DEVELOPING CARDIOMYOCYTE SPECIFIC VECTORS

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Certification of Maria García Gallardo MSc thesis work

The research entitled "Developing cardiomyocyte specific vectors" has been made under my supervision by Ms. María García Gallardo, a graduate student in the Master in Biotechnology of Environment and Health at the University of Ovieco. This thesis work took place in the Molecular Medicine Group at A.I. Virtanen Institute at the University of Eastern Finland (Kuopio).

I hereby state that I have read and corrected the present Master Thesis document and that I find suitable for its public defence by the student before the designated tribunal. Therefore, I authorize the submission of this Master Thesis to the University of Oviedo, MBEH academic commission.

On Friday, 8th July 2022

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ABSTRACT

Cardiovascular diseases (CVDs) are the leading cause of global death and new treatments are needed. Gene therapy stands out as a potential treatment option for many disorders, including CVDs, offering new solutions that are less invasive and longer lasting. Gene therapy for CVDs has had recent advances as many treatments are currently undergoing clinical trials. Delivering therapeutic genes to cardiomyocyte cells via viral vectors can improve the wellbeing of these cells, preventing cardiomyocyte from dying in instances of injury. However, specific targeting of cardiomyocytes is still a challenge. The aim of this study was to develop cardiomyocyte-specific lentivirus (LV) vectors using an enhancer element from a cardiomyocyte-specific superenhancer (SE), in order to restrict the expression of the transgene to cardiomyocyte cells. For the project 6 enhancers (CE) belonging to a cardiomyocyte-specific SE were chosen and 4 were successfully cloned into LV vectors and tested *in vitro* for reporter gene expression. Mouse cardiomyocyte cells and other cell lines were transduced with LV-CE(X), in order to assess their activity and specificity. Flow cytometry analysis of samples revealed high activation but low cardiomyocyte specificity of enhancers CE1, 2 and 3, whereas the activity for CE6 was low in every cell line. Further testing in human cardiomyocytes is necessary in order to determine if this enhancer is cardiomyocyte-specific. Nevertheless, so far no cardiomyocyte-specific enhancers were found in the context of LV vectors and the search is to be continued.

RESUMEN

Las enfermedades cardiovasculares (ECV) son la principal causa de muerte en el mundo y se necesitan nuevos tratamientos. La terapia génica destaca como una posible opción de tratamiento para muchas enfermedades, incluidas las cardiovasculares, ya que ofrece nuevas soluciones que son menos invasivas y tienen mayor duración. El uso de terapia génica en ECV ha experimentado recientes avances ya que muchos tratamientos se encuentran actualmente en ensayos clínicos. La entrega de genes terapéuticos en cardiomiocitos a través de vectores virales puede mejorar el bienestar de estas células, evitando que los cardiomiocitos mueran en caso de lesiones. Sin embargo, acceder solamente a los cardiomiocitos sigue siendo un desafío. El objetivo de este estudio fue desarrollar vectores basados en lentivirus (LV) que fueran específicos para cardiomiocitos utilizando un elemento potenciador obtenido a partir de un superpotenciador específico de cardiomiocitos, para restringir la expresión del transgén solamente en las células de cardiomiocitos. Para el proyecto, se eligieron 6 potenciadores pertenecientes a un superpotenciador específico para los cardiomiocitos y 4 de ellos fueron clonados con éxito en vectores LV y se testaron *in vitro* para determinar la expresión del gen reportero. Cardiomiocitos de ratón y otras líneas celulares se transdujeron con los vectores LV que incorporaron los diferentes potenciadores, para evaluar su actividad y especificidad. El análisis de citometría de flujo de las muestras reveló una alta activación pero una baja especificidad para los cardiomiocitos en los potenciadores 1, 2 y 3, mientras que la actividad del potenciador 6 fue baja en todas las líneas celulares. Es necesario realizar más pruebas en cardiomiocitos humanos para determinar si este potenciador es específico para los cardiomiocitos. Sin embargo, hasta el momento no se han encontrado potenciadores que sean específicos para los cardiomiocitos en el contexto de los vectores LV y, por tanto, la búsqueda debe continuar.

INTRODUCTION

Cardiovascular diseases

According to the World Health Organization, cardiovascular diseases (CVDs) are the leading cause of global death, taking approximately 17.9 million lives each year[1]. In addition, the prevalence of CVDs is increasing[2,3], since it is expected that by 2030 the number of people dying of CVDs will exceed 23.6 million[4]. CVDs are a group of disorders of the heart and blood vessels, including coronary heart disease, cerebrovascular disease, rheumatic heart disease and other conditions related to the heart, coronary circulation and the network of blood vessels[5].

The World Health Organization estimates that heart attacks are causing more than four out of five CVD-related deaths. And one third of these deaths occur prematurly in people under 70 years of age[1]. It is known that pharmacological and interventional therapies for CVD achieve symptom reduction and slow disease progression, however, these treatments are not suitable for everyone, therefore there is still an urgent need for alternative approaches to effectively treat CVDs[6].

Gene therapy

Gene therapy is a technique for the insertion and expression of a therapeutic gene in target cells[5]. Gene therapy stands out as a potential treatment option for CVDs[7] offering new solutions that are less invasive and can last longer. Potential targets for gene therapy include severe cardiac and peripheral ischemia, heart failure, vein graft failure, and some forms of dyslipidemias (Figure 1).

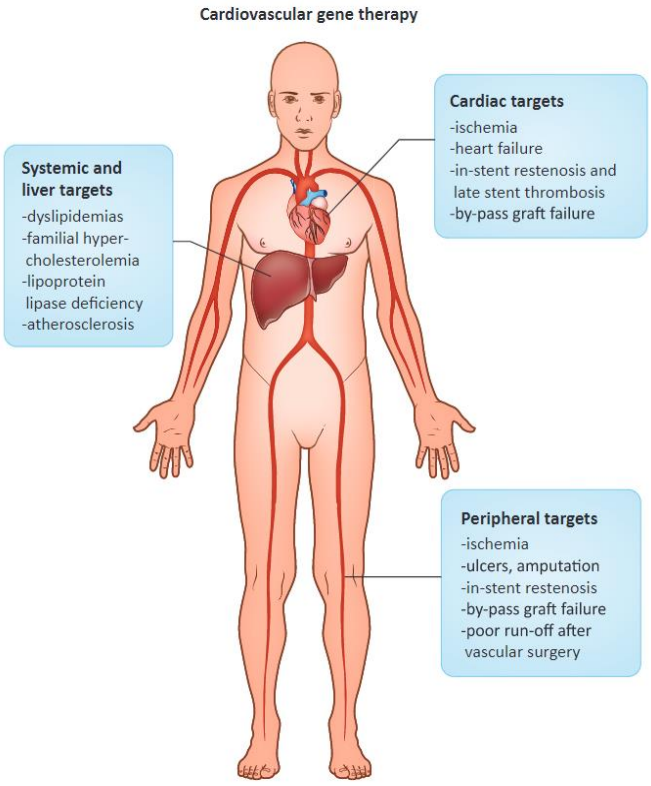


Figure 1. ***Potential targets for cardiovascular gene therapy****. Image obtained from S. Ylä-Herttuala, A. H. Baker. Molecular Therapy (2017).*

Testing of gene therapies for CVDs started in 1980, with a direct

intra-arterial gene transfer using endovascular catheter techniques[5,7]. At the same time, hyperlipidemias, instent restenosis, arrythmias, refractory angina and pirepheral vascular disease were recognized as possible targets for gene therapy[5,7]. However, despite positve preclinical results, clinical studies of CV gene therapies have not yet been very succesful. This is due to multiple factors such as insufficient gene delivery to the target site, short transgene expression duration and poor knowledge about pathophysiological mechanisms consequently leading to unsuitable strategies[7]. In spite of all the difficulties, in recent years some progress has been made in the field of gene therapy, for example the market authorization of Glybera[8–10], the first gene drug approved in the Westerns world, for the treatment of severe lipoprotein lipase deficiency.

Viral vectors

Gene therapy requires the use of vectors to carry the genetic material to the target cells[11]. The currently available methods for gene transfer are divided into two main classes: non-viral physico-chemical methods and viral gene delivery. The first group consists of the delivery of encapsulated genes mediated by chemical approaches[11,12] and the delivery of nucleic acids by the use of physical methods[11,13]. Non-viral approaches entail less safety risks compared with viral vectors and present some other advantages such as the simplicity of use and potential for large-scale production, the low cost of manufacturing and the large capacity of transgene uptake. However, the reason why they are not commonly used for gene therapy is that their transfection efficiency is much lower compared to viral approaches[11]. Therefore, viral vectors are the most popular approach for gene therapy (Figure 2). These viral vectors are composed of genetic material and protein coat and additionally a possible lipid envelope, they enter the target cell by identifying and binding a specific receptor located on the cell surface and once inside the cell they deliver the genetic material into the nucleus[11,14].

