase biological and environmental safety. Furthermore, due to their temperature sensitivity, they can be cleared from the host cells by incubation at 38°C for few hours.

SeV infects cells by attaching itself to the sialic acid receptor present on the surface of many different cells, it can infect a wide range of cell types of various animal species. Activation of F protein by a protease is required for the virus-cell fusion process to take place. After infection, the virus goes through genome replication and protein synthesis, and then daughter virus particles are assembled and released (**Figure 3**).

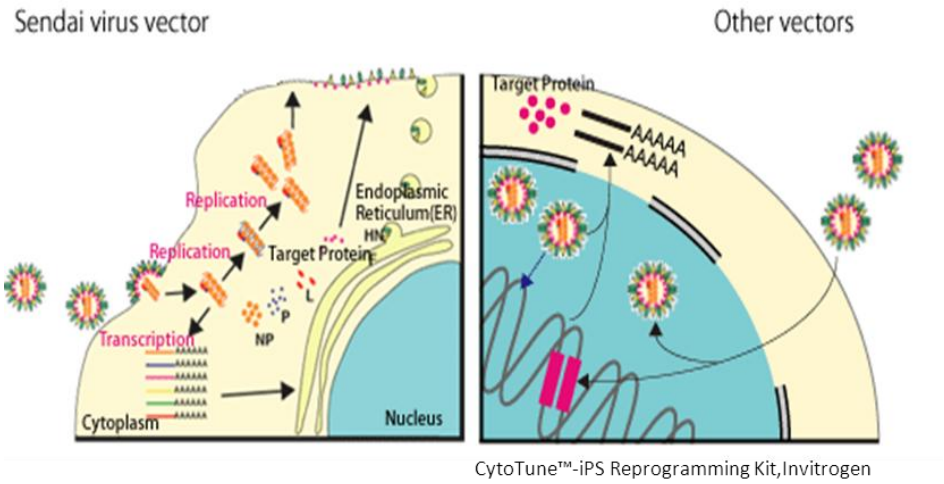


Fig.3: *Comparison of the lifecycles of non-integrating SeV vectors and other vectors*.

There are several advantages to use the SeV vectors:

- No genotoxicity: Sendai reprogramming vectors do not integrate into chromosomes of the target cells.
- Wide range of targets: the vectors are capable of transducing a wide range of cell types in proliferative and quiescent states.
- High transduction efficiency with low multiplicity of infection (MOI).
- Short contact time of virus with target cells is sufficient to establish transduction.
- High level of expression of the transgenes.
- Fast expression of the transgenes: expression is detectable as early as 6–10 hours after transduction, with maximum expression detected more than 24 hours after transduction.
- Zero footprint: the vectors and transgenes can be eliminated from the cells.
- No production of infectious particles by the transduced cells.
- Derived from a virus that is non-pathogenic to humans.
- Temperature sensitivity: virus can be easily removed from transduced cells with (38°C for three days)

iPSC to model human cardiac diseases: an overview

Cardiac arrhythmias and heart failure are major causes in the Western Countries. In younger patients, the majority of sudden cardiac deaths underlies Mendelian genetic causes. iPSC models have been described for some cardiac arrhythmia syndromes, including Brugada Syndrome, catecholaminergic polymorphic ventricular tachycardia (CPVT) and other cardiac diseases, such as Timothy syndromes and cardiomyopathies (CMPs – hypertrophic and dilated). In my project, I focused my interest on developing iPSC-based system to investigate recessive CPVT syndrome.

The discovery that somatic cells can be reprogrammed to pluripotency as iPSC has generated much interest since it presents an opportunity to generate patient- and disease-specific cell lines from which normal and diseased human cardiomyocytes (CMs) can be obtained *in vitro*.

Such possibility is of great interest, since it overcomes one of the major limitation related to the investigation of cardiovascular diseases, that is the limited source of cells from human heart.

In most cases, the iPSC-derived CMs have been shown to be able to recapitulate the disease phenotype and have provided opportunities for gaining novel insights into heart pathophysiology. These genetically diverse human model systems can be used *in vitro* to decipher mechanisms of diseases and to develop innovative strategies and reagents for personalized medicine approaches: iPSC-based models can be indeed employed as a platform for screening the efficacy of new therapeutic molecules, as well as their toxicity and side effects in a human context[31]. Preclinical data supporting the safety and efficacy of iPSC are also accumulating.

To give an example, in the laboratory has been recently described a model of CPVT due to the heterozygous mutation in the gene encoding RYR2 calcium release channel.

Results from these study demonstrated iPSC differentiation are able to give rise to an heterogeneous population of cardiac cells (i.e. atrial, ventricular and nodal-like cells) and showed that CMs derived from patient-specific iPSC recapitulate the disease phenotype, with occurrence of delay after depolarization and trigger activity after beta adrenergic stimulation through isoproterenol.

Furthermore, we also demonstrated that treatment with KN-93, an inhibitor of the calcium-calmodulin kinase pathway was able to restore the correct phenotype in the CPVT cells, indicating the feasibility of using iPSC-CM as a platform for drug testing.

Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT)

Disease characteristics

CPVT is a life-threatening form of arrhythmia (its prevalence in Europe is 1/10.000 so that it is considered a rare disease);characterized by ventricular tachycardia (VT)

induced by the adrenergic stress (exercise or acute emotion)which may degenerate into ventricular fibrillation and cause syncope and sudden death.

The onset of the disease is precocious (between 7 and 9 years of age) and shows similar frequency in both sexes. Affected individuals have normal ECG at the baseline and do not show any structural abnormalities of the heart. [33]

The mortality rate associated with the disease is extremely high, and about 30%-35% of the patients die by the age of 35 years, if not threated.

Therapy is mainly based on b-blockers that are ineffective in about 30% of the patients. In the most severe cases, use of implantable cardioverter defibrillators is necessary.

Genetics of CPVT

Two genetic forms of CPVT have been identified, one transmitted as an autosomal dominant (50%) disease caused by mutations in the gene encoding the cardiac ryanodine receptor (*RyR2*)[2,3] and the other with autosomal recessive(3%) way of inheritance that is caused by mutations in the cardiac specific isoform of the calsequestrin gene (*CASQ2*).[4,5]

Both genes encode for proteins that are responsible for calcium handling and storage within heart muscle cells (at the sarcoplasmic reticulum) and that are critically involved in cardiac excitation-contraction (EC) coupling by controlling calcium-induced calcium release. This suggests that abnormal intracellular regulation of Ca^{2+} may be crucial driving in arrhythmogenesis and may represent the central pathogenic pathway in CPVT[33].

Accordingly, it has been speculated that the electrophysiological mechanism underlying arrhythmias in CPVT is triggered activity initiated by delayed after-depolarizations (DADs).

Both proteins are part of a supramolecular Ca^{2+}- signaling complex in the junctional sarcoplasmic reticulum (SR) that also contains Triadin 1 (TrD) and Junctin (JnC), among other proteins.[6,7]

TrD binds specifically to the RyR/Ca^{2+}-release channel and CASQ2 and its binding to the C-terminal luminal loop of the RyR seems important for ensuring rapid Ca^{2+} release during ECC in the striated muscle.

JnC is an integral component of the junctional SR membrane in both cardiac and skeletal muscle. A short N-terminal region of this 26-kDa protein is located in the cytoplasm, and the bulk of the molecule projects into the SR lumen.[37]

More in details RyR2 serves as a Ca^{2+}-release channel in the SR; in fact it is located in the membrane of the sarcoplasmic reticulum. During the ECC process, RyR2 channels are activated by Ca^{2+} that enters the cell through voltage-dependent L-type Ca^{2+}-channels, causing the release of Ca^{2+}-from the SR into the cytosol, a mechanism known as Ca^{2+}-induced Ca^{2+}-release (CICR).[8,9] Increased cytosolic Ca^{2+}-levels activate the contractile apparatus. Ca^{2+}-release is terminated when SR luminal [Ca^{2+}] falls below a threshold level, causing a decline in RyR2 activity via a mechanism termed luminal Ca^{2+}-dependent deactivation.[10,11]

CASQ2 is a high-capacity Ca^{2+}-binding protein; its primary function is to store the releasable Ca^{2+} within the SR[12,9] and to control the local luminal [Ca^{2+}] in the vicinity of the RyR2 channels[11]; it has been also proposed to serve as a luminal Ca^{2+}- sensor for RyR2[13] : its buffering function allows the SR to store large amounts of Ca^{2+} ions near the releasing sites while preserving a low concentration of free Ca^{2+} [25]. Additionally, CASQ2 seems to play an important role in regulation the open probability of RyR2.[14] CASQ2 is thought to modulate RyR2 via TrD and JnC.[26]

CASQ2 may also importantly regulate the development of the SR ultrastructure along with these two proteins . Altogether, these observations have suggested that CASQ2 plays an essential role in the regulation of Ca^{2+} storage and release required for excitation-contraction (EC) coupling in mammalian hearts.[32]

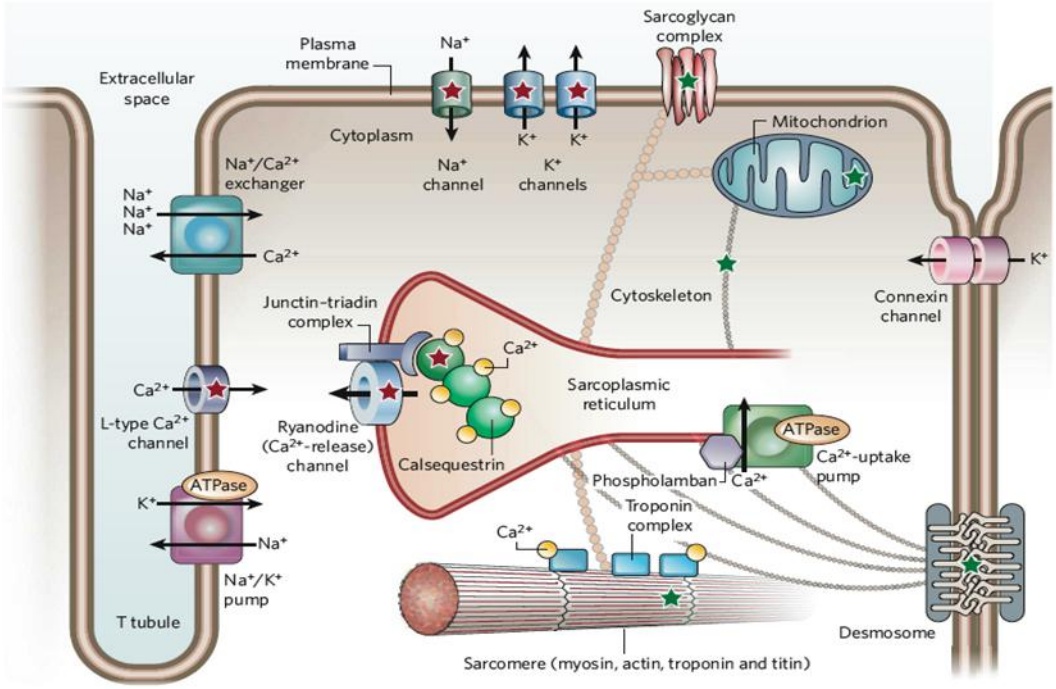


Fig.4: *Macromolecular complex in Junction Sarcoplasmic Reticulum* (jSR):

During muscle contraction the key role is played by the Ca2+ which is released into the sarcoplasmic reticulum after the opening of the channel Ryanodine, under the control of CASQ2, TrD-1 and JnC. The Ca^{2+} will bind to troponin-C, allowing slippage between the myofilaments (myosin and actin), allowing the contraction. During the relaxation is necessary that the channel ryanodine is closed does not allow the release of more Ca^{2+}.)

The precise mechanism for this regulatory function is still the subject of controversy.

Available evidence suggests that the clinical features of CASQ2 and RyR2-related CPVT are virtually identical.

Genothype/Phenotype correlation in CPVT-patients with CASQ2 mutation

CASQ2 gene maps on chromosome 1 at the 1p 13.1 locus and encodes the cardiac isoform of calsequestrin (CASQ2), a protein of 399 amino acid[25] located in the junctional sarcoplasmic reticulum (jSR) of mammalian cardiac muscle[32]. As already mentioned before, it is a calcium-binding protein[14]: CASQ2 appears as a densely staining protein in the lumen of the jSR and at this site forms a quaternary complex with the SR Ca^{2+} release channel RyR2 and with JnC and TrD.[15,16]

In my project I focused on a family case of the autosomal recessive form of CPVT is linked to the deletion from 339 to 354 (**G112**) in CASQ2 leading to a frame shift and a stop codon after 5aa **(5X)**[14]; the lack of CASQ2 stops the normal function of chelating calcium ion of this protein, leading to an increase of the diastolic Ca^{2+}-concentration in the cytoplasm and determining irregular and polymorphic arrhythmias.

In vitro examinations showed CMs with absence of CASQ2 are characterized by electrophysiological abnormalities (increase the frequency of Ca^{2+} sparks in isolated ventricular myocytes[14]; ventricular arrhythmias and abnormal T-wave alternans), ultra-structural variations to SR (increase in the volume and formation of electron-dense "clumps" of the SR cisterne[32] and a molecular changes (reduction in TrD and JnC expression)).

The patients present bidirectional arrhythmias, which can lead to a ventricular tachycardia and ventricular fibrillation.

Clinical Diagnosis and therapy of CPVT

The clinical evaluation of CPVT-patients includes the following main <u>diagnostic procedures</u>:

- ECG during a graded exercise (exercise stress test);
- Genetic testing;
- Evaluation of presence/absence of structural abnormalities of the right ventricle;
- Holter monitoring;
- Prenatal diagnosis (DNA from amniocentesis) when the mutation has already been identified in at least one member of the family.

<u>Therapies</u> are limited and rely on the following:

- Beta-adrenergic blockers (preferentially: Nadolol and Propanolol that are usually effective in preventing recurrences of arrhythmias in the majority of the patients);
- Implantable Cardioverter Defibrillator (ICDs);
- Sympathetic denervation (for patients who are resistant to the therapy with β-blockers);

- Flecainide (reduces the duration of channel opening and disrupts the propagation of calcium waves).

  However, available therapeutic options are ineffective in about 30% of the patients and identification of novel pharmacological and therapeutic approaches is of utmost important to ameliorate the survival and the quality of life of the affected patients.

  On this regard, development of patient specific iPSC-based model of CPVT may serve as a cardiac platform to identify new therapies and to test their efficacy in human cells.

