



UNIVERSIDADE  
DE LISBOA



# **Bolsas Universidade de Lisboa / Fundação Amadeu Dias**

**Edição 2011/2012**

**Relatório de Projeto**

## **Exploring RNA-targeted gene therapy approaches for Hypertrophic Cardiomyopathy**

Bolseira: Ana Carolina Freitas

Faculdade de Medicina, Universidade de Lisboa  
Curso: Mestrado Integrado em Medicina  
Ano: 4º ano

Tutora: Prof.<sup>a</sup> Maria Carmo-Fonseca

Julho de 2012

## FRAMEWORK

Hypertrophic Cardiomyopathy (HCM) is an autosomal dominant disease, which affects the myocardium leading to its hypertrophy. It occurs in 1/500 and is the most frequent hereditary disease of the heart, and a cause of sudden cardiac death in young adults and athletes. HCM-causing mutations affect genes that code for proteins of the sarcomere, which is the functional unit of the cardiomyocytes. There are currently 900 HCM-associated mutations identified in 13 different genes. MYBPC3 (Myosin binding protein C), MYH7 (Beta-myosin heavy chain), and TNNT2 (Troponin T), account for at least 60% of all cases of HCM. At present there is no effective treatment, only symptomatic treatment is available.

In the context of HCM, specifically disease associated to the mutation in TNNT2 gene, we proposed to explore RNA-targeted gene therapy approaches and thus establish a proof-of-principle for a novel RNA-based therapy for HCM at cellular level.

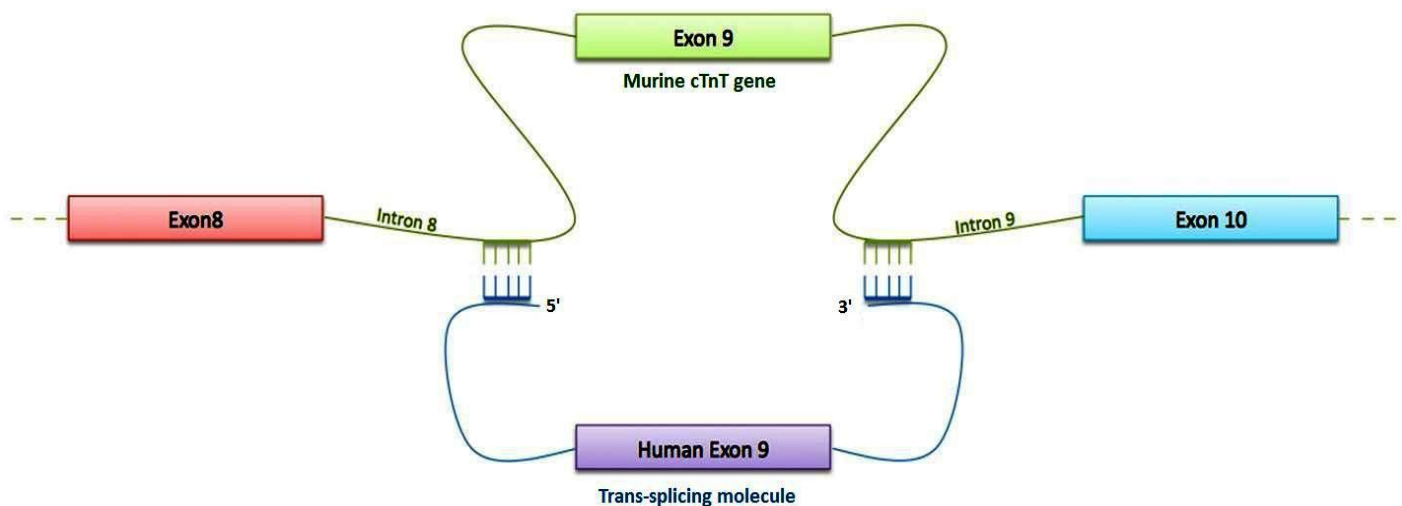
To achieve this, we planned to use spliceosome-mediated RNA trans-splicing to specifically replace exon 9 of the murine TNNT2 gene (Figure 1). Relative to antisense technologies, trans-splicing has the potential to treat more autosomal dominant genetic diseases and a larger population of patients affected by each disease. Several recent reports describe trans-splicing strategies to repair mutated exons. The first in vivo RNA repair by trans-splicing was performed in a mouse model of haemophilia A, the technique has been used to reprogram mRNA for cystic fibrosis, Duchenne muscular dystrophy, spinal muscular atrophy, frontotemporal dementia and various cancers. Our experimental approach is based on the transfection of mouse atrial cardiomyocytes (HL-1 cells) with “therapeutic” transcripts (trans-splicing molecules) designed to anneal to the introns flanking the murine TNNT2 exon 9, whose homologue in humans contains a cluster of pathogenic mutations associated to HCM. The success of this strategy will be evaluated by the replacement of murine exon 9 by the “therapeutic” human exon 9 via trans-splicing in murine TNNT2 mRNA.