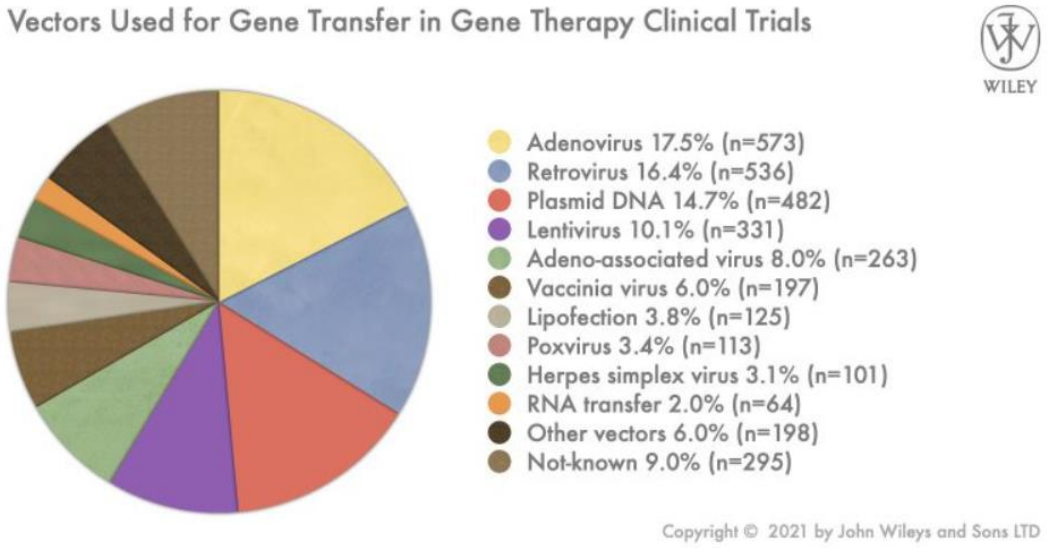


*Figure 2**. Different vectors used in gene therapy clinical trials worldwide between 1989-2018**. Graph from The Journal of Gene Medicine*[15].

The most applied viral vectors are based on adenoviruses (Ad), with a packaging capacity of 7.5 kb of foreign DNA providing short-term episomal expression of the desired gene in a broad range of host cells[15]. In addition, Ad vectors have high efficiency of transduction, high levels of expression and the possibility of transducing non-dividing cells. However, the main drawback is its transient transgene expression and Ad-associated immunity.

Other popular vectors in gene therapy are adeno-associated virus (AAV) vectors and lentivirus (LV) vectors. AAV vectors present many advantages because they are able to transduce a wide range of host cells[15], are safe to use since wild-type AAVs have not been demonstrated to cause any diseases in humans and AAV vectors are non-integrating. Despite their lack of integration, AAV are able to maintain long enough transgene expression to result in therapeutic effects. However, AAV vectors present some disadvantages as well. First of all, the packaging capacity is smaller than the other viral vectors presented above, around 4-5 kb. Second, the transduction of AVV vectors to specific types of cells is limited. And third, the prevalence of neutralizing antibodies in the human population increases the risks of immune reaction, which decreases the efficiency of these vectors[16].

Lentiviral (LV) vectors

LV are enveloped, spherical to pleomorphic in shape viruses that belong to the *Retroviridae* family. They are 80-100 nm in diameter with a single-stranded RNA genome of 9.75 kb, and they contain the reverse transcriptase enzyme that converts the RNA genome into DNA so that it can integrate into the host genome[17].

LV lifecycle starts with the binding of the LV to its target cell via interactions between its viral envelope glycoprotein and a specific cell surface receptor. Upon receptor recognition, viral transmembrane proteins change their conformation to facilitate membrane fusion of the virus with the host cell, resulting in viral entry. Once inside the cell, the viral genome and the viral proteins are released into the cytoplasm. Viral RNA is used as a template by the reverse transcriptase to synthesize viral DNA, which is transported into the nucleous and integrated into the host genome by the action of the viral integrase. Using the cellular machinery, the viral genome is transcribed into mRNA, which encodes for the viral proteins and is also the source of the viral vector genome. In the final stage, the exported viral genome and proteins are assembled at the plasma membrane and released from the host cell[18].

Vectors based on LV can carry transgenes of 12-15kb and have the ability to transduce dividing and non-dividing cells with high efficiency[16], allowing them to infect populations such as cardiomyocytes or stem cells[17]. LV vectors do not induce an immune response following infection, and due to their integration capability can provide a fast and long-term expression of the transgene[15]. In addition, LV vectors are compatible with multiple transcriptional promoters, including those of housekeeping genes and promoters for specific cells or tissues[16].

However, despite all the above-mentioned advantages, LV vectors present some drawbacks. The main issue is the uncertainty regarding safety since these vectors integrate into the host genome[17], therefore it can lead to non-specific and adverse effects, such as genotoxicity. In addition, insertional mutation can occur, leading to the activation of protooncogenes or inhibition of anti-oncogenes, which increases the risk of cancer[16]. Some of these issues have been addressed by ongoing work to target integration into safe harbours in the genome[19].

To sum up, LV vector stands out as a promising vector because of their ability to infect both dividing and non-dividing cells with high efficiency[20] and according to the

World Health Organization, the use of LV vectors in gene therapy clinical trials has increased significantly in recent years[21]. Moreover, LV vectors production takes around 2 weeks and they induce transgene expression more rapidly than other viral vectors like AAV vectors, and have shorter production time than Adenovectors, which can take approximatly 6 months.

Many attempts for the development of viral vectors for gene therapy have been made, however, there is still a lack of success in clinical trials[7]. To solve this issue, researchers have being trying to develop cell type specific viral vectors, to ensure that the gene is only expressed in the desired cell type, hence reducing unwanted side effects caused by the ectopic expression of transgenes in off-target cells[22]. In order to do that, tissue-specific promoters have been used to drive the expression of therapeutic genes in viral vectors constructs[23–26]. Cell type specific promoters are available for almost all tissues, a fact that makes this approach a promising one to achieve cell type specific viral vectors, however, an important drawback is often the low gene expression levels achieved by these promoters[15]. Alternatively, transductional retargeting strategies have been explored[27], which include the genetic incorporation of heterologous binding ligands to redirect vector tropism; or adapter conjugate strategies, based on the addition of an adapter molecule to crosslink the vector to a cellular target receptor. However, these modifications can compromise the levels of the transgene expression[28] and the viral vector production resulting in low titers[29]. Therefore, different approaches to achieve cell-specific targeting are needed. Enhancers, as gene regulatory elements, possess cell type specific activities and, hence, stand out as promising candidates in the development of cell type specific vectors in order to regulate transgene expression in target cells.

Enhancers and superenhancers

Enhancers are segments of the genome defined by the ability to activate gene expression over large genomic distances, independent of sequence orientation[30], by forming chromatin loops that bring the enhancer and the target gene into proximity. In 1981 the first enhancer was identified. It was derived from a 72bp repeat found in the simian virus 40 (SV40) genome and it increased the expression of β-globin gene in HeLa cells[31]. Shortly after this first discovery, similar transcriptional enhancers were found in

metazoan genomes, where they were implicated in cell-type specific stimulation of gene expression[32,33], since then the importance of enhancers in transcriptional regulation has been recognised universally in prokaryotes and eukaryotes. Studies have revealed that enhancers bind transcription factors (TFs), such as Oct4, Sox2, and Nanog[34], and the Mediator coactivation complex; and recruit RNA polymerase II[35,36], as shown in Figure 3. A loop in the chromatin serves to bring closer the enhancer to the promoter of the target gene, resulting in activation of the gene expression[37]. Moreover, the binding of TFs to the enhancer generates the recruitment of histone modifying enzymes[37], which causes decompaction of the chromatin fibre, increasing the accessibility of the DNA[38]. Between 400,000 and 1.4 million putative enhancers have been identified in mammalial genome thanks to several high-throughput techniques that are able to detect features of enhancers, such as specific histone modifications[34]and enhancer RNA. A typical mammalian cell contains thousands of active enhancers at any one time[39].

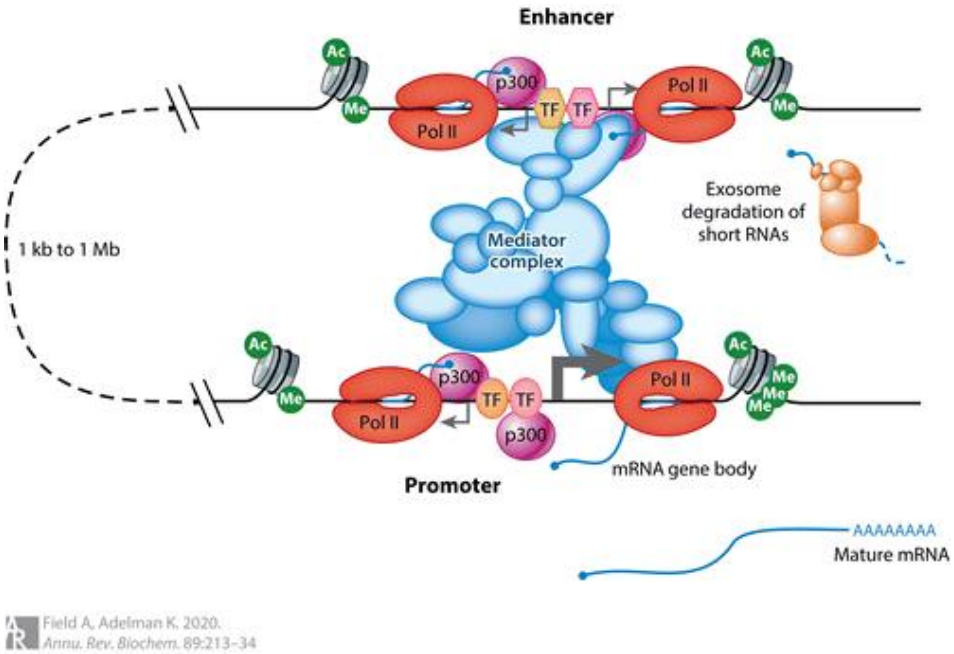


Figure 3. ***Features of an active enhancer.*** *Enhancers are regions of the genome capable of binding TFs, coactivator factors and RNA polymerase II. A loop in the chromatin brings the enhancer and the promoter of the target gene closer, resulting in the activation of the gene expression. Image obtained from Field A. Adelman K. 2020. Annu. Rev. Biochem. 89; 213-34.*

Advances in molecular techniques have allowed the discovery and study of clusters of transcriptional enhancers, named superenhancers (SEs), which are believed to play key roles in the control of cell identity.