GENE THERAPY OF CARDIAC ARRHYTHMIAS

Advantages of Adeno-Associated Viral Vectors

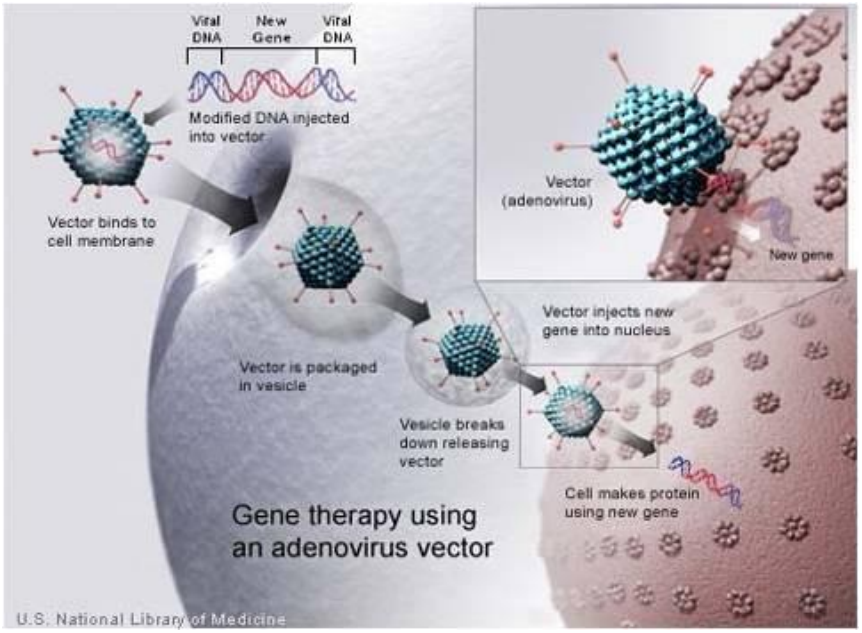


Fig.5:*Schematic representation of the use of adeno-associated vector for guiding expression of therapeutic genes:*
Viral gene therapy vectors entry into the mammalian cell. Viral vectors bind to cell surface receptors, initiating endocytosis via the clathrin-dependent process. Once internalized, the viral particles avoid degradation via the lysosomal pathway and direct endosome trafficking to the cell nucleus. Adenoviral and adeno-associated viruses are released from the endosome in the perinuclear region, and the viral particles ‘‘dock’’ on the nuclear envelope membrane pores. The viral particle capsid coat dissociates, and the genome is delivered into the cell nucleus. Retroviral and lentiviral vectors require further processing in the cytoplasm, including reverse transcription, prior to arrival in the nucleus.)

As discussed in the section above even though conventional therapies including pharmacological therapy, catheter ablation, and implantable devices have been shown to be effective in reducing morbidity and mortality of CPVT patient, a certain segment of these arrhythmias is still refractory to treatment.

“Gene therapy” approaches constitute an exciting option to threat human disease and to correct expression of a potential gene of interest.[31]

A study recently published by Denegri M *et al*, 2012 demonstrated that in vivo transduction of cardiac cells with adeno-associated vectors carrying the wild type

CASQ2 gene was sufficient to restore both expression and function in CASQ2 knock-out models.

Gene therapy is defined as the technology by which genes, small DNA or RNA molecules are delivered to human cells, tissues or organs to correct a genetic defect, or to provide new therapeutic functions for the ultimate purpose of preventing or treating diseases. The primary aim of gene therapy is to either increase or decrease the level of a specific protein within a target tissue, in order to modify cellular functions of cell of interest or to effect changes to surrounding tissues by altering secreted proteins. The development of successful gene therapy approaches is expected to have a great impact in reducing morbidity and mortality due to cardiac diseases.[36]

Of the diverse potential methods for gene therapy, the adeno-associated virus (AAV) is extremely advantageous. These are known to elicit a longer expression of the protein of interest; AAV are also expected to raise less immunological problems than others.

Among the several serotypes of AAV, AAV9 has a higher affinity to for cardiomyocytes than the other AAV serotypes. It has also been reported that AAV9 has a higher efficacy of transduction in the heart than in the other organs. Such properties combined with the low adverse effects related to the use of these vectors are fundamental to bring gene therapy applications closer to their clinical use.[31]

As already mentioned, a recent work from Serge Viatchenko K. *et al*.,2004 employed AAV9 vectors expressing CASQ2 to restore the expression of the mutated $CASQ2^{D307H}$ in adult rat myocytes. They showed that the Asp307 compromises the ability of CASQ2 to form high capacity Ca^{2+}-biding oligomers and/or to interact with RyR2 channel complex. Additionally, induction of CASQ2 expression by AVV9 in CASQ2-defective knock-out CPVT mice reverts the molecular, structural, and electric abnormalities associated with the absence of CASQ2 and prevents occurrence of life-threatening arrhythmia. In their experiments the authors used the pAVV2.1-CMV-CASQ2wt-eGFP vector and showed that infected myocytes (efficiency of infection was greater than 50%) exhibited increased levels of CASQ2, TrD and JnC (increased 80%-90% to controls); furthermore, ultra-structural abnormalities and

electrophysiological instability of CASQ-defective myocytes were restored, abolishing the ventricular tachycardia phenotype in such mice models[38.]

Importantly, AAVs have been recently approved for their use in the to threat patients affected by Leber's congenital amaurosis (LCA), a group of inherited blinding diseases with onset during childhood. One form of the disease, LCA2, is caused by mutations in RPE65 gene. Maguire AM and colleagues demonstrated the safety of sub-retinal delivery of a recombinant AAV carrying RPE65 cDNA: three patients with LCA2 manifested acceptable local and systemic adverse -events after delivery of AAV2.hRPE65v2, while each enrolled patient showed a modest improvement of the retinal function by subjective tests of visual acuity.

Testing revealed gains in visual acuity at 6 weeks; thereafter, there was a slower rate of improvement the clinical benefit to the patients has been sustained during the 6 months since the experimental treatment of LCA2 .[39]

These results provide the basis for further gene therapy studies in patients with LCA and support their potential applications to threat other diseases with an acceptable level of safety.

MATERIALS AND METHODS

REPROGRAMMING OF FIBROBLASTS INTO iPSC

Fibroblasts culture

Dermal fibroblasts biopsies (3-4 mm) obtained from the patient diagnosed with CPVT after written informed consent are isolated through enzymatic digestion as follow:

-Liberase (0,9U/mL)	345µL
-Dispase	1,25µL
-Trypsin+EDTA (0,05%)	10µL
-P/S	5µL
-DMEM:F12 (1:1)	640µL

-Incubate the sample at 37°C in agitation for approximately 3 hours.

-Mechanically dissociate the sample by pipetting up and down with a 10mL pipette,than a 5mL pipette and a 2mL pipette.

-Dilute the digestion mixture with PBS and filter the solution through a 100µm filter.

-Centrifuge the sample at 1700rpm for 10 min.

-Discard the supernatant than count and plate the cells in 12,5cm^2 flask.

(Ideally fibroblasts need to be densely populated to grow (approximately 2.5X10^4 cells/cm^2 are required).

Isolated fibroblasts were cultured in FB-MEDIUM, made of:

-DMEM–Low Glucose/F12 (1:3)

-10% FBS, 2mM Glutamax

-0.1 mM non-essential amino acids and antibiotics.

Identity and purity of isolated cells have been determined using fibroblasts specific markers by immunofluorescence and FACS analysis.

CytoTune™-iPS Reprogramming Kit

For the reprogramming, CytoTune™-iPS Reprogramming System has been used. This method is based on replication in competent Sendai virus (SeV) to safely and

effectively deliver the four transcription factors necessary for reprogramming of somatic cells into iPSCs. In contrast the other available protocols with comparable reprogramming efficiency, which mainly rely on viral vectors that integrate into the genome of the host cell, the CytoTune™ Reprogramming System uses vectors that are non-integrating and remain in the cytoplasm (i.e., they are zero-footprint). In addition to the cytoplasmic nature of SeV, the vectors have been genetically modified to become temperature-sensitive, so that the host cell can be cleared of the vectors and reprogramming factor genes by treatment at higher temperature (38°C). The CytoTune™-iPS Reprogramming Kit contains four SeV-based reprogramming vectors, each capable of expressing one of the four Yamanaka factors (i.e., Oct4, Sox2, Klf4, and c-Myc) and are optimized for generating iPSCs from human somatic cells. As already explained the reprogramming vectors have been engineered to increase biological and environmental safety.

Below is included the lists of the CytoTune™ Sendai reprogramming vectors included in the CytoTune™-iPS Reprogramming Kit and the detailed protocol used for inducing reprogramming.

Differentiation Medium	**Final Concentration**	**250 mL**	**Catalog Number**
RPMI 1640	-	212.5 mL	Life Technologies 11875-119
GlutaMAX	1X	2.5 mL	Life Technologies
B-27 Supplement without insulin or	50X	5 mL	Life Technologies 005012SA Life Technologies 17504044
B-27 Supplement with insulin	50X	5 mL	Life Technologies 005012SA Life Technologies 17504044

Reagents	**Final Concentration**	**Catalog Number**

**Y-27632 Rock inhibitor (5mM)	10 µM	Sigma Y0503-1MG
**CHIR-99021 (36mM)	8-12 µM	Selleck S1263-5MG
**IWR-1 (5mM)	5 µM	Sigma 10161-5MG
Growth Factor-Reduced (GFR) matrigel (10mL)	1:30	BD Biosciences 354230
Essential 8 Medium	-	-
Accutase	-	Life Technologies A1110501

PROTOCOL

Day -2: Prepare the cells for transduction

1. 2 days before transduction, plate human neonatal foreskin fibroblast cells into two wells of a 6-well plate at the appropriate density to achieve 5×10^5 cells per well on the day of transduction (Day 3).

   (**Note:** 80–90% confluence on the day of transduction is recommended.) Culture the cells for two more days, ensuring the cells have fully adhered and extended. To prepare 100 mL of complete MEF/fibroblast medium, aseptically mix the components listed in the table below. Complete MEF/fibroblast medium can be stored at 2–8°C for up to 1 week.

D-MEM with GlutaMAX™	89 mL
FBS-ESC Qualified (10%)	10 mL
MEM Non-Essential Amino Acids Solution (10 mM)	1 mL

Day 0: Perform transduction

2. On the day of transduction, warm 2 mL of fibroblast medium in a water bath.
3. Remove one set of CytoTune™ Sendai tubes from the –80°C storage. Thaw each tube one at a time by first immersing the bottom of the tube in a 37°C water bath for 5–10 seconds, and then removing the tube from the water bath and allowing it to thaw at room temperature. Once thawed, briefly centrifuge the tube and place it immediately on ice.
4. Add the indicated volumes of each of the four CytoTune™ Sendai tubes (3×10^6 CIU each; for the appropriate volume changes for each preparation and is provided in

the Certificate of Analysis) to 2 mL of fibroblast medium, pre-warmed to 37°C. Ensure that the solution is thoroughly mixed by pipetting the mixture gently up and down. Complete the next step within 5 minutes.

5. Aspirate the fibroblast medium from the cells, and add one half of the solution prepared in Step 5 to each of the two wells. Place the cells in a 37°C, 5% CO2 incubator and incubate overnight.

Day 1: Replace medium and culture cells

6. 24 hours after transduction, replace the medium with fresh fibroblast medium.
7. Culture the cells for 6 more days, changing the spent medium with fresh fibroblast medium every other day.

Day 5 or 6: Prepare MEF culture dishes

8. One to two days before passaging the transduced fibroblasts on to MEF feeder-cells, prepare 100-mm MEF culture dishes.

   Gelatin coating culture vessels:

   - Cover the whole surface of each culture vessel with gelatin solution (0,1%) and incubate the vessels for 30 minutes at 37°C or for 2 hours at room temperature.

   Thawing MEFs:

   - Remove the cryovial containing inactivated MEFs from the liquid nitrogen storage tank.
   - Briefly roll the vial between hands to remove frost, and swirl it gently in a 37°C water bath.
   - When only a small ice crystal remains in the vial, remove it from water bath. Spray the outside of the vial with 70% ethanol before placing it in the cell culture hood.
   - Pipet the thawed cells gently into a 15-mL conical tube.
   - Rinse the cryovial with 1 mL of pre-warmed MEF medium. Transfer the medium to the same 15-mL tube containing the cells.
   - Add 4 mL of pre-warmed MEF medium dropwise to the cells. Gently mix by pipetting up and down.

- Centrifuge the cells at 200 × *g* for 5 minutes.
- Aspirate the supernatant and resuspend the cell pellet in 5 mL of prewarmed MEF medium.
- Remove 20 µL of the cell suspension and determine the viable cell count

Plating MEFs:

- Centrifuge the remaining cell suspension (step 9) at 200 × *g* for 5 minutes at room temperature.
- Aspirate the supernatant. Resuspend the cell pellet in MEF medium to a density of 2.5×10^6 cells/mL.
- Aspirate the gelatin solution from the gelatin coated culture vessel.
- Add the appropriate amount of MEF medium into each culture vessel Into each of these culture vessels, add the appropriate amount of MEF suspension.
- Move the culture vessels in several quick back-and-forth and side-to-side motions to disperse the cells across the surface of the vessels.
- Incubate the cells in a 37°C incubator with a humidified atmosphere of 5% CO_2.
- Use the MEF culture vessels within 3–4 days after plating.

Day 7: Plate transduced cells on MEF culture dishes

9. Seven days after transduction (Step 6), fibroblast cells are ready to be harvested and plated on MEF culture dishes. Remove the medium from the fibroblasts, and wash cells once with D-PBS.
10. To remove the cells from the 6-well plate, TrypLE™ has been used following the procedure recommended by the manufacturer. When the cells have completely detached (1–3 minutes later), add 2mL of fibroblast medium into each well, and collect the cells in a 15mL conical centrifuge tube.
11. Centrifuge the cells at 200 × g for 4 minutes, aspirate the medium, and re-suspend the cells in an appropriate amount of fibroblast medium.
12. Count the cells and seed the MEF culture dishes with 5×10^4–2 × 105 cells per 100-mm dish and incubate at 37°C, 5% CO2 incubator overnight.

(We tried two different seeding conditions 5×10^4 and 1×10^5cells per 100mm dish).

(**Note:** Remaining cells have been harvested for RNA extraction to be used as a positive control in the RT-PCR detection of the SeV genome.)

Day 8 to 28: Feed and monitor the cells

13. 24 hours later, change the medium to iPSC medium or E8 and replace the spent medium everyday thereafter.