In the future, the best constructs will be delivered using Adeno-associated viral (AAV) vectors, to transgenic mice that express, specifically in the heart, a murine TNNT2 gene bearing a missense mutation R92Q (located in exon 9). This murine model recapitulates many of the aspects of the human disease and the ultimate goal is to determine whether repair of the mutation in the adult heart is sufficient to rescue the defects in cardiac performance.

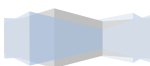


## OBJECTIVES

To repair a mutated exon using spliceosome-mediated RNA trans-splicing, we are using engineering transcripts designed to anneal the two introns flanking the mutated exon. Our target is the exon 9 of the endogenous Troponin T (cTnT) transcripts expressed in HL-1 cardiomyocytes. The first exon exchange construction containing specific annealing domains, spacer linkers, intronic splicing enhancers and three MSE2 array flanking exon 9, is being produced, transfected and tested for efficient replacement of exon 9 in the mRNA target (Figure 1). Different TNNT2 promoter sequences that ensure efficiency and specificity of cardiac gene expression are also being tested. The best annealing sequences to clone in the trans-splicing constructs were chosen by analysis of accessibility to specific FISH probes.



**Figure 1. Trans-splicing strategy.** Trans-splicing molecule, which anneals to both introns flanking the murine exon 9. As an initial approach, human TNNT2 exon 9 will be used as the trans-splicing molecule, at a cellular level; if so, wild-type murine TNNT2 exon 9 will be used as the “therapeutic” trans-splicing molecule in order to replace the mutated one in a cell line containing a specific HCM mutation.





Parallel to this, we are using FISH (Fluorescence In Situ Hybridization) techniques to characterize the cellular localization of trans-splicing constructs. For that, GFP-reporter trans-splicing molecules were also constructed containing a GFP sequence instead of the human exon 9. FISH provides a powerful tool for identifying the cellular distribution of a DNA or RNA sequence at cellular level. By using a labeled probe complementary to the specific GFP DNA sequence in the trans-splicing molecule, we were able to detect where this trans-splicing molecule is retained in the nucleus.

We are particularly interested to check if the trans-splicing constructs localize in the nucleus nearby the endogenous TNNT2 gene, which seem to be an essential step for the success of trans-splicing strategy, the nuclear localization and retention of trans-splicing constructs is a preliminary issue that should be clearly demonstrated.



## METHODOLOGY

### Human TNNT2-exon 9 trans-splicing construct: overall strategy

#### 1. Characterization and cloning of cardiac mTNNT2 promoters

##### 1.1 Cell model: HL-1 cells

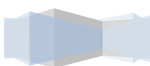
HL-1 cells: a mouse atrial cardiomyocyte cell line. Cells were obtained from Dr. William Claycomb's Laboratory. They are maintained in Gelatin/Fibronectin coated flasks in Claycomb Medium (Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich), Penicilin/ Streptomycin 100µg/ml (Sigma-Aldrich), Norepinephrine 0.1mM (Sigma-Aldrich) and L-Glutamine 2mM (Sigma-Aldrich). The medium is changed every 24h. The cells are split when 100% confluency is reached, and usually when they exhibit spontaneous contraction phenotype.