In 2013, Hnisz et al, generated a catalogue of SEs and their associated genes for 86 different human cells and tissues types[39], which provide a useful resource for further

transcriptional control of cell identity and reprogramming studies. The discoveries regarding DNA sequence variation associated with specific diseases being enriched in the SEs of cells related to those diseases[39]were very important. Moreover, cancer cells were found to acquire SEs at key oncogenes and other genes that play important roles in tumor pathogenesis[40], implicating them in the disease.

In recent years, several studies have been performed to better understand the role of SE in gene expression. Interestingly, it was found that an element of a SE, such as one enhancer from the cluster, can induce higher expression levels of the target gene compared with a typical enhancer[34,41]. This is due to the enrichment of the amount of master TFs in the SE area, increasing the binding of these factors to the DNA sequences[34]. Focusing on cardiomyocyte specific SEs, some studies provided a better understanding of the activity of these clusters of enhancers. J. C. K. Man et al. identified a cluster with SE characteristics downstream of SCN5A, which was involved in the chromatin architecture of the locus and Scn5 expression, a gene that encodes for the major cardiac sodium channel; suggesting that genetic variants or deletions, affecting its activity, can influence cardiac function[42]. In parallel, S. Ounzain et al. were studying another cardiac SE named CARMEN. They demonstrated that its knockdown inhibits cardiac specification and differentiation in cardiac precursor cells, showing the crucial role the SE has in the regulation of cardiac cell differentiation and homeostasis[43].

It is clear that the research to identify cell-specific SEs is very important for the improvement of gene therapy. Recently, a study performed in our research group showed functional and promising SEs for endothelial cell targeting[44]. In particular, one single enhancer from the cluster was observed to regulate many genes implicated in the cell cycle, proliferation and angiogenesis in endothelial cells. Moreover, this enhancer was cloned into LV vector, achieving the regulation of endothelial-specific expression of a reporter gene. Hence, a similar strategy to develop cardiomyocyte specific viral vectors may be feasible.

To achieve cardiomyocyte specific vectors using enhancers, a cardiomyocyte active SE needs to be selected. In addition to the cardiac-relevant SEs mentioned above, another SE regulating important cardiac genes, was characterised in mice[45]. This SE activates NPPA and NPPB genes, which encode for atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) respectively. These gene products are specific to

cardiac tissue, as demonstrated in the human RNA expression data derived from the GTEx project[46] (Figure 4 A and B). Hence, an equivalent SE in the human genome would be a putative candidate for the development of cardiomyocyte specific vectors.

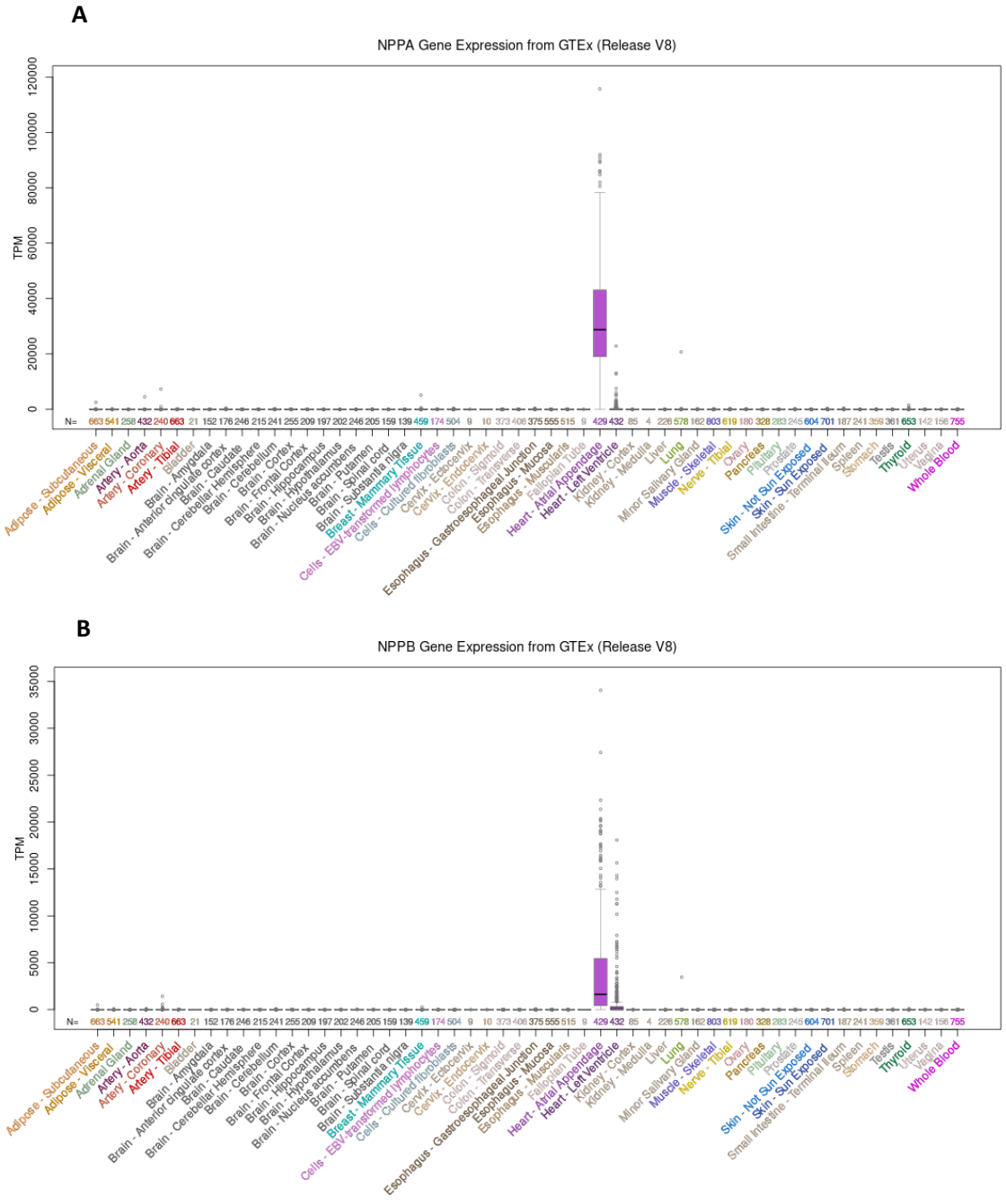


Figure 4. ***RNA expression data for NPPA (A) and NPPB (B) genes****. The RNA expression data shows all the tissues and confirms that expression of NPPA and NPPB genes are specific for the heart. The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. The data used for the analyses described in this manuscript were obtained from the GTEx Portal on 01/02/2022.*

OBJECTIVES

Gene therapy targeting cardiac diseases would benefit from cardiomyocyte specific vectors. Hence, our aim is to develop cardiomyocyte-specific viral vectors. To do this we will use enhancers from cardiac lineage-associated super-enhancers to develop viral vectors that specifically express their transgene cargo in cardiomyocytes. The enhancer elements will be delivered from a SE that was previously identified to activate cardiac specific NPPA and NPPB genes in mice.

We have identified an equivalent SE in the human genome and we aim to test the enhancers within this SE in the context of LV vectors. To do this the following specific aims will be addressed:

1) To clone cardiomyocyte-specific enhancers in LV vectors.
2) To determine the activation of these newly developed vectors in cardiomyocyte cells.
3) To investigate the activity of these vectors in other cell types in order to assess their specificity.

MATERIALS AND METHODS

From the data set of SEs identified in cardiac tissue by Hnisz et al 2013, a putative cardiomyocyte specific SE was selected for further study. This SE is located downstream of the NPPA and NPPB genes in chromosome 1. From within this SE, 6 enhancers were identified, and these regions (SEnppb_CE1 to CE6) were then used for cloning and testing in LV vectors.

PCR

To clone the putative cardiomyocyte enhancer regions, these regions were amplified by PCR from human genomic DNA. To do this, genomic DNA (gDNA) was extracted from HUVEC cells using the kit NucleoSpin Tissue Genomic DNA from tissue (Macherey-Nagel), and its concentation measured using Nanodrop 1000 (Thermo Fisher). Each PCR was performed using Doppio thermal cycler (VWR) in a total of 20µL reaction volume (20 ng gDNA, 5x Phusion HF green Reaction Buffer, 10mM dNTPs, 0.5µM forward primer, 0.5µM reverse primer and 0.02 U/µL Phusion HF DNA Polymerase). All PCR reagents used were from Thermo Fisher. As detailed in Table 1, six pairs of primers were used in the PCR reaction, one pair per enhancer. NCBI primer blast tool was used for primers' selection.