    To prepare 100 mL of human iPSC medium, aseptically mix the components listed below:

KnockOut™ D-MEM/F-12 (1X)	78 mL
KnockOut™ Serum Replacement (20%)	20 mL
MEM Non-Essential Amino Acids Solution (10 µM)	1 mL
GlutaMAX™-I Supplement (100X)	1mL
β-mercaptoethanol 55 mM (100 µM)	182 µL
Penicillin-Streptomycin (100X)	1 mL
Basic FGF* (20 µg/mL)	40 µL
N-supplement (100X)	1mL
B27-supplement without Vitamin A (50X)	5mL

    (* Prepare the iPSC medium without bFGF, and then supplement with fresh bFGF right before the use)

    Human iPSC medium can be stored at 2–8°C for up to 1 week.

14. Starting on Day 8, observe the plates every other day under a microscope.

    (**Note:** For BJ fibroblasts, the manufacturer reported colony formation on Day 12 post-transduction. However, depending on the cell type, culture for up to 4 weeks before seeing colonies may be needed).

15. Three to four weeks after transduction, colonies should have grown to an appropriate size for transfer. The day before transferring the colonies, prepare MEF culture plates using Attachment Factor-coated 12- or 24-wel plates.

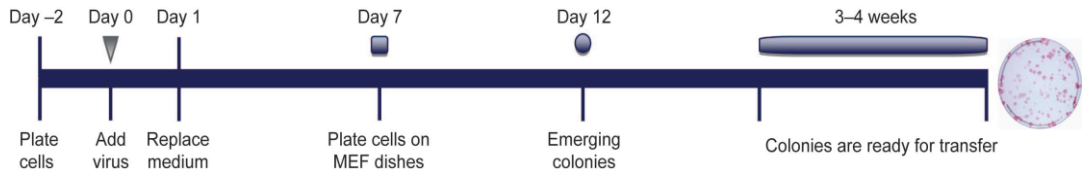
    By Day 21 post-transduction, the cell colonies on the MEF culture dishes will have become large and compact, covering the majority of the surface area of the culture dish. However, only a fraction of these colonies will consist of iPSCs, which exhibit a

hESC-like morphology characterized by a flatter cobblestone-like appearance with individual cells clearly demarcated from each other in the colonies.

16. Manually pick colonies and transfer them onto MEF plates prepared in step 15.

<u>Protocol for picking iPSC colonies:</u>

- Place the culture dish containing the reprogrammed cells under an inverted microscope and examine and take the photos to the colonies under 4X magnification.
- Mark and count (important to value the efficiency of reprogramming) the colony to be picked on the bottom of the culture dish.
- Transfer the culture dish to a sterile cell culture hood (i.e., biosafety cabinet) equipped with a stereomicroscope.
- Remove the colony slowly (paying attention to the contamination of FBs)
- Using a 200 µL pipette, transfer the cut pieces to a freshly prepared 12-well MEF culture plate containing human iPSC medium.
- Incubate the MEF culture plate containing the picked colonies in a 37°C incubator with a humidified atmosphere of 5% CO2.
- Allow the colonies to attach to the culture plate for 48 hours before replacing the medium with fresh human iPSC medium. After that, change the medium every day.
- Treat the reprogrammed colonies like normal human ESC colonies and passage, expand, and maintain them using standard culture procedures .



Once picked reprogrammed cell lines adapted to grow feeder free onto Matrigel coated dishes in Essential-8 medium (Life-technologies) appeared to be performed better in proliferation and maintenance of stability of iPSC colonies. Whenever such lines were splitted, we used Dispase or EDTA (if we want to single cells for the monolayer differentiation protocol).

CHARACTERIZATION OF THE iPSC

iPSC colonies that are obtained after a reprogramming process must be validated through a process of characterization, which allows us to test them as pluripotent stem cells. It consists in two main steps, the first we evaluate: morphology, pluripotency with alkaline phosphatase and RT -PCR that allows us to quantify markers of pluripotency. In second step will require testing the competence of differentiation: teratomas *in vivo* and embryoid bodies.

Immunohistological analysis and alkaline phosphatase activity

Cells were fixed in 4% paraformaldehyde (PFA) for 20 min and permeabilized with 0.2% Triton for 10 min. Blocking of unspecific sites was achieved by incubation with 10% donkey or goat serum (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. Cells were stained with several primary antibodies, specific for either 'stemness' or differentiation markers: human fibroblast surface protein (Clone 1B10, mouse monoclonal, 1 : 100; Sigma-Aldrich), human Oct4 (mouse monoclonal, 1 : 500; Millipore, Billerica, MA, USA), human TRA1–60 (mouse monoclonal, 1 : 100; Stem Cell Technologies), human SSEA-4 (mouse monoclonal, 1 : 100; Stem Cell Technologies), human a-sarcomeric actin (rabbit polyclonal, 1 : 400; Abcam, Boston, MA, USA) and cardiac troponin(mouse monoclonal, 1 : 200; Abcam, Boston, MA, USA) . Alexa-Fluor-488- and -555-conjugated secondary antibodies were used for specific detection, whereas nuclei were stained with 40,6-diamidino-2-phenylindole (DAPI). Coverslips were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA).

For the FB I have used the human fibroblast surface protein (Clone 1B10, mouse monoclonal, Sigma, 1:100). Alexa-Fluor-488- conjugated secondary antibodies (1h at RT) were used for specific detection, whereas nuclei were stained with DAPI.

Coverslips were mounted using 1 Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Confocal microscopy was performed with a Leica TCS-SP2 digital scanning confocal microscope equipped with a HCX PL APO

40x/numerical aperture =1.2 oil immersion objective. The pinhole diameter was kept at Airy 1. Images were exported to Adobe Photoshop (Adobe Systems, Mountain View, CA) and created with Adobe illustrator (Adobe Systems, Mountain View, CA).

Alkaline phosphatase activity of iPSC lines was determined using the Alkaline Phosphatase kit (Sigma-Aldrich). Lines were considered positive when alkaline phosphatase activity was detected in more than 95% of iPSC lines (two clones each condition were analyzed).

Flow cytometry analysis

iPSC were harvested and dissociated into single cells using Tryple Express (Invitrogen). Surface markers were assessed on fresh cell preparations: anti-SSEA4-FITC, and anti-TRA1-60-PE were from BD Pharmingen. Analyzes were carried out on a FACS Canto flow cytometer (Beckton Dickinson). Data were analyzed with DIVA software.

For the fibroblast I have used CD13 –APC, a fibroblasts specific surface antigen. Analyses were carried out as described above.

RNA EXTRACTION

Extract the total RNA from iPSCs using the TRIzol Reagent (Invitrogen) following the instructions provided with the reagent.

cDNA SYNTHESIS

Reverse Transcription was carried out using 2 µg of RNA in 20µL with "High-Capacity Reverse Transcription kit" from Applied Biosystems to obtain the corresponding cDNA for RT-PCR.

Step Temperature Time Cycles (RT)		
Denaturation	25°C	10'
Annealing	37°C	120'
Elongation	85°C	5'

Real-time PCR

Real-time PCR using either the Taqman Gene Expression Assay or the SybrGreen PCR Master Mix (Applied Biosystems), and data were analyzed with REST (Relative Expression Software Tool) software (http://www.gene-quantification.de/rest.html).42.

Primers used for the specific amplification are listed in the table:

Primers of Sendai Virus	
OCT-4 3'UTR	F:ccactagccttgacctctgg R:caaacatccttcgcctcagt
DNMT 3b	F:gagcccgaagaggagagaa R:aaagcccctgttcatgctc
REX-1	F:ccaggacaatggtgagtgc R:agctgtgcccatccactg
HGPRT	F:gaccagtcaacaggggacat R:ctgcattgttttgccagtgt

Primers of structural genes of myocytes	
TROPONIN	F:tccaactaccgcgcttatg R:tcgctccagctcttgctt
MHC-β	F:acaccctgactaaggccaaa R:tccagggatccttccagat
SCN5A	F:ggttctcgcctgcctttag R:aacttcacagcgctcaac
CACNA1C	F:ttgagctgaaatccatacg R:acaggcatctctggctcct
KCNQ1	F:cgcctgaaccgagtagaaga R:tgaagcatgtcggtgatgag

Primers of calcium-regulating genes	
CASQ2	F:ctgtgacattcaccacccca R:gttgcccgggacaatactga
RyR2	F:gctattctgcacacggtcatt

	R:atttccgtgccacttccttt
Trd	F:aacaagcttccagacccact R:tttcagaagcttttcccggc
JnC	F:accccactgggtccttctaa R:gggcgtctggacatctgtag

Primers of three germinal lineage	
MESODERM	
DESMIN	F:gtgaagatggccctggatgt R:tggtttctcggaagttgagg
SCL	F:ccaacaatcgagtgaagagga R:ccggctgttggtgaagatac
GATA-4	F:ttccagcaactccagcaa R:tcgcactgactgagaacgtc
ECTODERM	
NACAM	F:cagatgggagaggatggaaa R:cagacgggagcctgatctct
KRT-14	F:cacctctcctcctcccagtt R:atgaccttggtgcggattt
B-III TUBULIN	Sonda- FAM
ENDODERM	
SOX-17	F:ggcgcagcagaatccaga R:ccacgacttgcccagcat
GATA-6	Sonda -FAM

Genetic analysis of CPVT-iPSC lines

Genetic analysis of the exon 3 of the CASQ2 gene has been performed by direct sequencing on PCR products amplified by PCR from genomic DNA of control and CPVT-iPSC lines (three clones each). Analysis has been limited to the exon 3, since the mutations is located in this portion of the gene.

Genomic DNA was isolated using the following protocol:

Lysis buffer: (100 ml Recipe)

10 mM Tris-HCl pH7.5 - 0.5 ml of 2M

10 mM EDTA - 2 ml of 0.5M

10 mM NaCl - 0.2 ml of 5M

0.5% (w/v) Sarkosyl - 0.5gm (N-Lauroylsarcosine, Sigma # L-9150)

1mg/ml Proteinase K - add fresh each time (stored in freezer).

1. Add 250µl Lysis Buffer (with proteinase K added) to each well.
2. Incubate the plate in a sealed humid container at 60°C for 1 hour.
3. Transfer the contents of each well into a separate 1.5ml eppendorf tube .
4. Continue lysing the cells for 2 more hours. (60° C).
5. Add an equal volume (250 µl) of phenol:chloroform (phenol: chloroform: isoamylalcohol, 25:24:1).
6. Mix the contents of the tube until an emulsion forms. Shake by hand for ~1 min, (or vortex ~2sec),because vortexing shears genomic DNA.
7. Centrifuge 5-10 min 10.000g at in a microfuge at room temperature.
8. Transfer the aqueous phase (the top phase) to a fresh tube.
9. Repeat steps 5-8 until no protein is visible at the interface of the aqueous and organic phases.
10. Add an equal volume (250 µl) of chloroform and repeat steps 6-8.
11. Add 1/30th volume of 5M NaCl, for a final concentration of 0.2M NaCl. Mix well.
12. Add exactly 2 volumes of ice-cold ethanol and again mix the solution well.
13. Store the ethanolic solution on ice for 30 min to precipitate the DNA.
14. Centrifuge at 13.000xg for 10 min, 4°C.
15. Suck off the supernatant using the following protocol disturb the pellet. Vacuum droplets from walls of tube as well.
16. Half fill the tube with 70% EtOH and re-spin for 2 minutes at 4°ree;C.
17. Suck off supernatant as in step 15.
18. Store the open tube on the bench at room temperature until the last traces of fluid have evaporated.
19. Dissolve the DNA in 30µl-50 µl of water, depending on the size of the DNA pellet Let dissolve for at least an hour at 37°C
20. Control DNA quality on a 1% agarose gel .

    After isolation, CASQ2 exon 3 has been amplified and the PCR product purified from the agarose gel using the “QlAquick®Gel Extraction kit (50)” from QIAGEN,Cat.NO.28704.

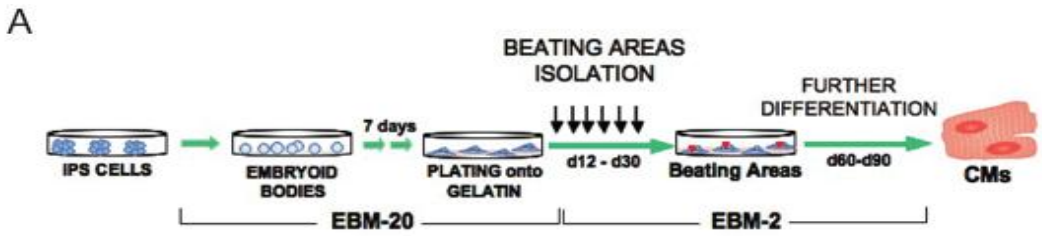
Sequencing has been performed both directions (forward and reverse primers) by Eurofins MWG Operon.

Spontaneous differentiation of iPSC and cardiac induction

EMBRYOID BODIES (EBs) PROTOCOL

Control and CPVT iPSCs were induced to differentiate by aggregation into EBs: iPSC colonies were detached using 1 mg/ml Dispase (Roche, Basel, Switzerland) and plated onto ultra-low-attachment plates (Corning, Incorporated, Corning, NY, USA) in EB differentiation medium, that is, DMEMF12 medium supplemented with 20% FBS, 0.1mM non-essential amino acids, glutamine and antibiotics. After 7 days, EBs were plated onto gelatin-coated dishes for further differentiation.

For cardiac lineage induction, ascorbic acid (50 mg/ml) was added to the medium. Spontaneously contracting areas, which appeared 12–30 days after EB plating, were manually microdissected and plated onto fibronectin-coated plates for further differentiation for an additional 45–90 days. Explants were maintained in EB differentiation medium supplemented with FBS at only 2% (**Figure 6A**).



(**Fig.6**: Schematic representation of the EBs protocol)

Alternatively, differentiation toward the cardiac lineage was more efficiently induced by a chemically-defined serum-free protocol, that has been recently published by Xiaojun Lian *et al.*,2012 and referred here as monolayer methods.

CARDIAC DIFFERENTIATION PF MONOLAYER METHOD:

This protocol efficiently directs iPSC to functional cardiomyocytes in a completely defined, growth factor (Activin A and BMP4) and serum free system by temporal modulation of regulators of canonical Wnt signaling. Appropriate temporal

application of a glycogen synthase kinase 3 (GSK3) inhibitor combined with the expression of β-catenin or a chemical Wnt inhibitor is sufficient to produce a high yield functional cardiomyocytes in 14 days from multiple iPSC lines without cell sorting or selection.