##### 1.2 Cloning mouse TNNT2 promoters in pcDNA3 vector

The promoters of the cardiac troponin T gene (TNNT2) of the chicken and the rat have been shown to possess cardiac gene specificity in rat and chicken cardiomyocytes, specifically a fragment of around 300 nucleotides (-303 to +6bp segment) of the rat promoter was identified as being the minimal sequence to allow it. The homologous sequence was identified in the mouse (*Mus musculus*) genome. Both the minimal and entire promoter sequences were amplified by PCR creating *Bgl*III and *Hind*III restriction sites, respectively, in the 5'-end and 3'-end. Two plasmid constructs (pcDNA3-L and pcDNA3-S) were created by cloning the minimal and entire promoter sequences in *Bgl*III and *Hind*III sites replacing the CMV promoter in pcDNA3 plasmid.

##### 1.3 Cloning EGFP reporter under the control of mTNNT2 promoters

The EGFP sequence was taken from the pEGFP-N1 vector by digestion with *Kpn*I and *Not*I, and then cloned in both pcDNA3-L and pcDNA3-S vectors in *Kpn*I and *Not*I restriction sites, generating the pcDNA3-L-EGFP and pcDNA3-S-EGFP constructs.



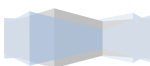
#### *1.4 Analysis of GFP expression under the control of L- and S- mTNNT2 promoters*

GFP expression under the control of both versions (L- and S-) of mTNNT2 promoters was analyzed in C2 and HL-1 cells. Briefly, C2 and HL-1 cells were transfected with 750ng of pcDNA3-L-EGFP and pcDNA3-S-EGFP constructs using Fugene 6 as a transfection reagent. As a control, pEGFP-N1 vector containing a CMV promoter was used. RNA was isolated every 24h during 3 to 5 days after transfection, and analyzed by RT-PCR using specific primers against GFP. In parallel, cells were fixed at each time point and GFP expression was analyzed by immunofluorescence.

Preliminary results reveal that the minimal sequence seems to induce a more immediate EGFP expression, whereas the entire promoter sequence seems to have a higher effect over time. GFP expression is higher in the HL-1 cells than in non-cardiac C2 cells. This data indicates that these TNNT2 promoters are cardiac specific. By immunofluorescence analysis, GFP is hardly detected probably due to very low levels of expression (we are testing more efficient methods of transfection at the moment).

## **2. Cloning a three MSE2 array in pTRE vector (Figure 2)**

pTRE vector was used as a platform to create a MSE2(3x) array. An oligo was designed with one copy of the MSE2 sequence flanked by specific restriction sites. One MSE2 copy was inserted in *PvuII* and *HindIII* sites of the pTRE vector. In order to insert the second MSE2 copy, pTRE-MSE2(1x) was digested with *PvuII* and *AvrII* to obtain the MSE2(1x) fragment, which was then cloned in *PvuII* and *SpeI* sites of the pTRE-MSE2(1x), generating the pTRE-MSE2(2x) construct (notice that *AvrII* and *SpeI* have compatible ends but are not digested by either one after ligation). The same approach is being performed to insert the third copy of MSE2 in the pTRE-MSE2(2x) and generate pTRE-MSE2(3x) construct.