Primer pair	Complementary	Sequence (5'-> 3')	Product length	Tm (°C)	GC%
SEnppb_CE1	Forward primer	AGGCCAGAGGAGGAGACAAA	776	60.18	55.00
	Reverse primer	GAATGTGACCCCAGGAAGCA		59.96	55.00
SEnppb_CE2	Forward primer	CTGCTTGTCGGCTTTGGTCTC	909	61.80	57.14
	Reverse primer	GGGGAAGTCCTTACAGAGCCG		62.18	61.90
SEnppb_CE3	Forward primer	AAACCTGGGGGTTCAGGCTA	1192	61.80	57.14
	Reverse primer	CATCAGCTTTGCTTGGCTGG		62.18	61.90
SEnppb_CE4	Forward primer	CTTGTTGCAGTAAGCAGGTGTC	1053	60.03	50.00
	Reverse primer	GCCCAGGCTTGATGAACGAT		60.75	55.00
SEnppb_CE5	Forward primer	TGCCCCAGCCATCTTTACTG	1297	60.03	55.00
	Reverse primer	TTTGGCTCTGTCCTCTGCTG		59.96	55.00
SEnppb_CE6	Forward primer	GGATTCAGACTCCTCTGTGCTG	1087	60.42	54.55
	Reverse primer	AGGCCTCCTTCTTCTACAGAGTGC		63.72	54.17

*Table 1. Summary of **primers' information** for each enhancer used in the PCR reaction.*

The cycle conditions used are detailed in Tables 2 and 3.

Cycle conditions for the PCR reaction
98°C for 30 s
30 cycles: 98°C for 10s Temperature gradient: 52, 54, 56, 58, 60, 62, 64, 66°C for 30s 72°C for 1 min 30s
Final extension 72°C for 5 min
Infinite 8°C

Table 2. ***Cycle conditions used in the PCR reaction*** *for amplifying the putative enhancers regions from gDNA.*

Cycle conditions for the PCR reaction with DMSO
98°C for 30 s
5 cycles: 98°C for 10s Temperature gradient: 52, 54, 56, 58, 60, 62, 64, 66°C for 30s 72°C for 1 min 30s
25 cycles: 98°C for 10s 60°C for 30s 72°C for 1 min 30s
Final extension 72°C for 5 min
Infinite 8°C

Table 3. ***Cycle conditions for PCR reaction*** *performed with 10% DMSO for enhancers number 4 and 5.*

PCR products were electrophoresed for 1 hour at 140 volts in a 1% agarose gel with Midori Green Dye (0.005%) alongside with 1kb Plus DNA Ladder (Thermo Fisher).

Molecular cloning

The molecular cloning process involved different steps including ligation, transformation, plasmid purification and digestion with restriction enzymes.

Ligation

DNA inserts obtained from the PCR reaction were extracted from the agarose gel using NucleoSpin Gel and PCR Clean-up kit (Machery-Nagel) and ligated into the pMiniT

2.0 plasmid (Figure 5) following the Ligation Protocol for NEB PCR Cloning Kit according to the manufacturer's instructions (NEB PCR cloning kit).

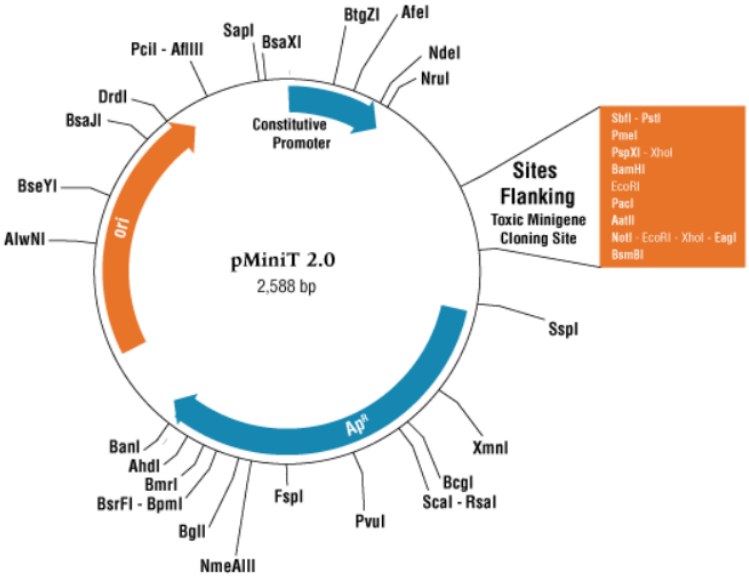


Figure 5. ***pMiniT 2.0 Vector Map*** *provided in NEB PCR Cloning Kit demonstrating the cloning site of PCR amplicons.*

DNA fragments were excised out of pMiniT 2.0 plasmid with a XhoI digestion and then ligated into previously constructed pLV-miniP-GFP[44] (100ng/µL; Figure 6). The plasmid was mixed with the corresponding volume of DNA insert, 5x Buffer, T4 ligase and up to 10µL of water. The mix was incubated at room temperature for 1 hour and transformed into *E. coli.*

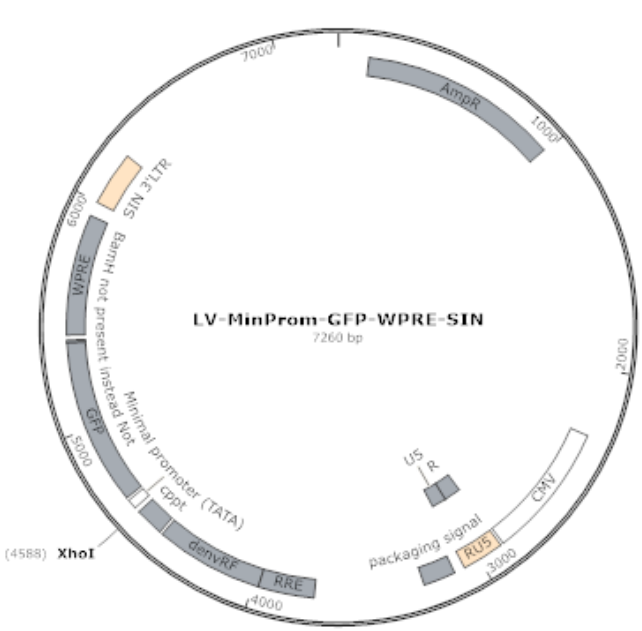


Figure 6. ***Plasmid map of pLV-miniP-GFP.*** *The plasmid is 7260bp and consists of green fluorescent protein (GFP), Posttranscriptional Regulatory Element (WPRE), Minimal promoter (minP), Ampicillin resistance (AmpR).*

Transformation

5 µL of ligation product was added to competent *E. coli* DH5α (NEB, C2987) bacteria which was kept in ice. The bacteria was transformed by heat shock by its immersion in a water bath at 42°C for 30 seconds thereafter its incubation in ice for 2 minutes. After the addition of 200µL of SOC media (New England BioLabs), the bacterial solution was shaken at 180 rpm at 37°C for 1 hour. 200µL of the bacterial solution were plated into an agar plate with Ampicillin and incubated at 37°C overnight.

Plasmid purification

Selected colonies from the transformation were grown in 3mL of LB+Ampicillin (0.1% from initial stock of 50mg/mL) medium, shaking at 230 rpm and at 37°C overnight. Plasmid purification was performed following the QIAprep Spin Miniprep Kit Protocol (QIAGEN Mini) for microcentrifuge. Final DNA concentration was measured using NanoDrop 1000 (Thermo Fisher).

For transfection and preparation of the virus like particles (VLP) a high concentration of the LV plasmid is needed. For that, one colony from the bacterial transformation was picked and grown in 1mL of LB medium with ampicillin by shaking at 230 rpm for 6 hours at 37°C. After the 6 hours, 200-500µL of the bacterial solution were added to 200mL of LB medium with ampicillin and incubated overnight at 37°C with shaking at 230 rpm. Plasmids were purified using the EndoFree Plasmid Maxi Kit (QIAGEN Maxi).

Digestion with restriction enzymes

Checking for plasmids with the desired insert was done by digestion with EcoRI for pMiniT 2.0 plasmid and with XhoI for the LV plasmid. Moreover, other restriction enzymes were used in digestion to verify the plasmids, as specified in the results. Digestion mixes were composed by the enzyme, 10x Fast Digest Green buffer, 0.5-1µg of plasmid and up to 20µL of water. The digestion mix was incubated at 37°C for 15 minutes and electrophoresed as stated previously.

Sequencing

To confirm the expected sequence of the PCR amplified plasmid inserts, plasmids demonstrating the correct size insert were sent for Sanger sequencing. 500ng of pMiniT 2.0 plasmid carrying the corresponding insert were mixed with 5µL of forward primer (5µM) (for bigger enhancers like CE3, CE4, CE5 and CE6 another tube was prepared with

the plasmid and the reverse primer (5µM)) and sent to sequencing (Macrogen Europe, The Netherlands). Primers sequences are indicated in Table 4.

Forward primer	ACCTGCCAACCAAAGCGAGAAC
Reverse primer	TCAGGGTTATTGTCTCATGAGCG

Table 4. ***Primers' sequences*** *used for the sequencing of purified pMiniT 2.0 plasmids.*

Cell culture

Six different cell lines were used for this study, HEK 293T (human embryonic kidney; ATCC CRL-11268™), TeloHAEC (aortic endothelial cell line; ATCC CRL-4052™), HeLa (uterus, cervix epithelial cell line; ATCC CRM-CCL-2™), MOVAS (*Mus musculus*, mouse; ATCC CRL-2797™), A549 (lung epithelial cell line; ATCC CRM-CCL-185) and HL-1 mouse cardiomyocites (Merck SCC065). All cell lines were cultured according to the manufacturer's instructions.