Protocol details and reagents are listed below:

Differentiation Medium	Final Concentration	250 mL	Catalog Number
RPMI 1640	-	212.5 mL	Life Technologies 11875-119
GlutaMAX	1X	2.5 mL	Life Technologies
B-27 Supplement without insulin **or**	50X	5 mL	Life Technologies 005012SA Life Technologies 17504044
B-27 Supplement with insulin	50X	5 mL	Life Technologies 005012SA Life Technologies 17504044

Reagents	Final Concentration	Catalog Number
****Y-27632 Rock inhibitor (5mM)**	10 µM	Sigma Y0503-1MG
****CHIR-99021 (36mM)**	8-12 µM	Selleck S1263-5MG
****IWR-1 (5mM)**	5 µM	Sigma 10161-5MG
Growth Factor-Reduced (GFR) matrigel (10mL)	1:30	BD Biosciences 354230
Essential 8 Medium	-	-
Accutase	-	Life Technologies A1110501

Aliquots for Stock Solution:

- **Y-27632 Rock inhibitor (5mM)**

  1 mg in 624.5µL of water; store 50 µL aliquots in -20°C

- **CHIR-99021 (36mM)**

5mg in 1 mL DMSO; store 20 µL aliquots in -80°C

- **IWR-1 (5mM)**

  5mg in 2.44 mL of DMSO; store 50µL aliquots in -20°C

- **Growth Factor-Reduced (GFR) matrigel (10mL)**

  Store 500µL aliquots in -20°C

  Preparation of GFR matrigel-coated 12-well plates.

PROTOCOL:

Day -4

1. Aspirate the old medium
2. Wash twice with 1mL PBS 1X per plate
3. Add 1 mL of pre-warmed accutase per plate. Incubate for 2-5 minutes at 37°C
4. Add 1 mL of E8 medium per well and pool all cells in a 15mL conical tube
5. Spin at 1000rpm for 4 minutes at room temperature
6. Aspirate the supernatant and resuspend the cells in E8 medium containing 10 µM of rock inhibitor
7. Count cells. Seed 100,000 cells per well of matrigel-coated 12-well plate. Bring the final volume to 1 mL per well with E8 medium containing rock inhibitor
8. Disperse the cells evenly by moving the plate in quick, short, back-and-forth and side-to-side movements and place in 37°C incubator.

Day -3

9. Aspirate the old medium
10. Add 1 mL of room-temperature E8 medium

Day -2

11. Aspirate old medium
12. Add 2 mL of room-temperature E8 medium

Day -1

13. Aspirate old medium
14. Add 2 mL of room-temperature E8 medium

Day 0

15. Aspirate old medium

16. Add 2 mL of room temperature RPMI/B-27 without insulin containing a final concentration of 10μM CHIR-99021. Record the time.

    Note: 8-12μM CHIR-99021 can be used to optimize for the sensitivity of each cell line. In our experiments we used a concentration of 10 μM

    Day 1

17. After 24 hours, aspirate the medium gently with p1000 tip

    Note: a lot of cell death will be observed

18. Very gently add 2 mL of room temperature RPMI/B-27 without insulin

    Day 3

19. Prepare 1.5 mL per well of fresh RPMI/B-27 without insulin containing IWR-1 at a final concentration of 5 μM. Collect and add 1.5 mL of medium from each well into the 15 mL conical containing RPMI/B-27 without insulin and IWR-1.
20. Gently rock the plate back and forth to collect the debris in suspension. Aspirate the remaining media gently with p1000 tip.
21. Add 3 mL of the combined medium containing IWR-1 to each well

    Day 5

22. Aspirate old medium gently with p1000 tip
23. Add 2 mL of room temperature RPMI/B27 without insulin

    Day 7

24. Aspirate old medium gently with p1000 tip
25. Add 2 mL of room temperature RPMI/B27 without insulin

    Day 8

26. Aspirate old medium gently with p1000 tip
27. Add 2 mL of room temperature RPMI/B-27 **with insulin**

    Continue to change the media every 2-3 days with RPMI/B-27 **with insulin.**

Teratoma assay

iPSC lines were harvested by dispase treatment, resuspended in X-VIVO medium (Lonza) and matrigel(1:1); the cell preparation was then injected subcutaneously into immunodeficient mice (NOD-SCID,NSG or Rag-/- can be used). Teratomas

formed 6-9 weeks after injection; after their formation these were collected and processed according to standard procedures for paraffin embedding and hematoxylin–eosin staining.

Karyotype

Chromosomal G-banding analysis was performed by Humanitas Research Hospital Cytogenetics Laboratory, using standard procedures.

Western Blot analysis for CASQ2

Normal and mutant CASQ2 protein levels were determined by immunoblot analysis as described previously.14 Briefly, 10 g of cell lysate proteins was subjected to 12% SDS-PAGE, blotted onto PVDF membranes (Santa Cruz Biotechnology, Inc), and probed with antibodies specific for CASQ2 (1:2500, PA1-913, Affinity Bioreagents). Blots were developed with Super Signal West Pico (PIERCE) and quantified using a Visage 2000 Blot Scanning and Analysis system (BioImage Systems Corporation).

Construction of Recombinant Adenoviruses

The wild type CASQ-2 gene was cloned in the CIS pAAV2.1-eGFP3 adeno-associated vector through standard cloning procedures using DH5α cells. After the cloning, correct insertion of the CASQ2 gene and accuracy of its amplification have been checked by direct sequencing. Large-scale production of viral particles will be carried out by the Vector Core Unit (TIGEM, Naples).

In detail, isolated exdotoxin-free plasmid DNA endotoxins-free is transfected together with pAd helper plasmid, pAAV rep-cap in 293T cells and cell media collected for viral particle purification. The virus is isolated through two cycles of purification with ultracentrifugation in CsCl2 gradients and dialyzed in sterile PBS in single aliquot of about 400-500µL.[41] The virus is stored at -80°C. The order of magnitude of the title of the virus is 1.4 E+13 GC/mL.[41]

RESULTS

Clinical history and genetic analysis

The proposed study is focused on a family with recessive form of CPVT that came to the attention of our collaborators, the Unit of Cardiology of the Salvatore Maugeri Foundation in Pavia. The pedigree of the family is shown in the **Figure 7A**: the proband is a male child homozygous for the 16bp deletion at position 339-354 in the exon 3 CASQ2 gene, leading to a frame shift and stop codon after 5aa(CASQ2G112+5X) while the asymptomatic parents are both heterozygous for the same mutation[33]. This deletion (G112+5X) lacks the second, the third and part of the first domain and is devoid of most of the acidic residues at COOH-terminal tail, responsible for the ion binding and for the dimerization (the amino acids involved in either front-to-front or back-to-back interactions) of the protein. The consequences of this mutation compromises its ability to bind Ca2+ and its dimerization capability should be compromised.[34,35]

The proband, now 17 years old suffers of syncope since the age of 3 ½ years, when he was diagnosed of CPVT. He is currently in therapy with β-blockers.

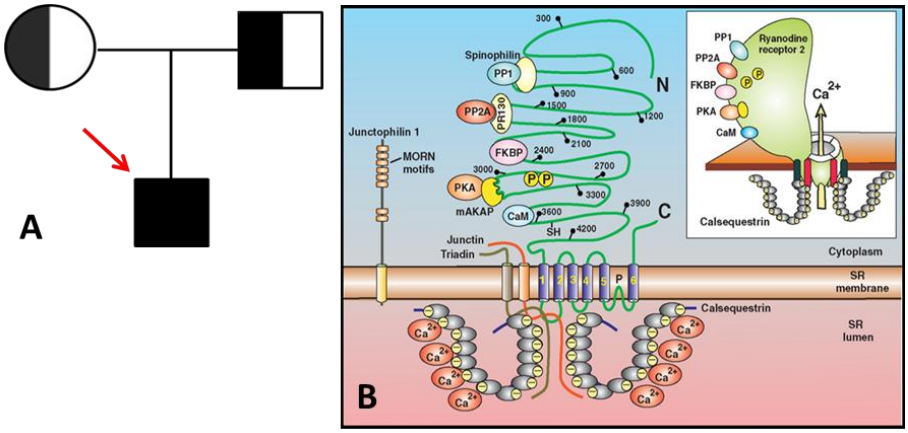


Fig.7: **A.** The pedigree of the Casq2 mutation CPVT family studied in this work: Proband CASQ2$^{-/-}$ (III-2) is indicated by the arrow. Filled symbols indicate clinically and genetically affected subjects. Shaded symbols indicate heterozygous individuals. White symbols is the individuals without CASQ2 mutations; grey symbols is the individuals with del.G112+5X. Typing for the 5 markers used for haplotype analysis is shown below symbols.
B.The structure about Calsequestrin-2 with macromolecular complex.

Generation of patient-specific CPVT-iPSC

The purpose of our study was to create a patient-specific iPSC -based system that could be used as an *in vitro* model to facilitate the validation of gene therapy approaches for the treatment of CPVT and to study the molecular, biochemical and functional features due to mutation of CASQ2 gene in human cardiac cells. To this end, we generated iPSC and iPSC-derived CMs from both the proband and his father, carrying either the homozygous or heterozygous G112+5X mutation in CASQ2 gene respectively

CPVT-iPSC were generated from primary fibroblasts (FB) isolated from a skin biopsies of the two patients (3-4mm) after informed consent using Sendai virus-based reprogramming method as schematically represented in the (**Figure 8**).

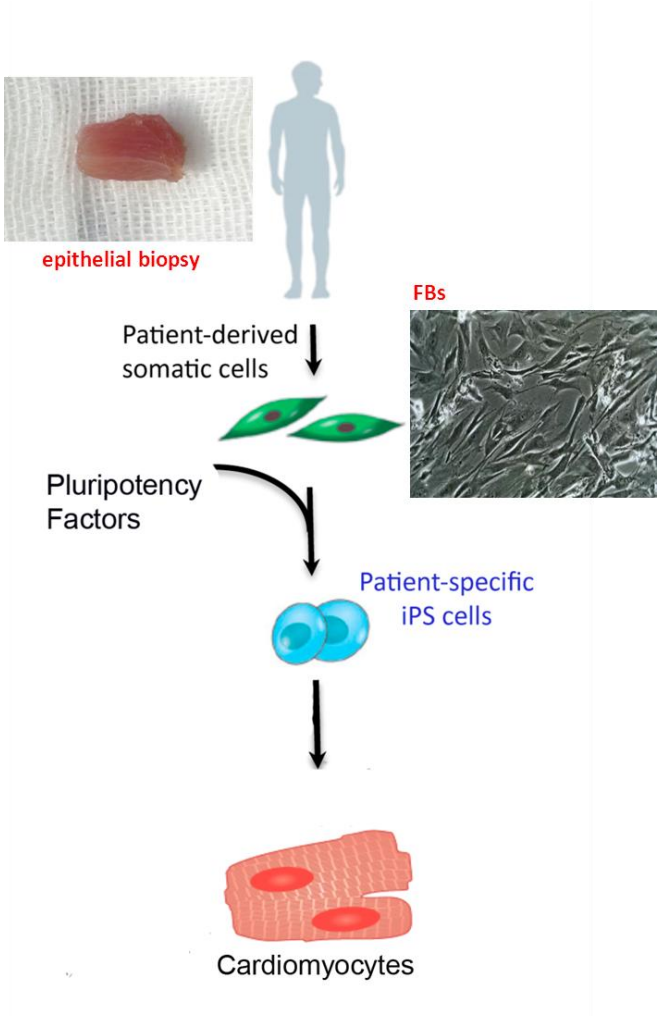


Fig.8: *Reprogramming and differentiation strategy*
We start from the patient-FBs and after the reprogramming with Sendai vectors expressing the 4 Yamanaka's factors we obtained patient-specific CPVT-CMs).

Prior to reprogramming induction, purity of the isolated primary FB has been validated by analysis of expression of markers typical of these cells using FACS and

immunofluorescence techniques. The results, represented in the **Figure 9**, show that the totality of the isolated fibroblasts are positive for the specific CD13 markers and correctly express and localize the IB10 fibroblast-specific surface antigen by the plasma membrane of the isolated cells

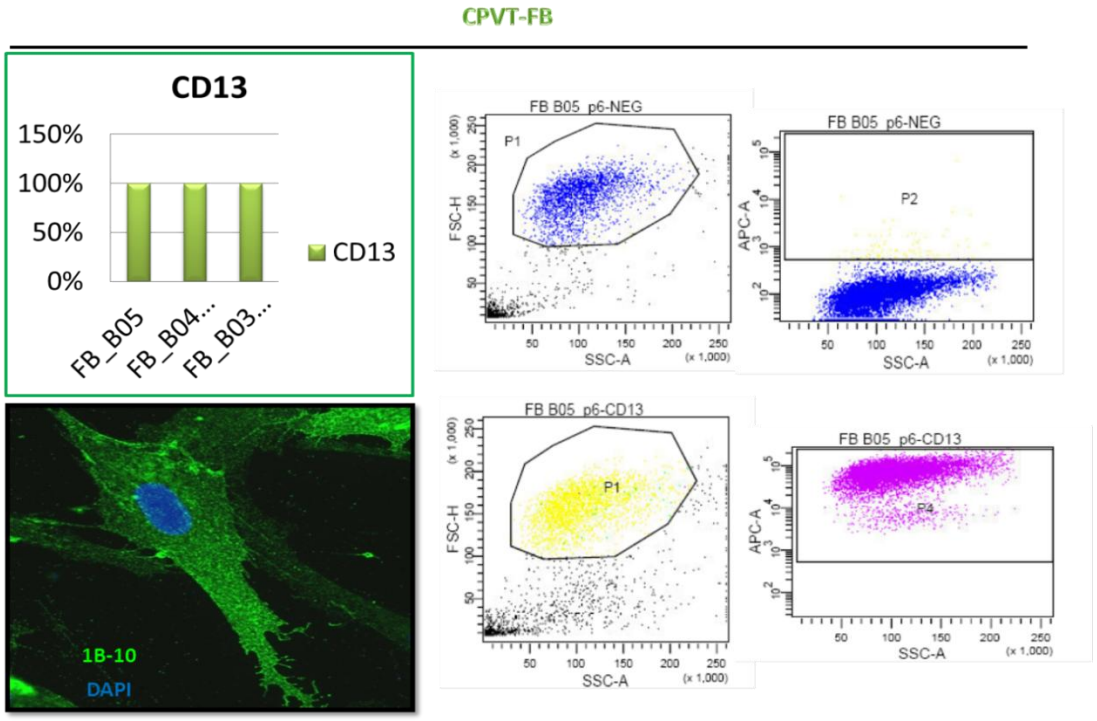


Fig.9: *Characterization of isolated fibroblasts*:
Upper right panel: FACS analysis showing expression of CD13 marker in the patient fibroblasts. Cell with no antibody staining have been used as negative control. Putting together the results obtained by FACS for the whole family we observe an expression equal to 100% (left table) of CD-13 antigen.
At the bottom left we see an immunofluorescence on patient-FB using another surface antigen: 1-B10 (green).