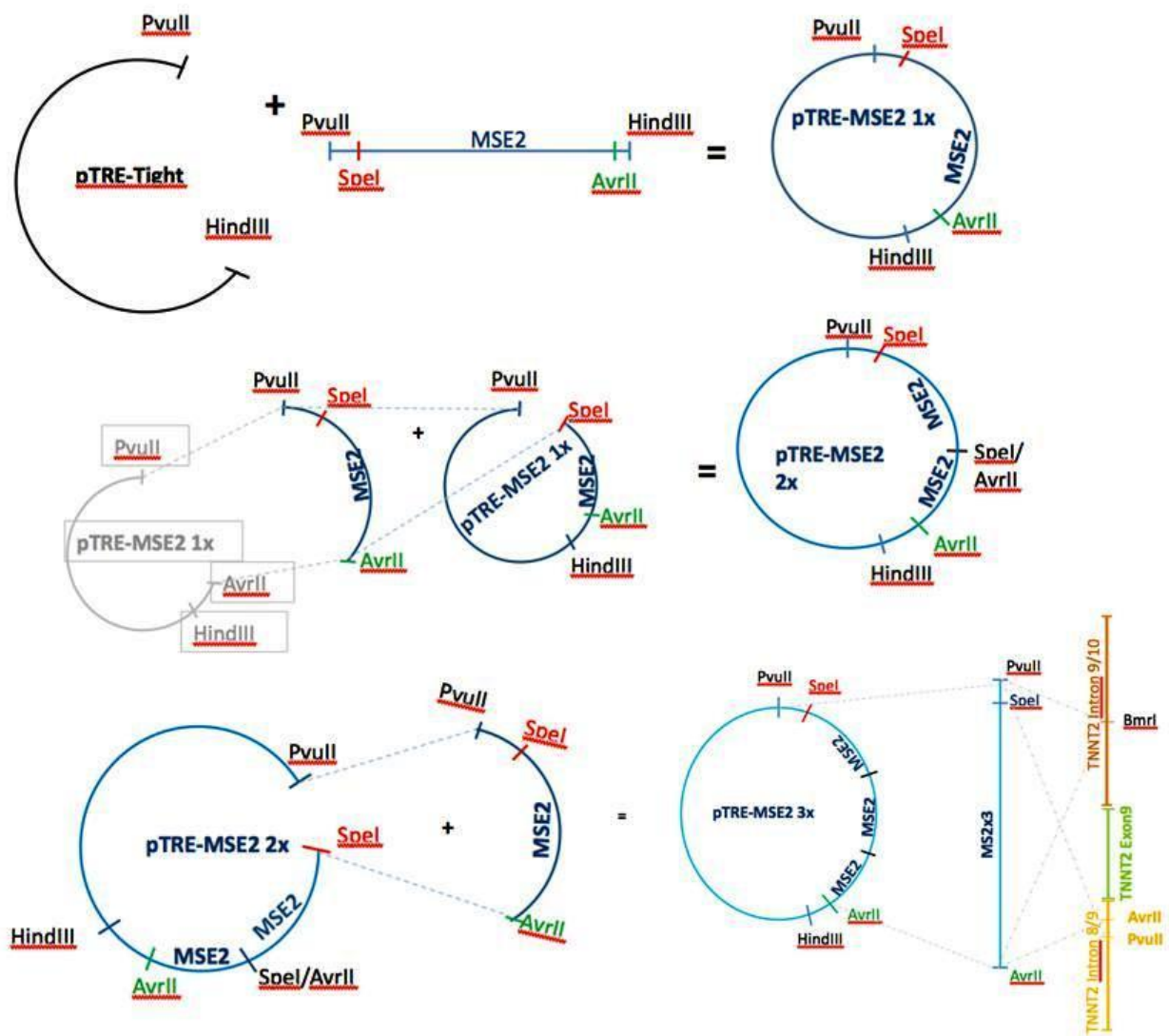


Figure 2. Strategy to insert three copies of MSE24 downstream and upstream of the human exon 9 trans-splicing molecule. The use of three MSE2 intronic sequences, downstream and upstream of an exon, has been described as sufficient for the inclusion of that exon in the processed transcript. According do that, a cloning strategy was developed in order to introduce MSE2 sequences into the intronic regions of trans-splicing molecules.



### **3. Cloning and mutagenesis of the Human Intron8/Exon9/Intron9 TNNT2 fragment**

#### *3.1 Cloning the Human Intron8/Exon9/Intron9 TNNT2 fragment in pTRE vector*

The Intron8/Exon9/Intron9 of hTNNT2 gene was amplified by PCR and inserted in the EcoRI/HindIII restriction sites of the pTRE vector.

#### *3.2 Mutagenesis of Int8/Ex9/Int9 hTNNT2 fragment*

To insert the MSE2(3x) array in the pTRE + Int8/Ex9/Int9 hTNNT2 construct, mutagenesis was performed on the intron 8 and 9 to create specific restrictions sites. The first mutagenesis created *PvuII* and *AvrII* restriction sites in intron 8 of the human TNNT2 and the second one generated the *NaeI* site in intron 9 of the human TNNT2.