TeloHAEC cells were cultured in Vascular Cell Basal Medium (ATCC, PCS-100-030) supplemented with Vascular Endothelial Cell Growth Kit-VEGF (ATCC, PCS-100-041) and 100 mg/ml Penicillin, 100 U/ml Streptomycin (PS).

HEK 293T, HeLa, MOVAS and A549 were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) and PS. In addition, for MOVAS cells, the medium was also supplemented with 0.2 mg/mL geneticin selective antibiotic (Thermo Fisher Scientific). HL-1 cardiomyocytes (Merck) were cultured in Myocyte Growth Medium (PromoCell) in T75 flasks previously coated with 0.02% gelatin / 0.5% fibronectin.

All cells were incubated at 37°C and 5% CO_2 in a humidified incubator. For passaging, cells were washed with Dulbecco's Phosphate-Buffered Saline (D-PBS, Thermo Fisher Scientific), followed by incubation with 0.25% (v/v) trypsin (Sigma) at 37°C for 3-5 minutes to detach the cells from the bottom of the plate. After the incubation, the trypsin was neutralized with cell type matched complete media. Similar detachment protocol was followed for HL-1 with the exception of washing with prewarmed D-PBS followed by a wash with trypsin. The cells were centrifuged at 500 x g for 5 minutes afterwhich they were resuspended in Myocyte Growth Medium (PromoCell). Cells were counted using CountessTM Automated Cell Counter (Invitrogen) according to the manufacturer's instructions (Invitrogen CountessTM).

Transfection of 293T cells

Human embryonic kidney 293T cells were seeded in 6-well plates the day before the plasmid transfection, at a density of 1-1.2x10^6 cells/well, to ensure a high confluence (70-90%). Transfection was done using Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific) and following the manufacturer's instructions. Briefly, a total of 2.5µg of DNA/well was diluted in OptiMEM and 10µL of P3000 reagent were added. A mastermix of 6% (v/v) L3000 reagent and OptiMEM was added to the DNA dilution and incubated at room temperature for 15 minutes. In the meantime, 1mL of media was removed from every well, and after the 15 minutes the transfection mixture for each plasmid was added dropwise to the respective wells. Cells were incubated at 37°C for 4 hours, after which 2mL of fresh media were carefuly added, followed by a 3 days incubation period at 37°C.

Viral transduction

Cells were seeded, the day before transduction, at 7.5x 10^4 cells/well for TeloHAEC, A549, MOVAS and HeLa and at 5.0x 10^4 cells/well for 293T HEK and HL-1 cardiomyocytes in 12-well plates, and allowed to reach 20-40% confluency before transduction.

For LV vectors, virus like particles (VLP) encapsulating the putative cardiomyocyte specific vector were prepared by Nihay Laham Karam as described previously[47]. Titration of the LV was determined by using reverse-transcription and quantitative PCR (RT-qPCR).

On the day of transduction, the LV vector media was diluted with culture media, the media was removed from the wells and 0.5mL of diluted viral media was added per well. Cells were incubated at 37°C for 3-4 hours. After that time the transduction media was replaced with 1mL of fresh media. Cells were harvested 72 hours after transduction and they were analysed using flow cytometry.

All transduction experiments were performed 3 times independently for each cell line using duplicate wells for each viral preparation in any individual experiment.

Flow cytometry

For transfection, cells were harvested by removing the media and resuspending the cells in 1 mL of PBS. Cells were centrifuged at 800 x g for 5 minutes at room

temperature. The PBS was discarded and the pellet was resuspended in 200µL 1% formaldehyde in PBS and stored at 4°C until flow cytometric analysis.

For transduction, cells were harvested by trypsinization (as detailed in the cell culture section) and centrifuged at 800 x g for 5 minutes for TeloHAEC, HEK 293T, A549, HeLa and MOVAS; and at 500 x g for 5 minutes for HL-1. Cell pellets were then resuspended in 1% formaldehyde (in 1X PBS) and stored at 4°C until analysed by flow cytometry.

GFP expression was quantitated using CytoFLEX S flow cytometer (Beckman Coulter) with a 488nm laser and 525/40 BP fluorescent channel (FITC/GFP channel) and analysed using the CytExpert software (v.2.4). For transfection, non-transfected cells were used as negative control and cells transfected with pLV-minP-GFP were used as positive control. For transduction, non-transduced cells and cells transduced with empty VLP were used as negative control and cells transduced with VLP containing the pLV-minP-GFP were used as experimental control. The plasmids contained the green fluorescent protein (GFP) as a reporter gene.

RESULTS

To address our overall aim of developing cardiomyocyte-specific viral vectors, the project was divided according to three specific objectives, therefore the results are divided in three parts, according to each objective.

Cloning cardiomyocyte-specific enhancers in LV vectors

To clone the putative cardiomyocyte enhancer regions, these regions were amplified by PCR from human genomic DNA. Genomic DNA was extracted from HUVEC cells and subjected to PCR with a temperature gradient (Figure 7) in order to determine the best temperature for each pair of primers (Table 5).

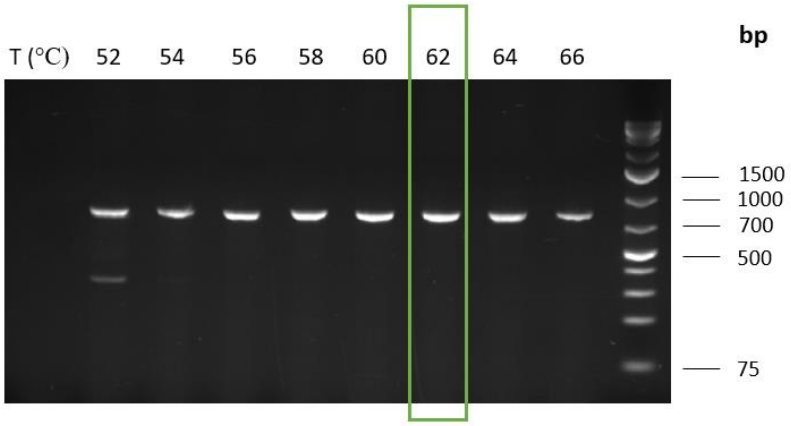


Figure 7. ***PCR reaction performed with a temperature gradient for enhancer 1****. Bands correspond to the PCR products, and the optimal temperature is determined to obtain single and bright bands. PCR of enhancers 1, 2, 3 and 6 gave clear band profiles with almost no secondary bands at any temperature tested.*

Enhancer number	Optimal temperature for the selected primer pair (°C)
CE1	62
CE2	66
CE3	64
CE4	-
CE5	52
CE6	62

Table 5. ***Temperature selection for each pair of primers.*** *Temperature was chosen according to the brightness of the bands and the absence of secondary bands.*

For enhancers 4 and 5 it was not possible to obtain specific bands after the PCR, therefore reactions were repeated with the temperature gradient and 10% DMSO included in the PCR mix (Figure 8).

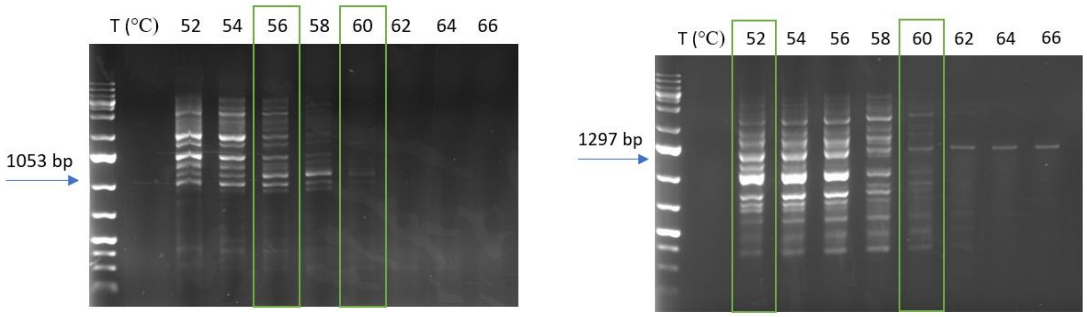


Figure 8. ***PCR reaction with temperature gradient for enhancers 4 (left) and 5 (right).*** *PCR performed with 10% DMSO. Many non-specific PCR products are visible at the lower temperature as indicated by the many bands.*

Gel extracted PCR products were ligated into pMiniT 2.0 plasmid and EcoRI verified the presence of insert in all the cases (Figure 9). However, in some of these a different amplicon size was observed from the expected fragment (Table 1), only correct size plasmids were continued with for sequence verification by Sanger sequencing.