As a second step, reprogramming has been induced using the CytoTuneTM-iPS Reprogramming Kit. We initially generated iPSC lines from the proband. Colonies appeared 13 days after the plating of FB on MEF feeder with an efficiency of reprogramming of the 0.2%, calculated as the ration between the number of cells positive for the alkaline phosphatase activity and the number of the infected cells plated onto MEF ($5x10^4$ cells) (**Figure 10**).

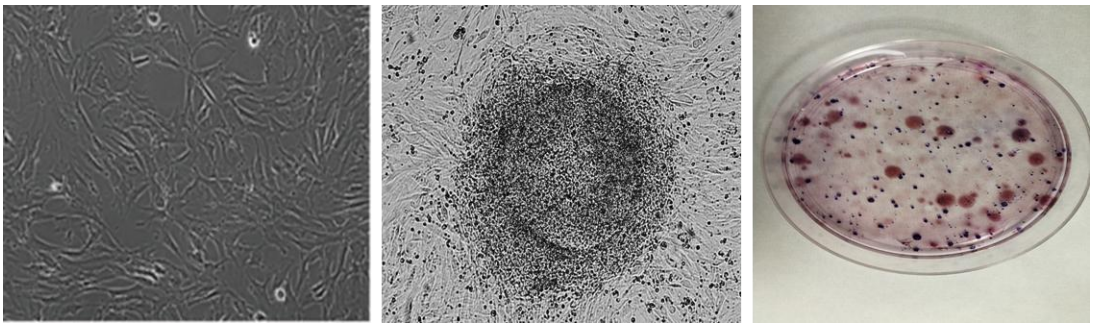


Fig.10: *Reprogrammed-FB, colonies of iPSC on MEF and Alkaline Phosphatase on reprogrammed-FB:* Through AP kit we show the violet colonies that are positive to the pluripotency

We then picked 24 colonies based on their morphology for their amplification and further characterization.

Morphological changes of FB during reprogramming following transduction with by Sendai Vectors are shown in the

CPVT-iPSC characterization

Of the 24 isolated colonies, 3 were selected for the molecular characterization. It is indeed necessary to verify the actual pluripotency of the obtained induced cells, since the process may be incomplete and give rise to intermediate of reprogramming with impaired ability to proliferate or to correctly differentiate or degenerating during passages.

Characterization of the iPSC lines entails two main steps, one aimed at verifying the expression of markers of pluripotency and the other to evaluate the developmental competence of the generated cells (**Figure 11**).

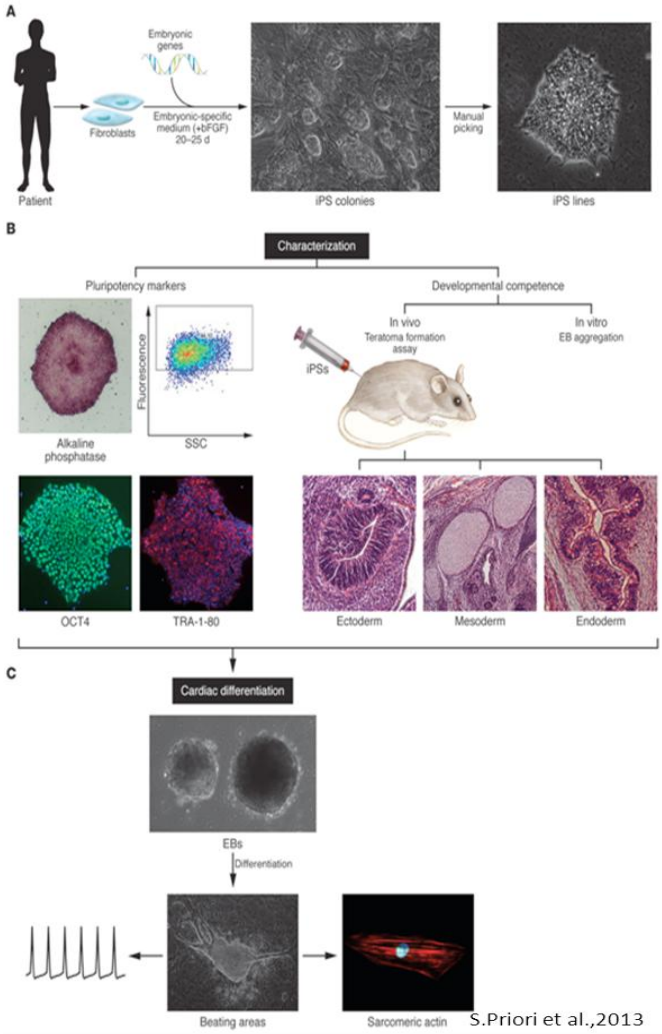


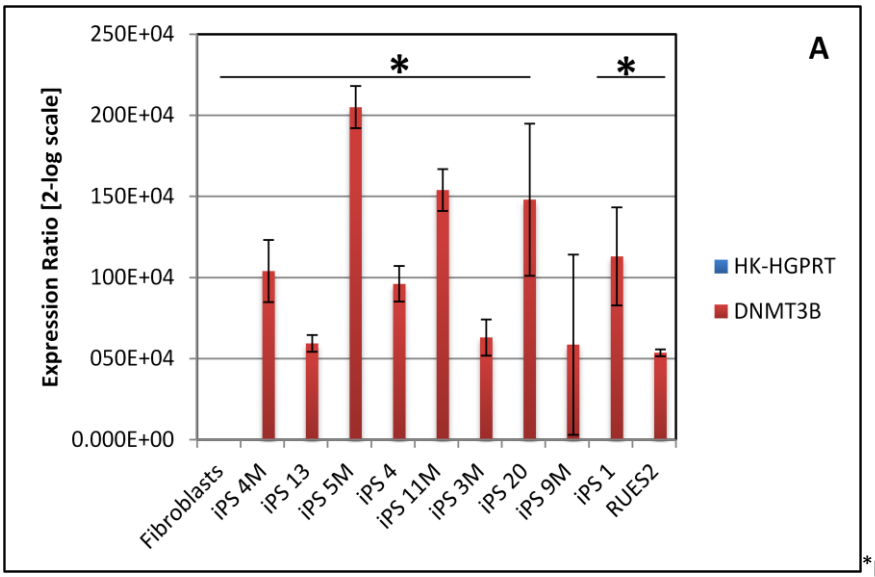
Fig.11: *Workflow for iPSC- generation, characterization, and derivation of beating cardiomyocytes*.

In the first step, alkaline phosphatase activity detection and expression of markers typical of the pluripotent state are used. The second part of the characterization process entails the assessment of developmental competence of the generated lines, defined as the ability to give rise to derivatives of all the three germ layer, both in vitro by EBs aggregation and in vivo by teratoma assays.

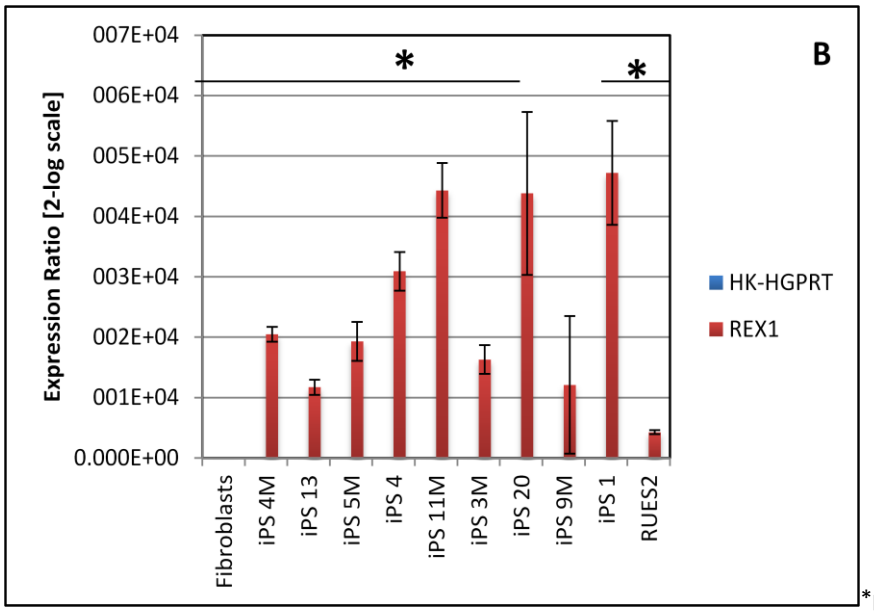
Cardiac differentiation is then induced in three characterized lines for each patient.

Alkaline Phosphatase activity and expression of marker of pluripotency by Real-Time PCR and FACS analysis have been initially employed for a major screening of the lines. Pluripotency markers expression has been then confirmed by immunofluorescence analysis on 3 selected lines.

Analyzed markers include: OCT4 (Octamer-binding transcription factors 4 involved in the self-renewal of undifferentiated embryonic stem cells), REX1 (RNA exonuclease 1 homolog, indicator of pluripotency, coexpressed with Oct4 in the inner cell mass of the blastocyst), DNMT3b (DNA cytosine-5 methyltransferase 3b,is essential for successful nuclear reprogramming of the somatic cells **Figure 11**) and the surface markers SSEA4 (**B**)(stage-specific embryonic antigen-4,sometimes also presents at the perinuclear) and (**C**) TRA1-60 (tumor rejection antigen1-60) whose expression lay to disappear during differentiation (**Figure 12**).



*p<0,05



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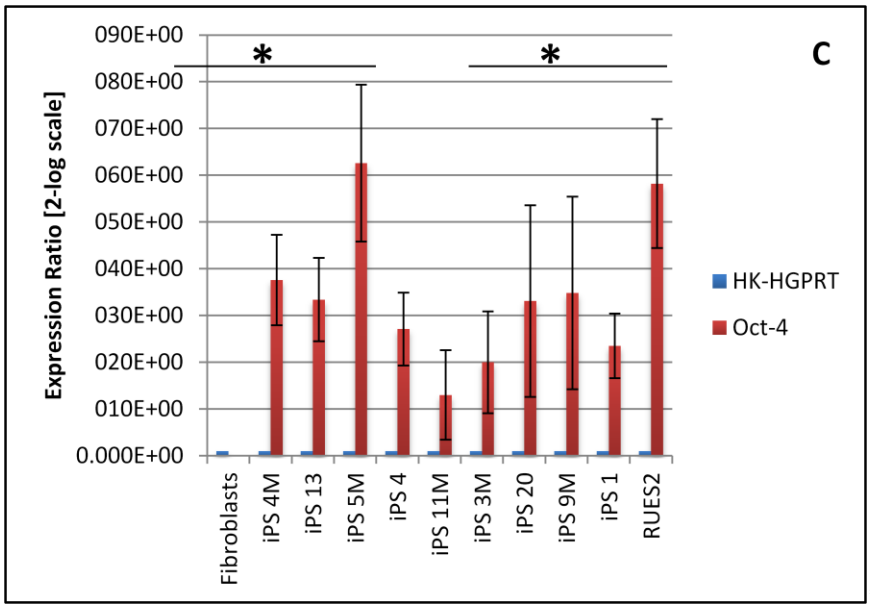
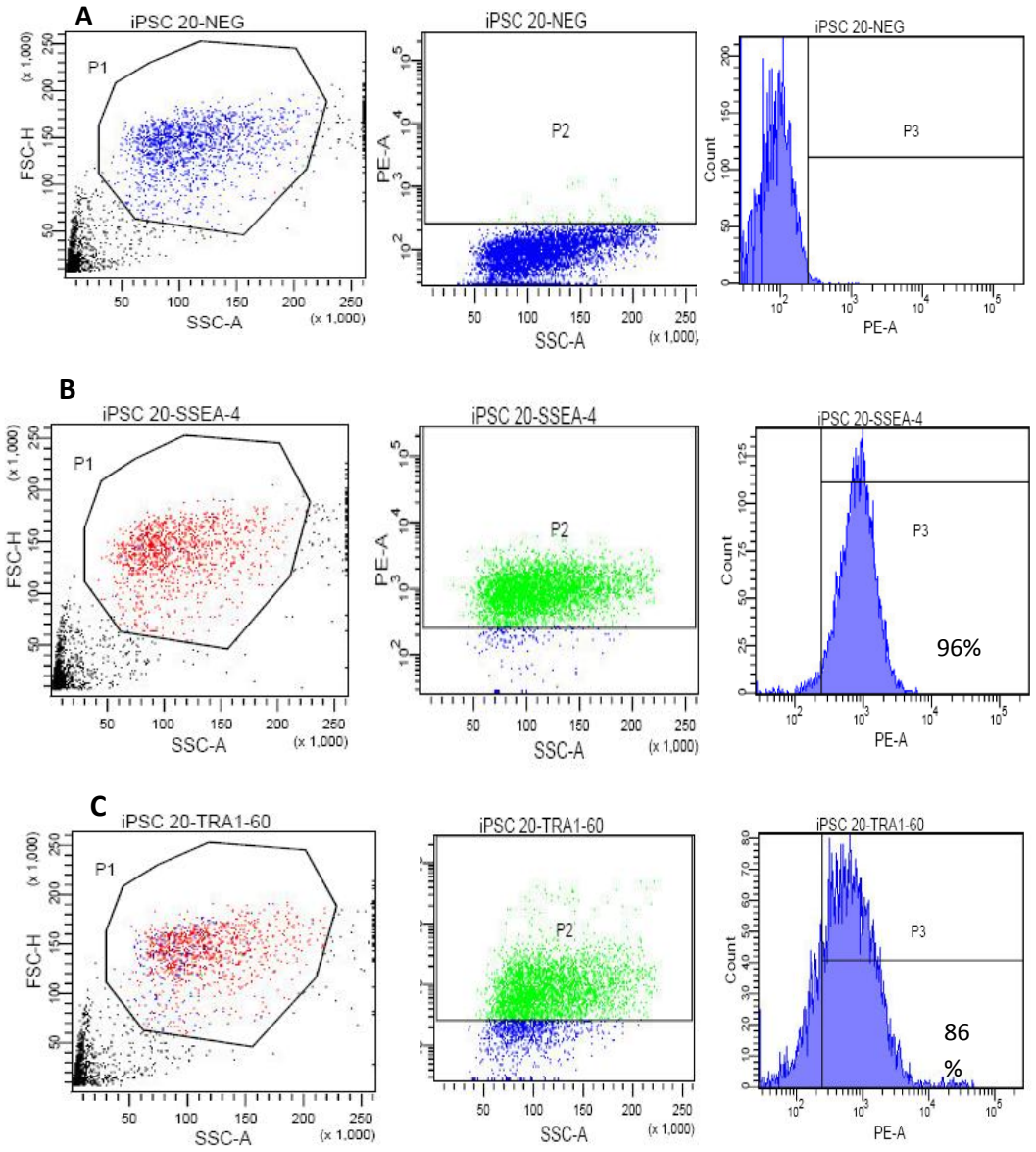


*p<0,05

Fig.12: *RT-PCR for 3 marker of pluripotency: Oct4,Rex1,Dnmt3b:*

In each RT-PCR HGPRT (gene housekeeping) was used for linearization and fibroblasts (somatic cells) as calibrator. The positive control is Rues (embryonic stem cells).