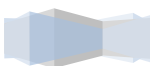
### **4. Insertion of MSE2(3x) array in both intron 8 and intron 9 of the Int8/Ex9/Int9 hTNNT2 construct**

pTRE-MSE2(3x) was digested with *PvuII/AvrII*, and then the MSE2 (3x) array was cloned in the intron 8 of the Int8/Ex9/Int9 hTNNT2 construct in the same restriction sites. In Intron 9, the same MSE2(3x) array was cloned in *NaeI*, which generates blunt ends.

### **5. Selection of annealing sequences for the trans-splicing constructs**

To determine the best annealing sequences to clone in the trans-splicing constructs, several FISH probes were designed to recognize different intron regions in the endogenous mTNNT2 transcripts. In this way, we analysed which regions are more exposed to hybridization and indirectly to the annealing of the trans-splicing construct.

Four pairs of primers were selected in order to amplify non-conserved intronic regions to avoid potential regulatory elements. For intron 8, only one pair of primers was chosen due to the small size of this intron. For intron 9, two pairs of probes were selected. As a control, a pair of primers for exon 9 was also designed.





The reverse primers bear a promoter sequence (T7: 5'TAATACGACTCACTATAGGG3') located in the 5' end, in order to perform *in vitro* transcription with T7 RNA polymerase.

PCR reaction was performed using the aforementioned primers and DNA from HL-1 cells. PCR products were separated by gel electrophoresis, detected by gel red and sequenced to confirm the amplification of the correct fragments.

All probes were made by *in vitro* transcription by incorporation of digoxigenin-UTP (Roche), or Chromatide Alexa488-UTP (Molecular Probes), using as a template PCR products with a T7 promoter as explained above.

For fluorescence *in situ* hybridization (FISH) experiments, HL-1 cells were fixed in 3.7% paraformaldehyde in PBS for 10 min and permeabilized with 0.5% Triton X-100/ 2mM VRC in PBS for 10 min. The cells were incubated over night with the already described probes diluted at 5ng/ $\mu$ l in hybridization buffer. Washes were performed with 50% Formamide in 2xSSC and in 2Xssc-0.05% Tween. Digoxigenin detection was carried out with anti-digoxigenin labeled antibodies (Jackson Immuno Research Labs).

Images were acquired on a Zeiss LSM 510 confocal microscope using a PlanApochromat 63x/1.4 objective.

## 6. Cellular localization of trans-splicing GFP reporter constructs

GFP-reporter trans-splicing molecules were constructed containing a GFP sequence instead of the human exon 9. By using a labeled probe to the specific GFP DNA sequence, we will be able to localize this reporter molecule inside the cell.

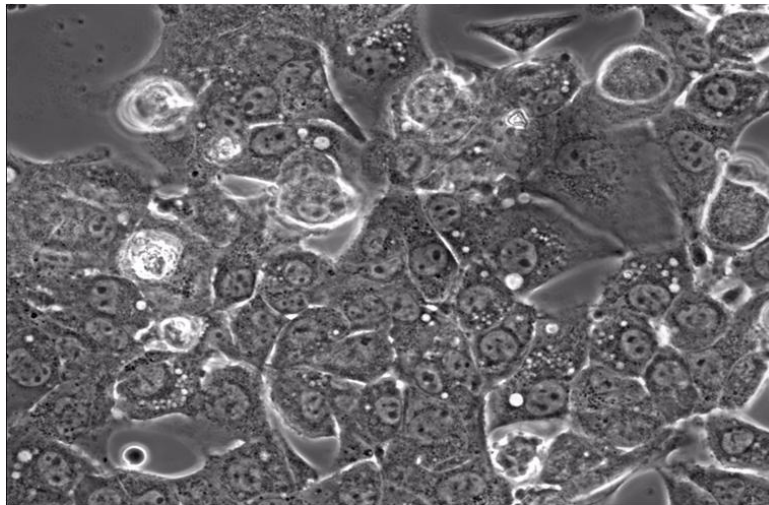
Perfoming double FISH, using the intronic TNNT2 aforementioned probes together with the anti-GFP probe, we expect to be able to detect a nuclear co-localization between the reporter molecule and the endogenous TNNT2 transcripts. This step of the project is in progress, and we don't have results yet.