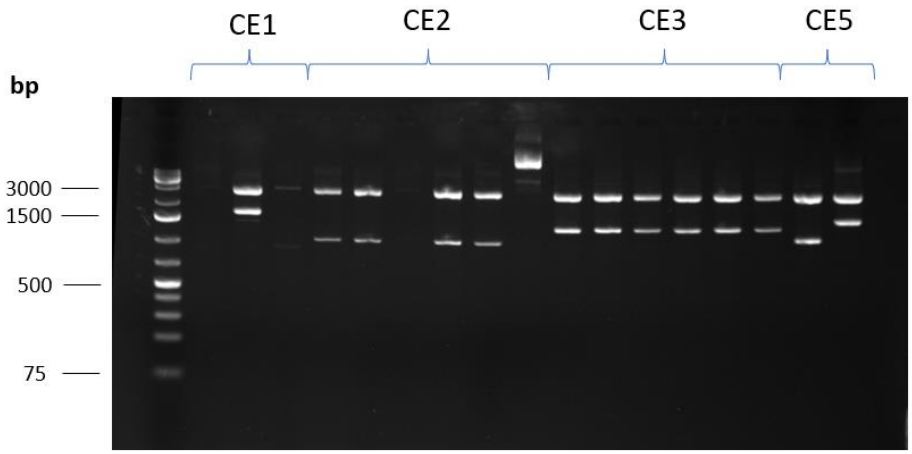


Figure 9. ***Example of plasmids' screening with EcoRI.*** *EcoRI was used to verify the presence of insert. There were two restriction sites for EcoRI in the plasmid, giving two bands as a result of the digestion, the backbone plasmid and the insert.*

Consistent with the difficulties in PCR amplification for enhancers 4 and 5, although many colonies were screened, no positive colonies were derived (Table 6). Sequencing verified that plasmids containing enhancers 1, 2, 3 and 6 were positive for the desired insert, whereas plasmids for 4 and 5 were negative for enhancer, having incorporated a fragment of chromosome 4 and 6 respectively.

Therefore, work discontinued for these enhancers and enhancers 1-3 and 6 proceeded to cloning into LV plasmid.

Enhancer	Screened plasmids	Plasmids positive for desired insert
1	12	3
2	6	5
3	6	6
4	32	0
5	26	0
6	6	3

Table 6. ***Screening for positive plasmids****. The table shows the number of screened plasmids and the number of plasmids positive for the desired insert found among the total for each enhancer.*

Enhancers number 1, 2, 3 and 6 were cloned into pLV-minP-GFP and purified plasmids were screened with XhoI (Figure 10) to verify the incorporation of the insert (Table 7). Many plasmids were screened but only a few were positive for the insert, this may be due to the fact that LV plasmid tends to cause rearrangements.

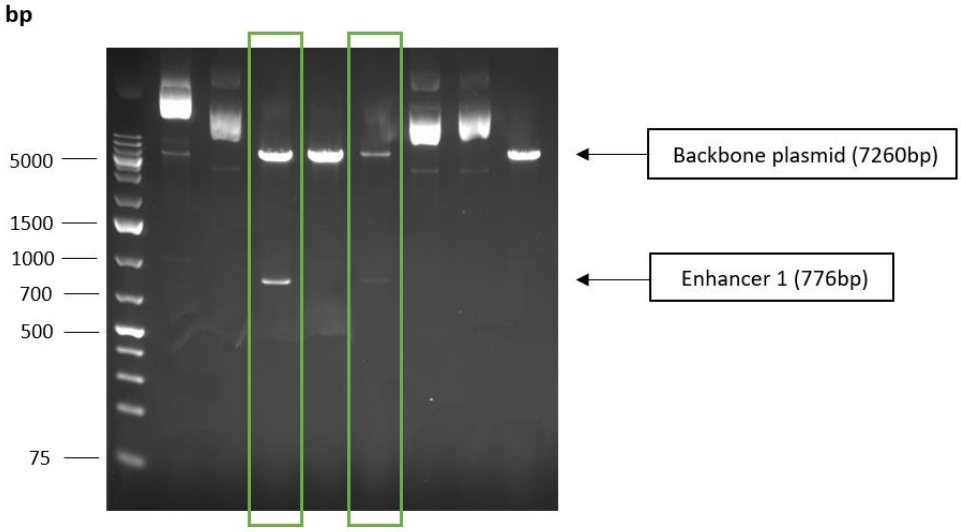


Figure 10. ***Screening of CE1 plasmids through digestion with XhoI.*** *The digestion gives two bands: one with the size of the backbone plasmid (7260bp) and other with the size of the corresponding enhancer (776bp in the case of enhancer 1). Positive plasmids are highlighted in green.*

Enhancer	Screened plasmids	Positive plasmids	Plasmid with enhancer in forward orientation	Plasmid with enhancer in reverse orientation

1	50	2	2	0
2	42	1	1	0
3	34	2	2	0
5	12	0	0	0
6	42	8	6	2

Table 7. ***Screening for positive LV plasmid****. The table shows the number of plasmids that were screened for each enhancer, the number of positive plasmids among the total number and how many of these were in the forward or reverse orientation.*

A panel of restriction enzymes was used to examine the successful ligation of the inserts within the viral vectors for all plasmids, the results of which are shown for pLV-CE2-minP-GFP and pLV-CE3-minP-GFP in Figure 11. The correct fragment sizes were obtained as compared to the DNA ladder and the expected bands generated by SnapGene software (from Insightful Science; available at snapgene.com).

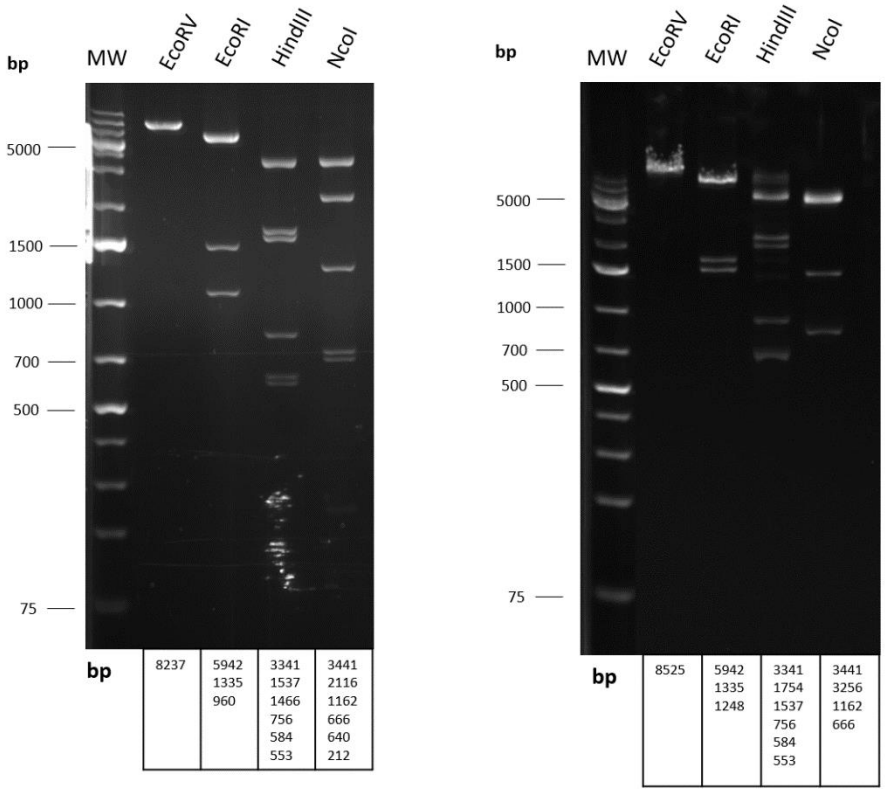


Figure 11. ***DNA gel images for pLV-CE2-minP-GFP (left) and pLV-CE3-minP-GFP (right) digested with restriction enzymes (EcoRV, EcoRI, HindIII and NcoI)****. Expected DNA fragment sizes are indicated below the gel image.*

Restriction enzyme NcoI was used for assessing the orientation of the enhancer in the LV plasmids (Figure 12).

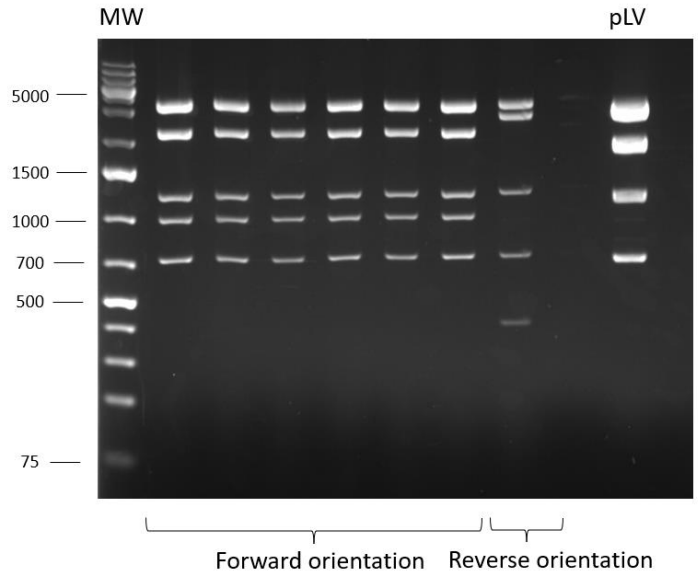


Figure 12. ***Digestion of CE6 plasmids with NcoI****. Restriction enzyme NcoI was used to assess the orientation of the enhancer within the LV plasmid. A clear difference can be seen in the band profile for enhancers in forward or reverse orientation.*

To check the integrity of the GFP open reading frame of the plasmid constructs, 293T cells were transfected with pLV-CE1-minP-GFP, pLV-CE2-minP-GFP, pLV-CE3-minP-GFP, pLV-CE6for-minP-GFP, pLV-CE6rev-minP-GFP. As a control, cells were also transfected with the parent plasmid pLV-minP-GFP. All the transfected plasmids showed GFP-positive cells from within the live cell population as detected by flow cytometry 72h after transfection. In addition, the average level of GFP expression across those cells was also measured (Figure 13).