D

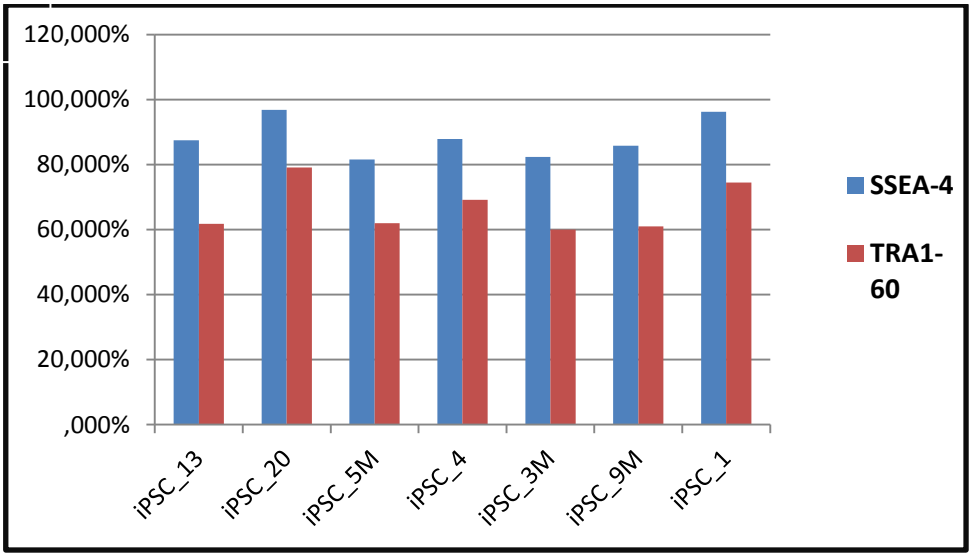


Fig.12: *FACS analysis of iPSC lines:*
A.B.C.: Images of plots from FACS-analysis from one representative line, showing expression of "stemness"-associated antigens (TRA1-60 (80%) and SSEA4(96%)
D:Histogram summarizing the analysis of the generated CPVT lines, indicating the percentage of cells expressing either SSEA4 or TRA1-60.

Results from these experiments show up-regulation of the expression of markers associated to the pluripotent cells in all the selected lines compared to the parental fibroblasts.

Based on these results, three lines (CPVT-iPSC #1, #13 and #20) were chosen with expression levels similar to those of embryonic stem cells control (RUES2 cell line) and further characterized.

Characterization of the iPSC lines derived from the father of the proband is still in process.

As first step, we validated selected iPSC lines possess alkaline phosphatase activity and their morphology (**Figure 13**) and confirmed the expression of Oct4, SSEA4 and TRA1-60 markers by immunofluoresce (**Figure 14**). Our results showed the totality of the cells were positively stained for alkaline phosphatase and correctly pluripotency markers by immunofluorescence.

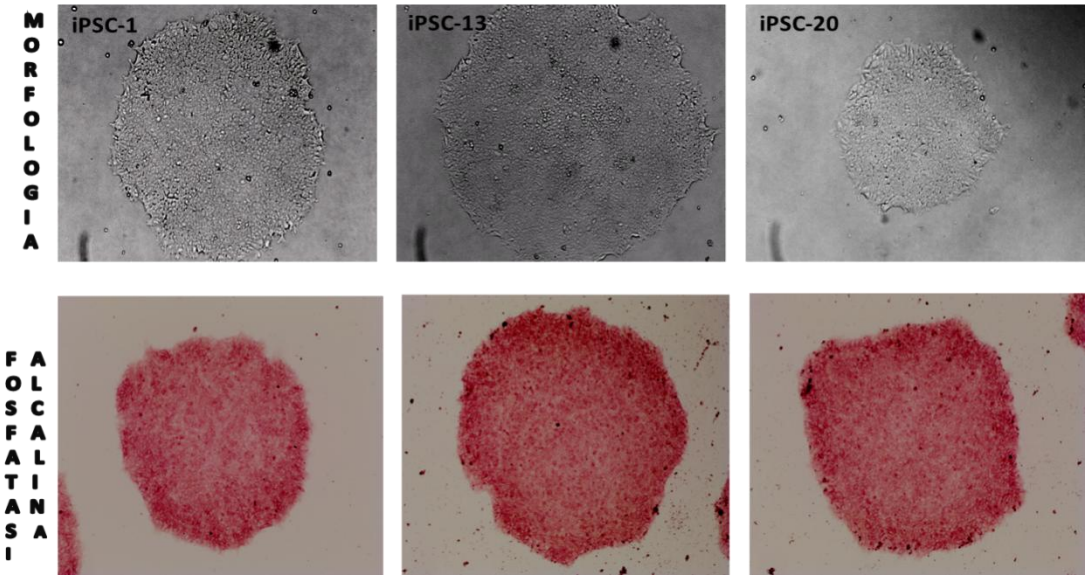


Fig.13: *Morfology and AP test*:
iPSC colonies derived from the patient's fibroblasts showing alkaline phosphatase activity and positivity for the pluripotency, it's important that the positive colonies are >90%

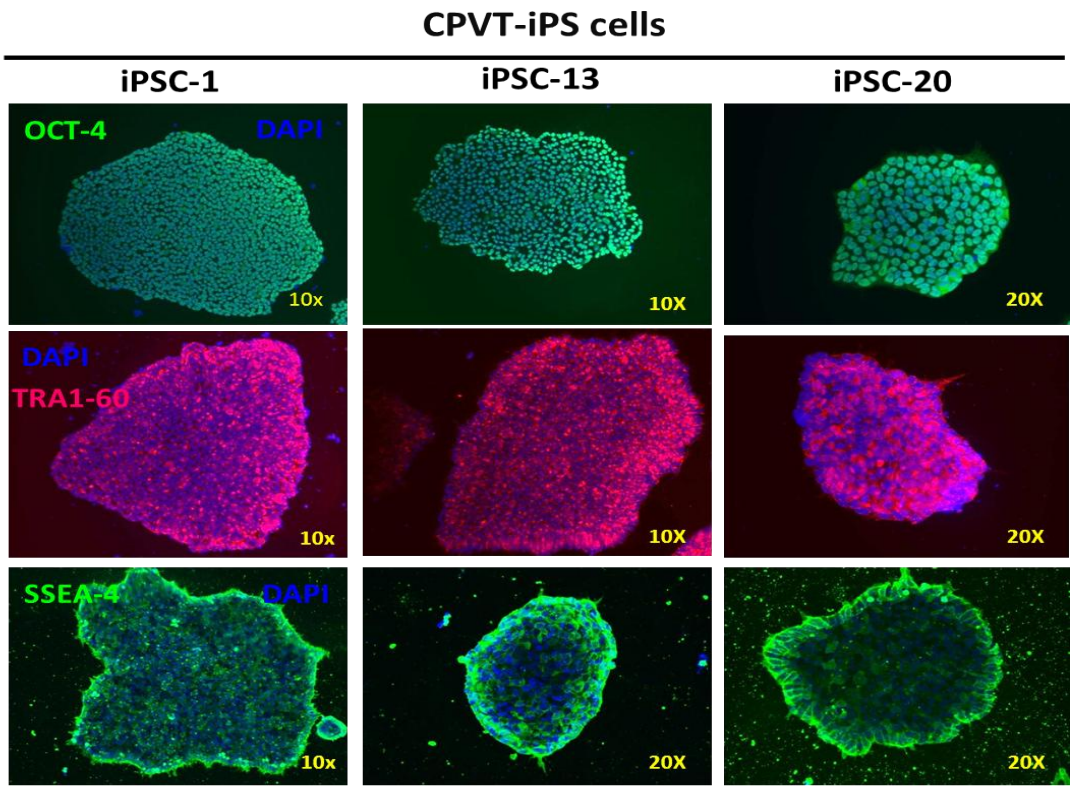
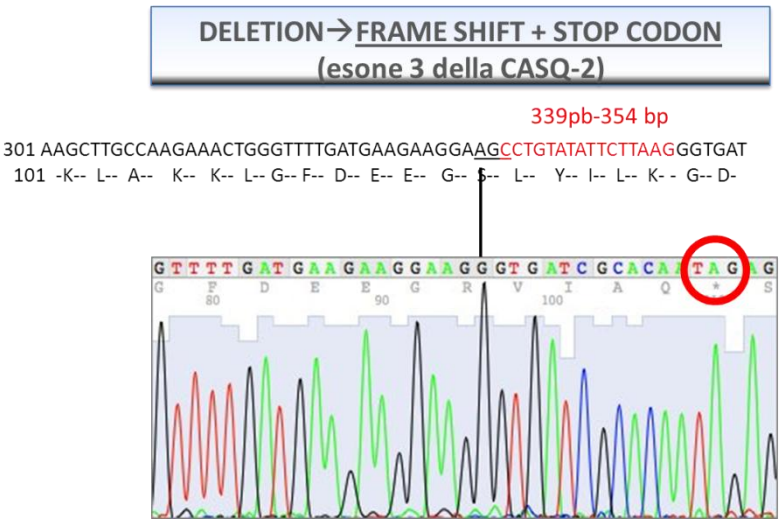


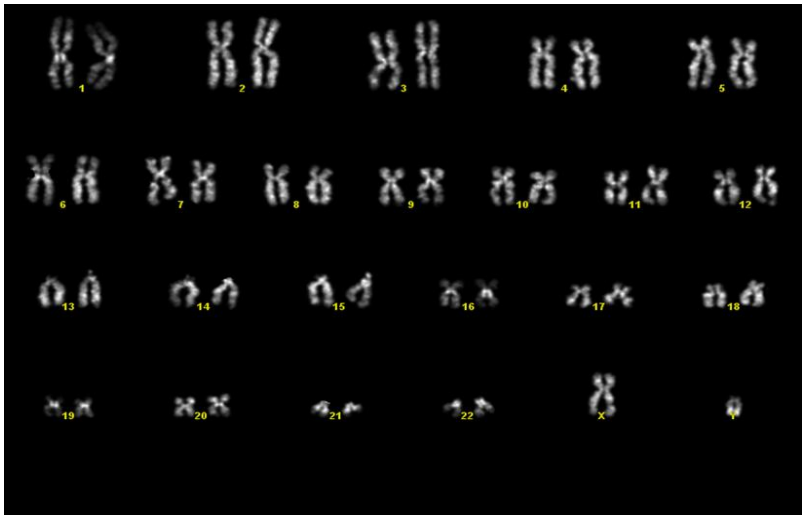
Fig.14: *IF for the Chracterization of iPSC generated from a CPVT patient skin biopsy:*
Immunofluerescence utilized three pluripotency markers:Oct-4 (inside the nucleus) and TRA1-60 and SSEA-4 (surface antigen membrane.

We then verified that iPSC lines carry the genetic mutation of the patient by direct sequencing. Results are shown in the **figure 15** and confirm iPSC lines possess the same genetic mutation of the patient.

Analysis of the karyotype also confirmed that CPVT-iPSC lines possess a normal 46,XY karyotype indicating that no chromosomal abnormalities occur during the reprogramming process.



A



B

Fig.15: **A**. Sequencing of proband-lines-DNA to confirm the presence of the deletion from 339pb to 354pb in exon 3 of the CASQ2.
B. Karyotype of the proband iPSC lines.

As second step of the characterization process differentiation potential of the iPSC lines have been validated by EBs aggregation and teratoma formation assay, that

represent the most stringent test of pluripotency for human cells. Results from these experiments showed CPVT-iPSC lines are able to differentiate into cells of ectodermal, mesodermal and endodermal origin in both experimental settings (**Figures 16** and **17**). Semi-quantitative real-time PCR (**Figure 17**) revealed that differentiating EBs contain cells expressing markers of the ectodermal (NCAM1, KRT14, βIII-tubulin), mesodermal (SCL, desmin, GATA-4 and cardiac genes) and endodermal (GATA6 and Sox17) lineages. Accordingly, analysis of teratomas formed after injection of iPSC into immune-compromised mice shows presence of tissue from the three germ layers (i.e. neural and adipose tissues and intestinal epithelium).