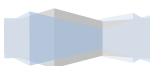
## RESULTS AND CONCLUSIONS

### *1. HL-1 cells show cardiac phenotype*

Our preliminary results show that HL-1 cells exhibit a cardiac phenotype making them a suitable cell model for HCM (Figure 3).

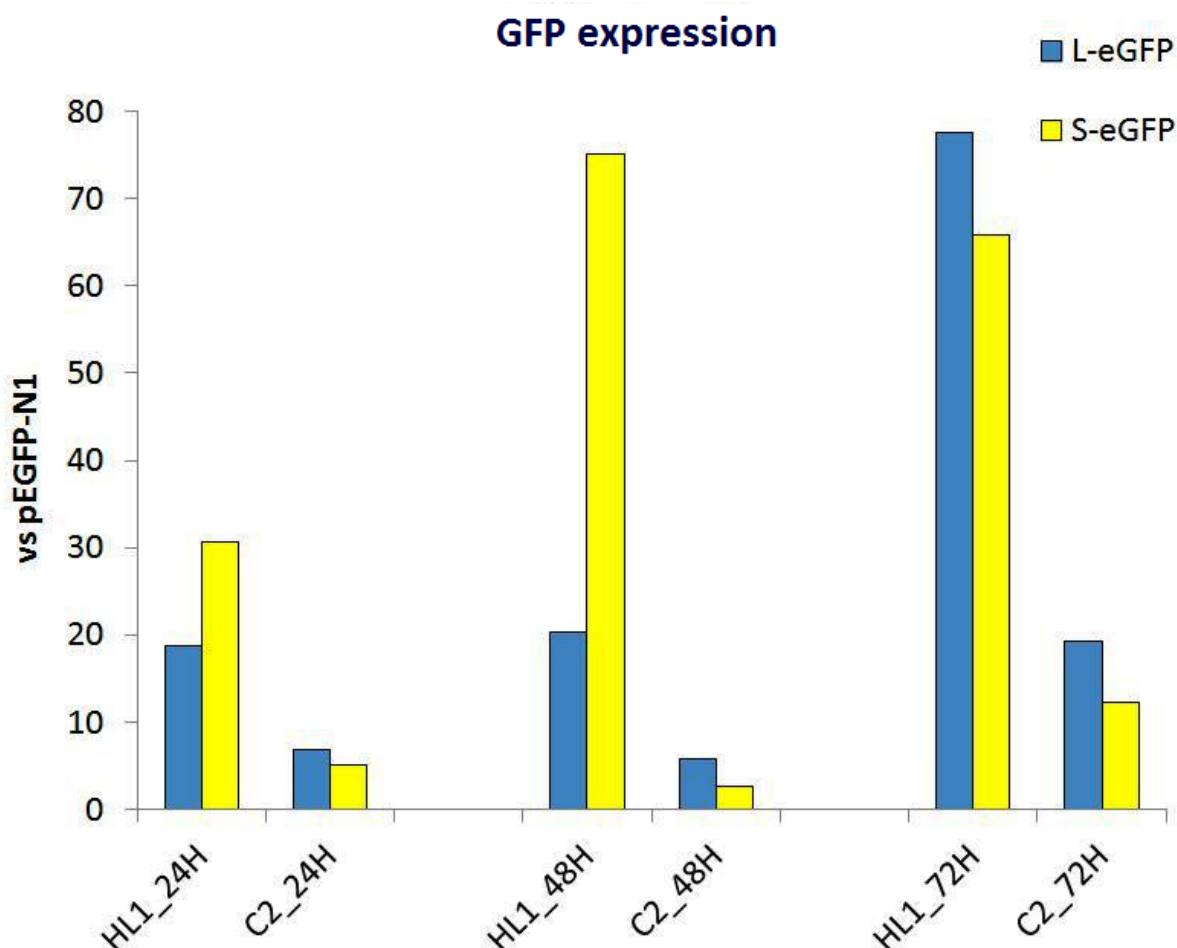


**Figure 3. HL-1 cells.** HL-1 cells are mouse atrial cardiomyocytes, where cardiac mTNNT2 promoter specificity was tested. These cells show to maintain cardiac phenotype (namely spontaneous contraction) in our culture conditions, proving to be an adequate cell model for further trans-splicing assays.