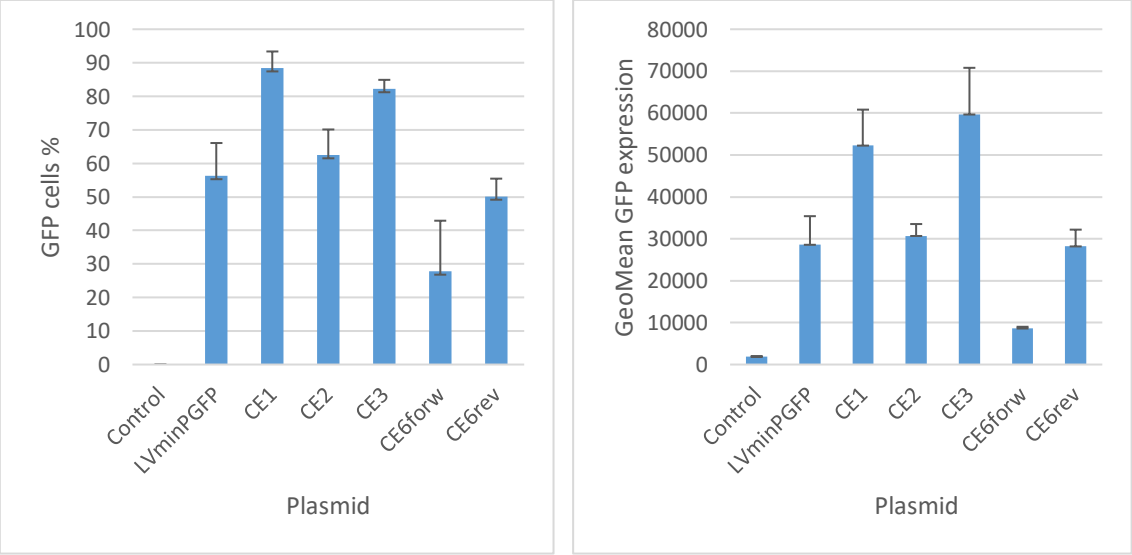


Figure 13. ***GFP is expressed from newly cloned viral vector plasmids.*** *GFP expression in 293T cells is analyzed by flow cytometry at day 3 post-transfection.* ***Left****. The percentage of GFP-positive cells within the viable cell population in transfected cells.* ***Right****. The geometric mean of GFP expression in the same samples. The plasmid LV-minP-GFP served as control.*

The level of GFP expression varied between the constructs likely due to differences in transfection efficiency between plasmids. Overall, this data showed that our constructs were functional *in vitro*, capable of expressing GFP in 293T cells.

Assessment of the activation of cardiomyocyte specific vectors in cardiomyocyte cells.

To verify that mouse cardiomyocyte cells can be transduced by LV vectors, HL-1 cells were transduced with LV-GFP vector, a control vector at different multiplicities of infection (MOI). HL-1 were highly transduced with LV-GFP even at MOI of 10, close to all the cells were GFP positive (Figure 14). These results suggest that mouse cardiomyocytes can be transduced with LV vectors.

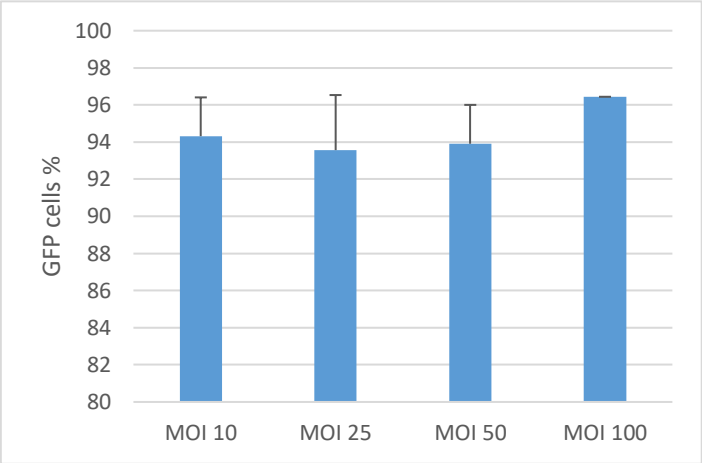


Figure 14. ***GFP expression in LV-transduced cardiomyocyte cells at different MOI.*** *Quantitation of flow cytometry results showing the % of GFP expressing cells at different MOI (10, 25, 50 and 100). Data is presented as Mean±SEM, n=3.*

To evaluate the activity of the enhancers, LV vectors were transduced into HL-1 mouse cardiomyocytes.

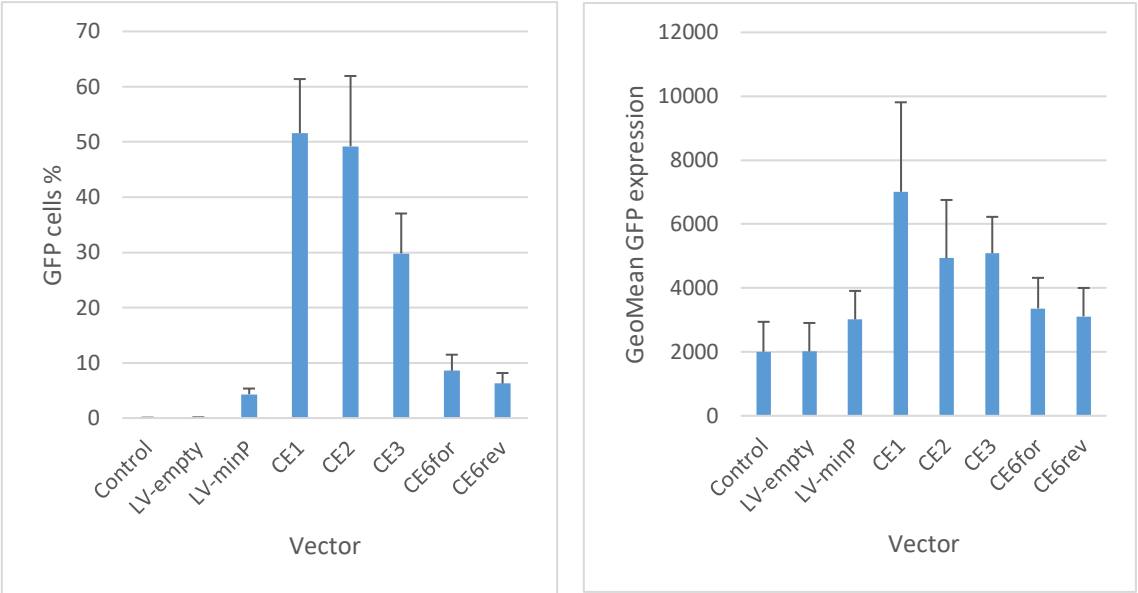


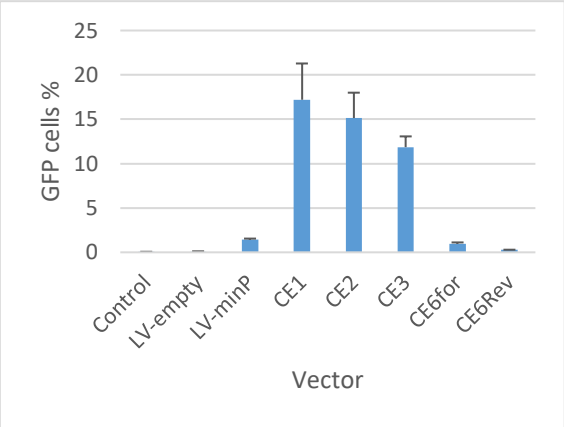
Figure 15. ***GFP expression in LV-transduced cardiomyocyte cells.*** *Quantitation of flow cytometry results showing the % of GFP expressing cells for each vector (left graph) and the corresponding geometric mean GFP expression (right graph). Data is presented as Mean±SEM, n=3.*

Interestingly, enhancers number 1, 2 and 3 resulted in a high percentage of GFP positive cells, unlike cells transduced with enhancer number 6, which shows the lowest activity (Figure 15, left graph). Moreover, average levels of GFP expression in live cells (Figure 15, right graph) also demonstrated variable activation of GFP by the enhancers, following the same trend as for percentage of GFP positive cells. These data show that enhancers number 1, 2 and 3 have high activity in cardiomyocyte cells, while enhancer number 6 shows very little activity in these cells since the percentage of cells expressing GFP is at the same level as the cells transduced with the LV-minP-GFP.

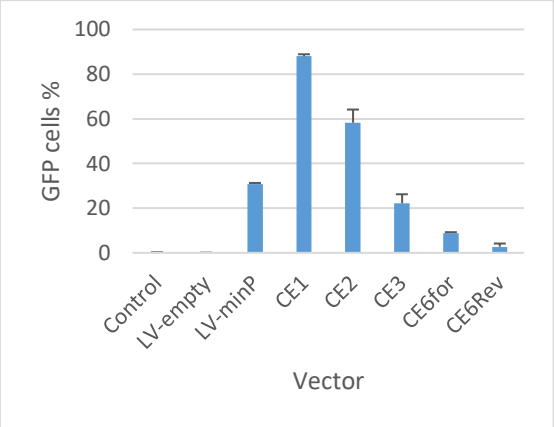
Assessment of the activity of cardiomyocyte specific vectors in other cell types in order to evaluate their specificity.