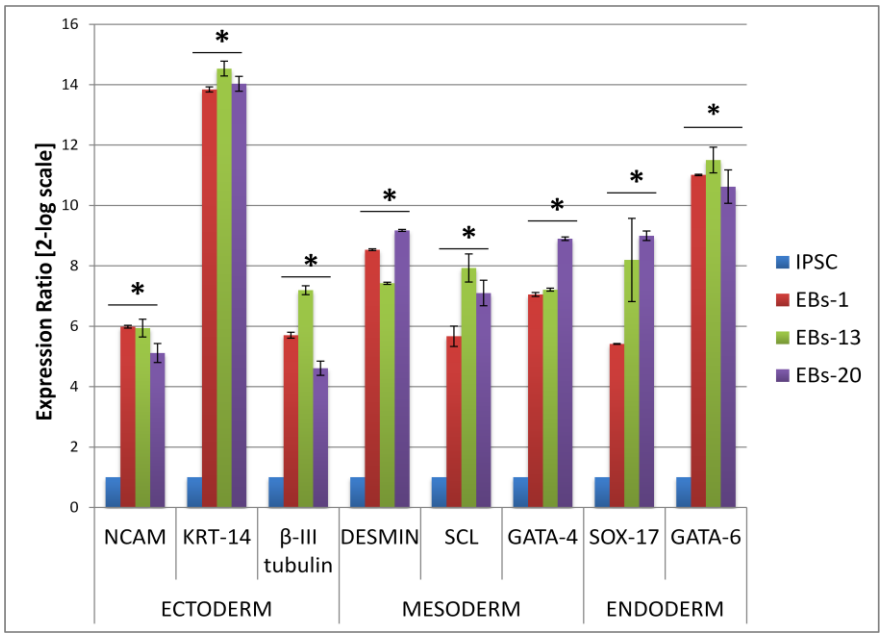


Fig.16: RT-PCR from *Embryoid Bodies, indicating the presence of cells that derive fromall the three germ layers*

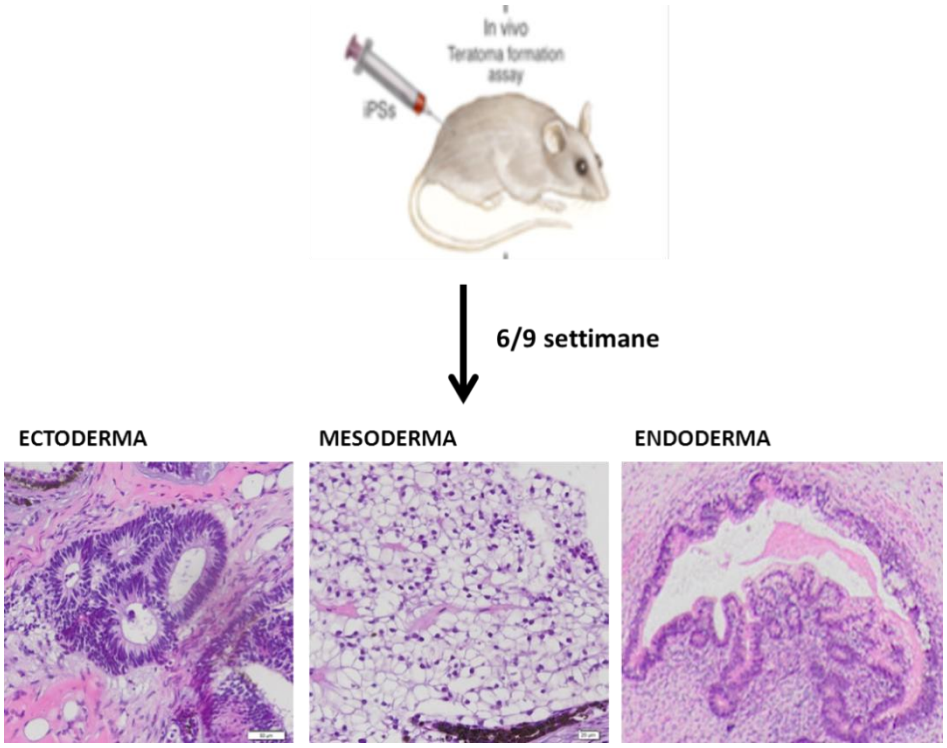


Fig.17: *Teratoma assay*:
CPVT-iPSC were injected (about 500.000 cells) into immuno-compromised mice and showed the ability to form teratomas containing derivatives of all the three germ layers.

Cardiac differentiation induction and analysis of iPSC-derived CPVT-CMs

As a next step, we induced iPSC to differentiate toward the cardiac lineage.

When iPSC are removed from differentiation suppression conditions and grown in suspension aggregates (EBs) in presence of serum, differentiation into cells from the three germ layers occurs, including spontaneously beating CMs. The inductive process is enhanced in the presence of specific medium and ascorbic acid.

Tipically the first sign of successful differentiation to CMs is the appearance of areas of contraction.

However, using this methods we obtained very low efficiency in cardiac differentiation; furthermore, the EBs-based protocol is quite long and contracting cells do not appear before 20 days of differentiation. It is also well known that CMs obtained from such protocol possess a very immature phenotype, so that maintenance in culture for additional 60-90 days is required to further differentiate them.

Based on these premises, we decided to employ a protocol that has been recently published[40] and that allows to obtain an almost pure population of CMs (greater than 80%) in 15 days.

The protocol is based on subsequent activation and inhibition of the Wnt pathway that is a key player in driving heart development. A schematic representation of the protocol, that we called "monolayer method" is given in the **Figure 18** .

Major advantages of this approach are the use of defined concentrations of chemicals and the absence of serum. The protocol will enable efficient production of human cardiomyocytes for development and disease research, drug screening and testing and advancing cardiac cellular therapies.

As a first step, we set up such protocol in the laboratory using control iPSC lines that were already available in the laboratory[39,15].

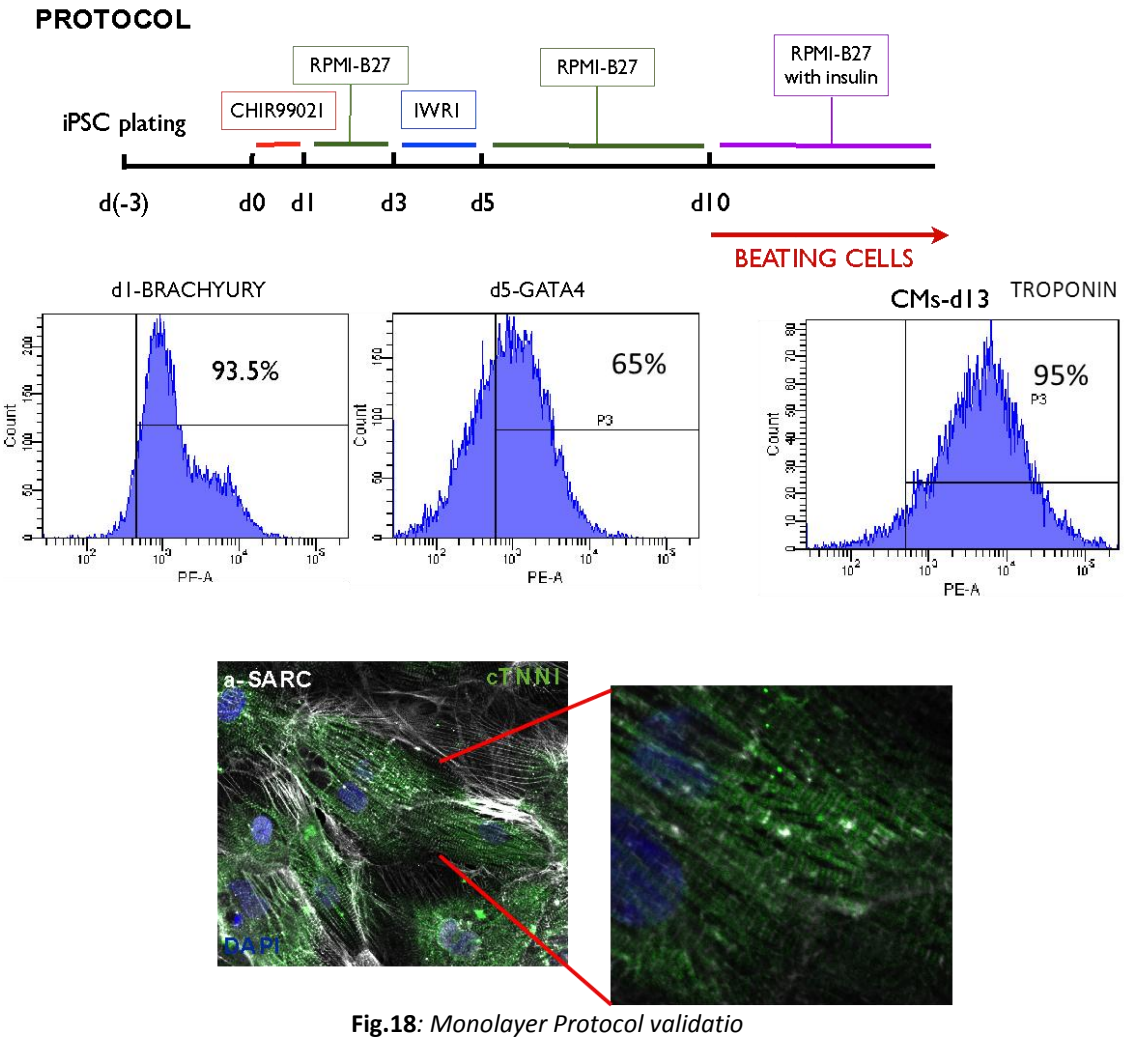


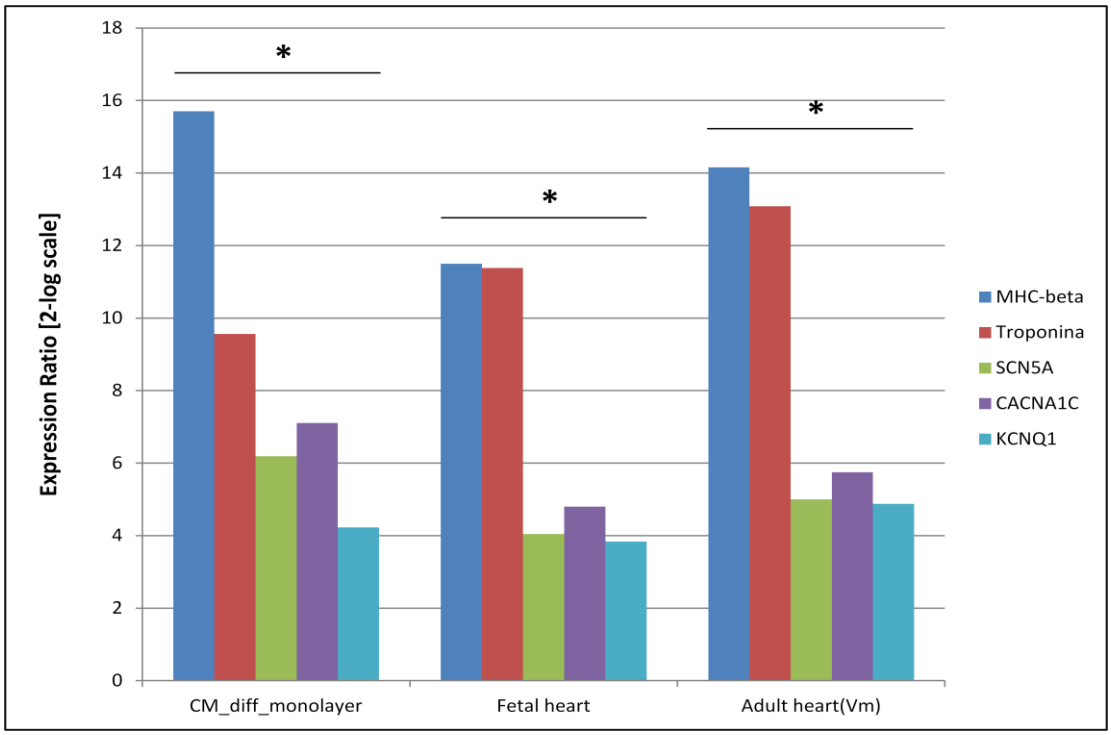
Fig.18*: Monolayer Protocol validatio*

Differentiation cells have been analyzed by FACS at d1, d3 and d13 after induction and result showed we were able to respectively obtain mesodermal precursors (93,5% of Brachiury that is transcription factor important in the mesodermic lineage), cardiac progenitors (65% of GATA, it's transcription factor evolves in embryonic development and in the cardiac differentiation) and differentiated CMs (95% of Troponin that is integral to muscle contraction in skeletal and cardiac muscle).

Immunofluorescence staining for two structural markers a-sarcomeric actin and cardiac troponin also showed that induced cells possess an organized sarcomeric organization.

Finally, in order to establish the maturity of the obtained cells, we checked the expression of both structural markers and ion channel, fundamental for contraction and action potential propagation.

We found that CMs differentiated from iPSC possess expression levels of these genes (MHC-β, Troponin, SCN5A, CACNA1C and KCNQ1) comparable with both fetal and adult CMs. (**Figure 19**).



*p<0,05

Fig.19: *RT-PCR about the structural genes of CMs*

Given the importance of calcium handling in the CPVT, we also specifically investigate the correct expression of genes that are important in this process (i.e. CASQ2, RyR2, JnC and TrD) at this differentiation stage. Results are shown in the **Figure 21** and indicate that calcium-regulating genes have a lower expression level compared to fetal CMs.

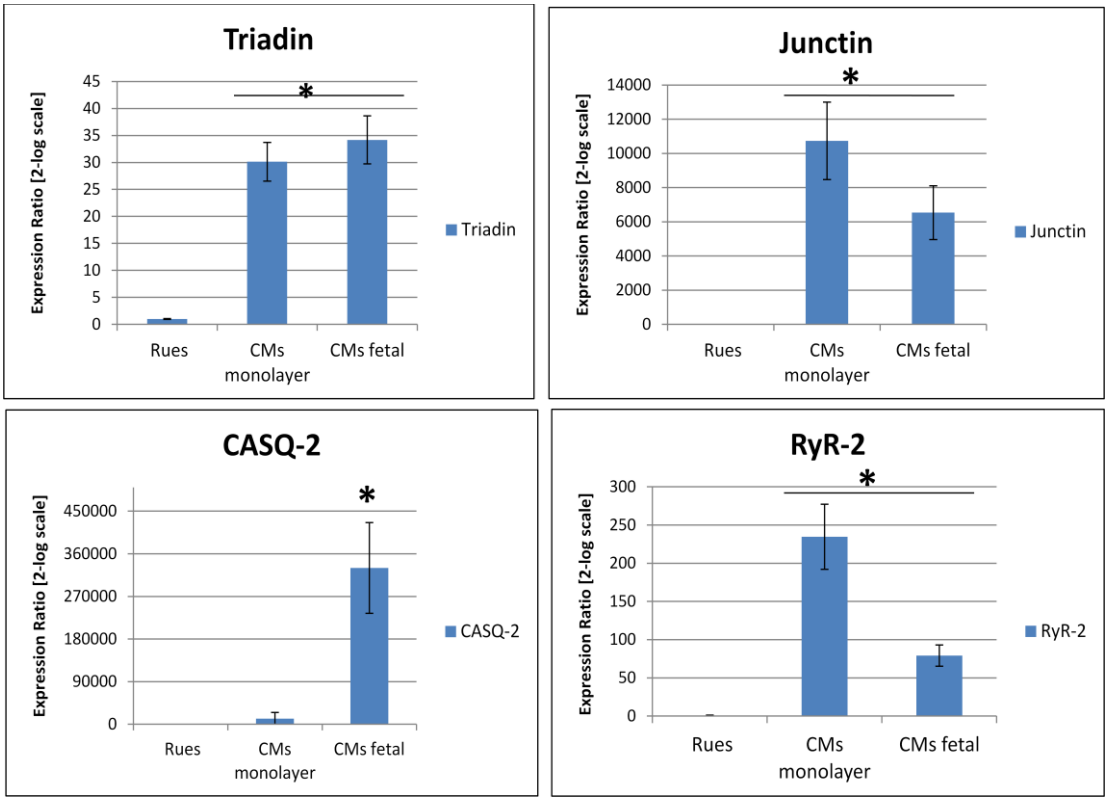


Fig.21: *RT-PCR for calcium-regulating genes*

These results suggest that, despite the structural maturity of the cell, the obtained CMs still have a immature calcium machinery. It is therefore important to further differentiate the cells before the electrophysiological and molecular investigation of the CPVT phenotype.

Next steps of the study will indeed entail the definition of the molecular profile of CPVT-CMs and their electrophysiological properties by path clamp analysis.