## 2. *TNNT2 promoter sequences drive specific expression of reporter gene in HL-1*

Our data also show that the minimal and the entire TNNT2 promoter sequences drive efficient and specific expression of GFP-reporter genes in HL-1 cells (Figure 4), allowing the use of those promoter sequences in our “therapeutic” constructs and confirming that HL-1 cells are a potential model to perform our trans-splicing assays.

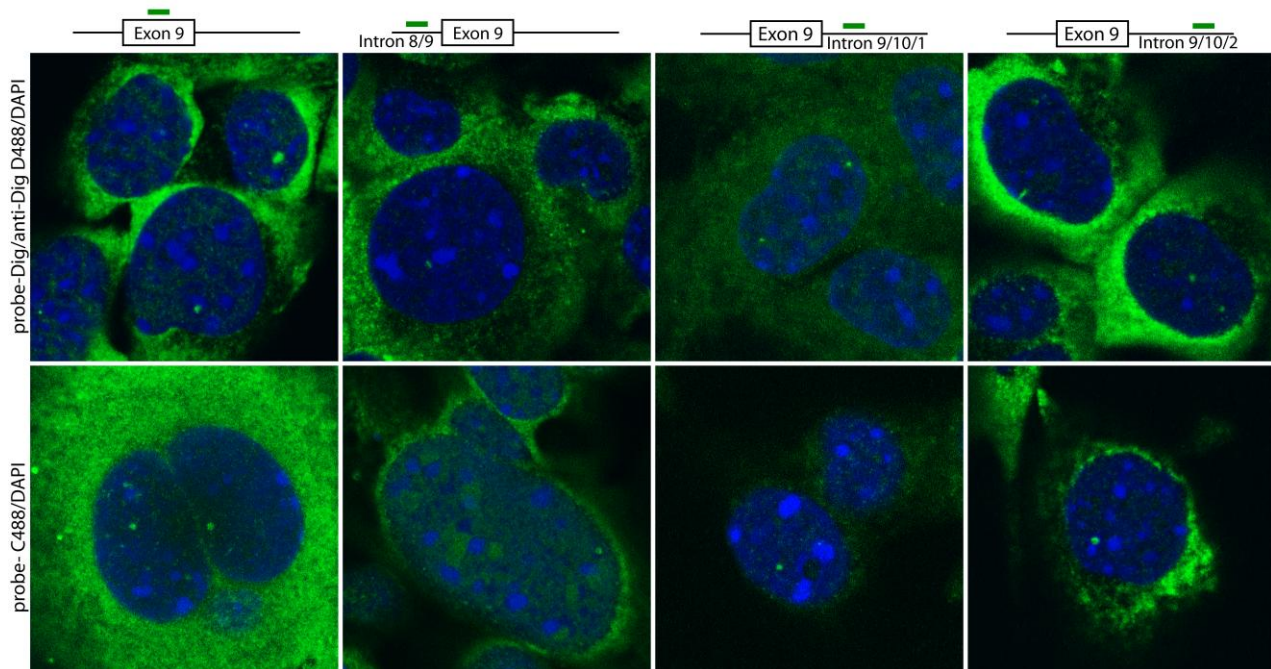


**Figure 4. Cardiac mouse TNNT2 promoters specifically drive GFP expression in HL-1 cells.** RT-PCR analysis of GFP expression in HL-1 (cardiomyocyte cell line) and C2 cells (myoblast cell line), upon transfection with minimal (S-eGFP) and entire (L-eGFP) mouse TNNT2 promoter constructs. As a control, GFP expression under the control of a CMV promoter was monitored. Briefly, cells were transfected with 750ng of each plasmid DNA using Fugene 6 as a transfection reagent. RNA was isolated 24h, 48h, and 72h after transfection and analyzed by RT-PCR using specific primers against GFP. The results were normalized against GFP CMV-dependent expression.