To evaluate the specificity of the enhancers, vectors were transduced into different cell lines (293T, TeloHAEC, A549, HeLa and MOVAS).

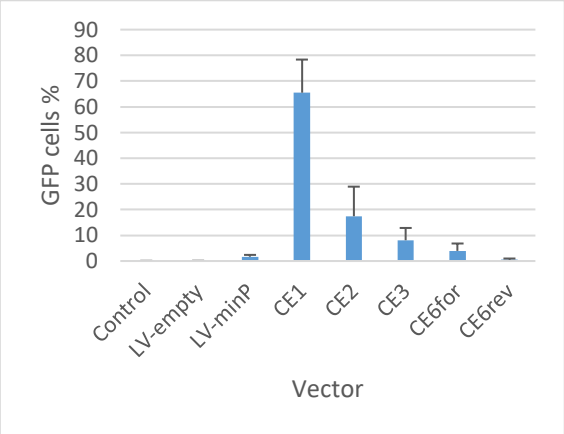
293T



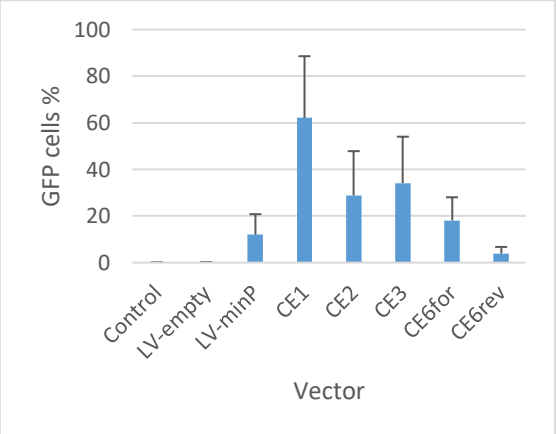
TeloHAEC



HeLa



A549



MOVAS

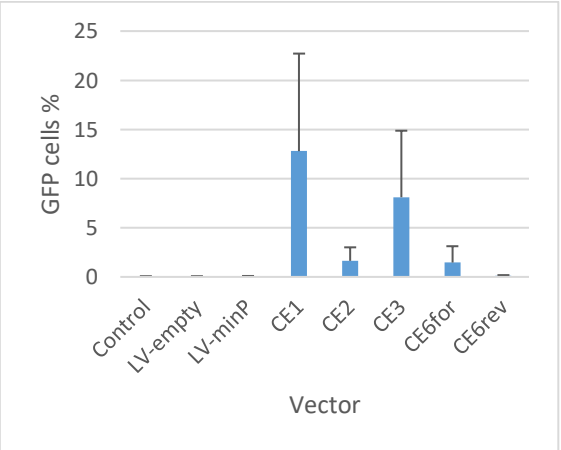


Figure 16. ***GFP expression in LV-transduced cells (293T, TeloHAEC, HeLa, A549 and MOVAS).*** *Quantitation of flow cytometry results showing the % of GFP expression cells for each vector. Data is presented as Mean±SEM, n=3.*

As shown in Figure 16, cells transduced with LV-minP-CE1 showed a high percentage of cells expressing GFP in all cell lines tested, indicating activation by this enhancer in many different cell types and not specific to cardiomyocytes. Likewise, LV-

minP-CE2 and LV-minP-CE3 were not specific to cardiomyocytes, however they demonstrated more selective activation in different cell lines ranging from no to high activation, for example MOVAS vs TeloHAEC, respectively. Moreover, cells transduced with LV-minP-CE6for and LV-minP-CE6rev showed a low percentage of cells expressing GFP in these five different cell lines. However, as shown before, they also have a low activity in mouse cardiomyocyte cells, therefore it cannot be stated that they are cardiomyocyte specific.

DISCUSSION

The aim of the study was to develop cardiomyocyte-specific LV vectors for their future use in cardio-targeted gene therapies. These vectors were developed using different enhancers belonging to an active cardiomyocyte-specific SE. Within the cluster of enhancers that form the SE, 6 enhancers were selected for further evaluation of the cardiomyocyte specificity.

Cloning the different enhancers in LV vectors was successful for most of the enhancers (CE1, CE2, CE3 and CE6) and the constructs were functional *in vitro*, being able to express GFP in 293T cells. However, cloning was not successful for CE4 and CE5. Already at the PCR stage these genomic regions were problematic and designing new primers is necessary.

The activity of enhancers CE1, 2, 3 and 6 was evaluated in mouse cardiomyocytes and CE1-3 demonstrated different levels of activation in cardiomyocytes, CE1 having the highest activation. However, when these same vectors were checked for their specifity to cardiomyocytes then disapointingly none of them demonstrated this specificity. These vectors transduced all the cell lines (293T, TeloHAEC, A549, HeLa and MOVAS) that were tested. Interestingly, although CE1 was active in all cell types tested, CE2 and CE3 showed some specificity. CE6, in both directions (forward and reverse), showed the lowest expression of GFP in every cell line. The lack of specificity of the developed vectors may indicate that the TFs needed for the enhancer activity are available in all cell types and are not unique to cardiomyocytes. It is known that TF binding is a requirement for enhancer's activity and, moreover, many different enhancer states are defined based on combinations of histone post-translational modifications, which are deposited by transcription co-regulators recruited to enhancers and promoters by TFs[48,49]. Co-regulators are large proteins that have several interaction sites for TFs, and are believed to act as facilitors and integrators of TFs binding and intracellular signals to enhancers. Recruitment of co-regulators to a given enhancer is more frequent when more transcription factors are co-bound to that enhancer[50,51], meaning that if the availability of TFs in the region of the enhancer is high, is very likely that enhancer activity is higher as well[48,52].

Since the different activities of the enhancers in this study are believed to be affected by the availability of specific TFs in the different cell lines, then those required for CE1 activation are likely to be widely available since it showed the highest activity in every cell line. The same could be applied to CE2 and 3, while the poor activity of CE6 suggests a poor availability of specific TFs.

Moreover, it is known that enhancers function is generally independent of their orientation, however, Y. Guo et al. demostrated that enhancers with proximal CTCF (transcription factor involved in various aspects of gene regulation[53]) binding sites can have different activities *in vivo* if the DNA fragment containing the enhancer is inverted[49,54], showing that the orientation of the enhancer could also have some role in gene expression. For our study, enhancers 1, 2 and 3 were only cloned into LV vectors in the forward orientation since, despite the exhaustive screening for positive plasmids, only a few positive plasmids were found and all of them were in forward orientation. Therefore, it would be interesting for the future to derive reverse-orientation clones in order to evaluate if there is any significant change in their activity in the different cell lines.

Concerning CE6, this enhancer was the only one showing low expression of the reporter gene in all cell lines tested. This could mean that this enhancer is also not specific for cardiomyocytes due to the lack of high activity in mouse cardiomyocytes as well. However, the lack of specificity towards cardiomyocytes is a statement that cannot be made until the LV vector carrying enhancer 6 is tested in human cardiomyocytes, where the activity could change in relation to the activity shown in mouse cells. The initial plan for this project included testing LV vectors in human cardiomyocyte cells, however, due to external problems regarding bacterial contamination in the primary cell laboratory and media availability, these tests could not be performed, remaining as future work.

Regarding the results achieved so far in this project we can state that no cardiomyocyte specific enhancers were found in the context of LV vectors. This is not completely surprising since only 6 enhancers within the cardiomyocyte specific SE were evaluated. In the literature there are several examples of the large number of screenings needed to find some cell type-specific enhancer within a specific superenhancer. I. Mushimiyimana et al., tested the activity of 18 enhancers. Out of which only three

endothelial-specific enhancers were found[44]. Therefore, as is consistent with previously published data, a larger screen is required to find an active cardiomyocyte-specific enhancer.

Finally, several studies that are performed nowadays consist in the deletion, through molecular tools such as CRISPR/Cas9[44,55], of a single enhancer or a combination of them to evaluate the consequent effect in the target gene. Results are very variable, sometimes resulting in target gene expression reductions between 12% and 92%[55], depending on the influence the particular enhancer or cluster of enhancers has over the target gene. Therefore, in some instances, a single enhancer is capable of regulating the expression of a target gene but at other times a cluster of enhancers is neccesary for gene regulation. This could explain why these 6 enhancers, obtained from a cardiomyocyte-specific SE, are not cardiomyocyte-specific themselves. It is possible that, in this particular case, enhancers are only cardiomyocyte-specific in the context of their cluster activity[56].

CONCLUSIONS

The main conclusions obtained in this study were:

- LV vectors carrying the different enhancers were successfully constructed.
- None of these vectors demostrated cardiomyocyte specificity according to the transduction results.
- CE1, 2 and 3 induced high level of GFP expression in mouse cardiomyocyte cells, but they also induced high levels of GFP expression in other different cell lines, demonstrating lack of specificity.
- CE6 demonstrated low level of GFP expression in every cell line that was tested, however, this remains to be tested in human cardiomyocytes, where it may demonstrate increased activity.

Overall, the final conclusion is that no cardiomyocyte-specific enhancers were found so far in the context of LV vectors. Therefore, the aim of achieving cardiomyocyte-specific viral vectors continues and the next step should be to test these vectors in human cardiomyocytes. Moreover, other cardiomyocyte-specific SE should be targeted in the search for cardiomyocyte-specific enhancers in order to obtain specific vectors that can contribute to the improvement of the treatment of patients with CVDs.

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