On this regard, we performed preliminary investigation on the expression the CASQ2, JnC and TrD on CPVT-CMs by Real Time-PCR and found that, compared to control lines, mutated CMs show a marked down regulation of CASQ2 and JnC, whereas RyR2 and TrD do not show any significant modulation. However, even if not significant, TrD showed a trend of up-regulation. (**Figure 22**).

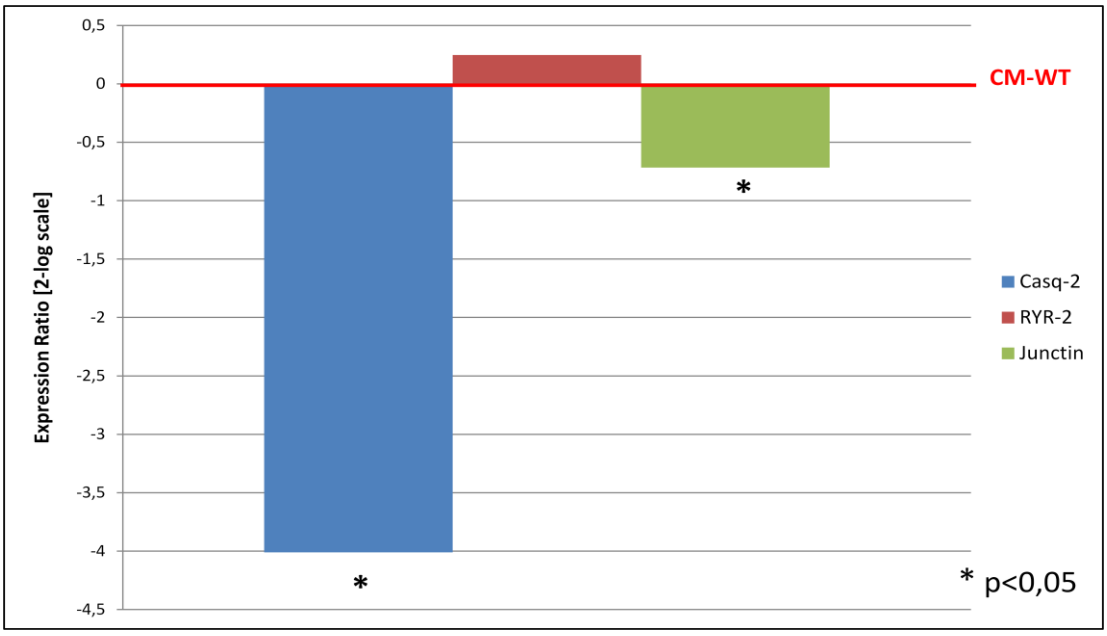
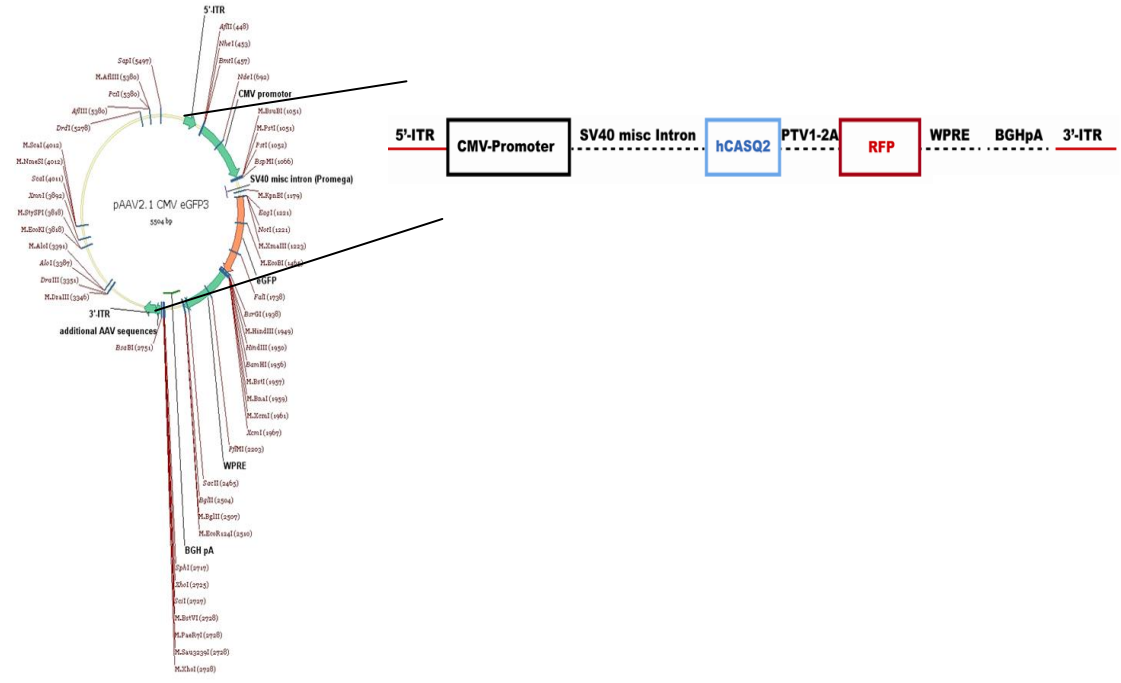


Fig.22: *RT-PCR showing the calcium handling genes in CPVT-CMs*

Our results and previous evidence showed impairment of CASQ expression in CPVT-CMs. In order to normalize the expression levels of intracellular CASQ2, we plan to use of Adeno-associated virus (AAVs) carrying the CASQ2 and to test weather this can also correct the pathogenic phenotype typical of the disease.

In collaboration with the Laboratory of Molecular Cardiology of the Salvatore Maugeri Foundation in Pavia, we cloned the cDNA of the wild-type CASQ2 into the pAAV2.1-eGFP vector, an AAV deleted of its protein :rep and cap (**Figure 23**).



(**Fig.23**: *Schematic representation of the cloning of the gene of the human CASQ 2* in the viral vector Cis pAAV-2.1-eGFP. Component of the plasmid: 5' ITR: sequences of regulation to 5' of the virus AAV (inverted terminal repeat"). CMV-Promoter: promoter of Citomegalovirus. SV40 : Sequence intronic of viral origin of necessary SV40 for the regulation of the genic expression. CASQ2-WT: sequence coding the gene of the mouse CASQ 2. PTV1-2A: sequence biscistronic of viral origin. RFP: sequence coding of the gene of the Red Fluorescent Protein. WPRE: regulation sequence post-transcriptional of the genic expression of viral origin (WHP). BGHpA: sequence of poli-A of bovine origin. 3'ITR: sequences of regulation to 3' of the virus AAV (inverted terminal repeat").

Cloned vector has been checked by sequencing (not shown) and by transfection in HEK293T cells, to verify the correct expression of the CASQ2 protein.

Results show that cells transfected with the generated vector correctly express CASQ2 protein by immunofluorescence and western blot analyses (**Figure 24**).

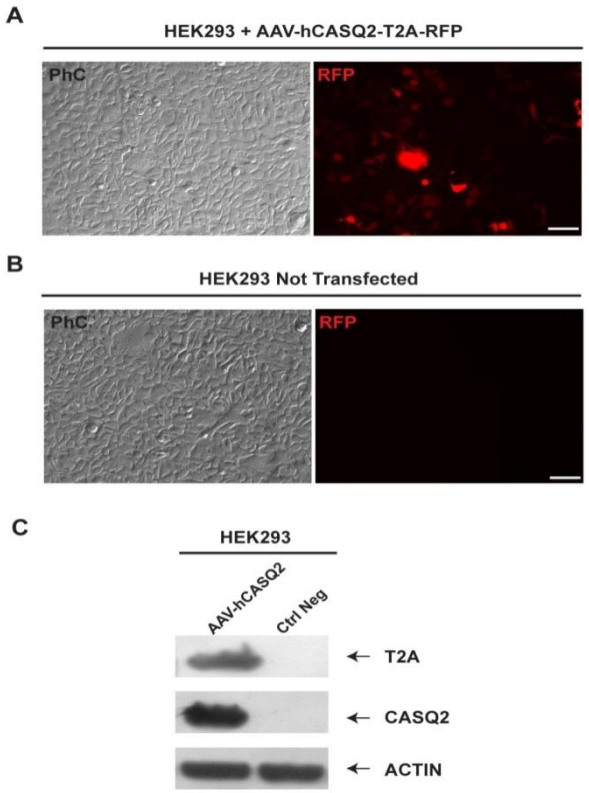


Fig.24: *HEK293 trasfected:*
we can observer to the HEK cells trasfected with pAAV-hCASQ2-T2A-RFP,viewed in phase contrast (PhC) and in channel of Roidamina due to the protein RFP (Red Fluorescent protein) in red, and the HEK don't transfected.
To confirm everything has been done the Western Blot with respect to Ab:anti-T2A,anti-CASQ2 and anti-Actinin (as a normalizer) to see that the cloning is done correctly and you can see the expression of the reports protein in tail to the gene of interest (previously checked by sequencing) and the gene hCASQ2 is expressed regularly as well as the tag in C-terminal position)

CONCLUSION and DISCUSSION

Recessive form of CPVT is a inherited arrhytmogenic disease associated with absence or drastic reduction of the gene encoding cardiac calsequestrin-2 (CASQ2), a protein critical for the regulation of cytoplasmic calcium homeostasis in cardiac myocytes. The distinguishing clinical and molecular features of recessive CPVT include: (1) severe reduction of two proteins TrD and JnC that, together with CASQ2, regulate RyR2-mediated release of calcium from the SR; (2) development of adrenergically mediated diastolic delayed after-depolarizations and triggered activity;(3) development of sustained polymorphic or bidirectional ventricular tachycardia elicited by stress or emotion and (4) lack of amino acids essential for dimerization and activity of biding to calcium by the CASQ2.

The objective of the present study was to create a model of CPVT to investigate the functional consequences of the deletion of 16pb in exon 3 in CASQ2 and to experiment corrective therapies.

To this aim, we decided to employ iPSC, a novel technology that allow the generation of a patient-specific cell-based system that could be used as an in vitro model to facilitate the screening of new therapeutic molecules for the treatment of CPVT.

Here we described the generation and characterization of this iPSC-based model from a patient carrying a mutation in the gene encoding CASQ2 and with phenotypic manifestations of the disease.[17]

Among the several available reprogramming methods, we decided to reprogram the patient's skin fibroblasts using viral vectors based on modified sendai virus, for their efficiency and their safety of use.

These are important issues, since our final goal is to employ such model for gene therapy approaches.

In the study I specifically carry out the generation and characterization of the patient’s iPSC lines and their differentiation toward the cardiac lineage.

Results showed here demonstrated we were able to derive iPSC lines from the selected CPVT patient (and his asymptomatic father – not shown) with high

efficiency (0.2%). Furthermore, analysis for pluripotency and developmental competence with different techniques confirmed the pluripotent nature of the generated lines.

Data on cardiac differentiation show that CMs generated from control lines exhibited, in a first instance, a quite immature phenotype for the expression of the calcium handling machinery, despite the correct expression and morphology of the structural proteins.

Previous report indicated that differentiation of stem cells into cardiomyocytes leads to the generation of immature cardiac cells that resemble fetal myocytes. It is therefore necessary to consider such issue for the future experiments and to further differentiate these cells.

Preliminary results obtained on differentiated CMs after 20d of induction have confirmed CPVT cells down regulate CASQ2, together with JnC, while TrD and RyR2 do not show any significant modulation. However, even if not significant, TrD showed a trend of up-regulation. We were surprised by these findings that will be further investigated in more mature cells and with different techniques (i.e. Western blot). It is therefore possible that protein levels do not respect the level of transcription and other mechanisms may be involved. Also maturity of the cells may affect transcription of some genes.

As a general note, to progress toward the clinical use of iPSC-derived myocytes in predicting susceptibility to arrhythmias and response to therapy, the development of improved differentiation protocols able to give rise to produce iPS-derived myocytes with the electrophysiological properties of adult cardiomyocytes and with a fully functional calcium-handling apparatus is of paramount importance.

Here we compared two methods, one based on aggregation into EBs that was very poor efficient in my hands, and the other, relying on chemically-defined medium and cells grown as monolayer, that gave better results in term of efficiency. We are now working to improve maturity of the cells: longer time in culture is surely a possibility to accomplish this goal and to obtain more CMs at the molecular and functional level.

Definition of the electrophysiological properties of these cells is in progress and will be performed by the collaborators of this project in Pavia.

We expect CPVT-CMs will exhibit delayed afterdepolarizations (DADs), a prominent feature of CPVT phenotype, after treatment with isoproterenol, a beta-adrenergic agonist.

If this will be confirmed, our intent will be to explore whether viral gene transfer using AAV9 engineered to carry the cDNA-CASQ2-wt it is able to revert such phenotype, as described in a mouse model of the disease,.

On this regard, we already have generated an AVV9 vector expressing the wild-type form of CASQ2 that will be used for overexpression of this protein in the patient-derived CPVT-CMs.

Ultimate goal of the study is to validate AVV9 as new therapeutic approach for the disease.

Drug therapies have been demonstrated ineffective in some patients; for this reason, we though GENE THERAPY approach may be an alternative strategy. In this case, since the disease phenotype is dependent by the lack of CASQ2. As already discusses, previous results obtained in the mouse are promising and showed an improvement in the levels of expression of CASQ2 (80%) and the respective proteins to it associated (TRD and JNC) after AVV-CASQ2 transduction.

Obtainment of positive results of human CMs will be the first step toward the clinical applicability of this therapeutic strategy.

On this regard it is worth noting that AVVs have been already approved for Clinical trials to treat the LCA disease, a genetic form of retinopathy, showing a beneficial effect of the view of the patients with no adverse effects. This indicates the feasibility of using AVV in therapy.

However, there are some limitations on the application of AVV to treat human diseases and that should be taken into account. In treating cardiovascular disease, for example, targeting requires the AVV particles to pass physical barriers and to reach the heart; also, within the organ, transduction should preferentially target a specific cell type. Determination of the most appropriate way of administration and the generation of AVV targeted to infect or to express the target gene in the cell type of interest can potentially overcome these limitation.

On this regard, there is an active phase II clinical trial (CUPIS) using intracoronary perfusion to drive expression of AAV-SERCA into the heart in the treatment of heart failure patients.

To conclude, in this study we created the basis for the development of a cardiac platform to investigate molecular and functional dysfunctions of CPVT and for the testing of innovative therapeutic strategies. The generation of such experimental models, together with the improvement of the specificity of the targeting vectors and the methods for inducing reprogramming and differentiation, constitutes the prerequisite for the application of iPSC technology in the clinical practice.

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