### 3. Intronic regions of HL-1 TNNT2 gene are accessible for FISH

Preliminary FISH data in HL-1 cells show that both intronic regions flanking mTNNT2 exon 9 are accessible for annealing with trans-splicing molecules (Figure 5).



**Figure 5. Intronic regions flanking mTNNT2 Exon 9 are accessible for annealing with trans-splicing molecules.** HL-1 cells were fixed and analysed by fluorescent in situ hybridization (FISH) using probes against mTNNT2 exon 9 (exon 9) and its two flanking introns: intron 8/9 (intron 8/9) and intron 9/10 (intron 9/10/1; intron 9/10/2). Notice that two different probes against intron 9/10 were tested. The relative position of each probe is represented. Probes were either labelled directly with Chroma Tide 488 UTP (C488) or with DIG-11-UTP, followed by anti-Dig Dye 488 detection. Nuclei were stained with DAPI (in blue).

All together, these preliminary results suggest that we will be able to construct a “therapeutic” transcript for efficient replacement of mTNNT2 exon 9 in cardiomyocyte HL-1 cells.



## FINANCIAL IMPLEMENTATION IN ACCORDANCE WITH THE BUGDET PLAN SET

- ✧ General reagents and disposable lab ware - 450 €
- ✧ Reagents for molecular biology, including, DNA ligase, restriction enzymes, oligonucleotides for plasmid construction, purification kits - 500 €
- ✧ Media and reagents for mammalian cell culture and cell transfection - 500 €
- ✧ Reagents for RT-PCR, i.e., primers, cDNA kits and Taq polymerase - 450 €

## PRESENTATION

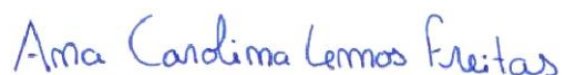
The presentation of the project for display at public forum to be held in October 2012 will be in poster format.

We would like to thank to Amadeu Dias Foundation for the fund provided, which allowed the development of this work.

Prof. Maria Carmo-Fonseca



Ana Carolina Lemos Freitas



## REFERENCES

1. Wang, L., et al, Harnessing Molecular Genetics for the Diagnosis and Management of Hypertrophic Cardiomyopathy, *Ann Intern Med.*, 152(8): 513–W181, 2010
2. Watkins H, Ashrafian H, Redwood C., Inherited cardiomyopathies, *N Engl J Med.* Apr 28;364(17):1643-56, 2011
3. Chao H, et al, Phenotype correction of hemophilia A mice by spliceosome-mediated RNA trans-splicing., *Nat Med.*, 9(8):1015-9, 2003
4. Hammond, S.M., Matthew, J.A.W., Genetic therapies for RNA mis-splicing diseases, *Trends in Genetics*, Vol. 27, No. 5, 2011
5. White SM, Constantin PE, Claycomb WC., Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function, *Am J Physiol Heart Circ Physiol.* Mar;286(3):H823-9, 2004
6. Ma, H., Cell-specific expression of SERCA, the exogenous Ca<sup>2+</sup> transport ATPase, in cardiac myocytes, *Am J Physiol Cell Physio* , 286: C556–C564, 2004
7. Wang, G., Yeh, H., Jung-Ching Lin, J., Characterization of cis-Regulating Elements and trans-Activating Factors of the Rat Cardiac Troponin T Gene, *The Journal of Biological Chemistry*, Vol. 269, No. 48, pp: 30595-30603, 1994
8. Cooper, T.A., Muscle-Specific Splicing of a Heterologous Exon Mediated by a Single Muscle-Specific Splicing Enhancer from the Cardiac Troponin T Gene, *MOLECULAR AND CELLULAR BIOLOGY*, Vol. 18, No. 8 , p. 4519–4525, 1998





# ANEXOS

