REWIRING THE HEART

MIGHT ENDOGENOUS NANOCARRIERS BE THE ULTIMATE TOOL TO ACHIEVE IN VIVO CARDIAC REPROGRAMMING?

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ABSTRACT

Electronic pacemakers constitute the state-of-the-art treatment for cardiac rhythm disturbances which slow down its rate (bradyarrhythmias). Nonetheless, their implantation and/or presence often poses significant risks for the patient. As an alternative, over the last years new gene and cell therapies have been developed with the objective of creating self-excitable cellular units able to mirror the performance of natural cardiac pacemaker cells in which we know as biological pacemakers. In the present study, we propose a cellular reprogramming strategy through an exosome-mediated gene therapy with the objective of generating pacemaker cells from working cardiomyocytes. The proposed study design is a preclinical twofold experimental assay: the noted strategy will be firstly tested *in vitro* to determine its feasibility and subsequently *in vivo* to evaluate its therapeutic benefit for a large animal model of bradyarrhythmia.

Key words: Bradyarrhythmias, biological pacemaker, somatic reprogramming, tbx18, exosomes.

RESUMEN

Los marcapasos electrónicos constituyen el mejor tratamiento disponible para las anomalías del ritmo cardíaco que conllevan su enlentecimiento (bradiarritmias). Sin embargo, su implantación y/o presencia con frecuencia supone riesgos significativos para el paciente. Como alternativa, nuevas terapias génicas y celulares han sido desarrolladas en los últimos años con el objetivo de crear unidades celulares autoexcitables que replicaran el funcionamiento de las células marcapaso naturales del corazón en lo que se conoce como marcapasos biológicos. En el presente estudio se propone una estrategia de reprogramación celular con terapia génica mediada por exosomas con el objetivo de generar células marcapaso a partir de cardiomiocitos contráctiles. El diseño del estudio corresponde a un ensayo experimental preclínico en dos fases, una *in vitro* inicial para determinar la viabilidad de dicha estrategia y una *in vivo* posterior en un modelo animal de bradiarritmia para evaluar su beneficio terapéutico.

Palabras clave: Bradiarritmias, marcapasos biológico, reprogramación somática, tbx18, exosomas.

RESUM

Els marcapassos electrònics constitueixen el millor tractament disponible per a les anomalies del ritme cardíac que en suposen l'enlentiment (bradiarítmies). No obstant, la seva implantació i/o presència freqüentment suposa riscs significatius per al pacient. Com a alternativa, en els últims anys s'han desenvolupat noves teràpies gèniques i cel·lulars amb l'objectiu de crear unitats cel·lulars autoexcitables que repliquessin el funcionament de les cèl·lules marcapàs naturals del cor en el que es coneix com a marcapassos biològics. En el present estudi es proposa una estratègia de reprogramació cel·lular amb teràpia gènica mitjançant exosomes amb l'objectiu de generar cèl·lules marcapàs a partir de cardiomiòcits contràctils. El disseny de l'estudi correspon a un assaig experimental preclínic en dues fases, una *in vitro* inicial per a determinar la viabilitat de l'esmentada estratègia i una *in vivo* posterior en un model animal de bradiarítmia per a avaluar el seu benefici terapèutic.

Paraules clau: Bradiarítmies, marcapassos biològics, reprogramació somàtica, tbx18, exosomes.

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BACKGROUND

About the importance of keeping up the pace and the origins of pacemakers

The normal initiation of the heartbeat in mammals relies critically upon two factors: the spontaneous depolarizations of an exceedingly rare cell subpopulation of <10.000 constituents[1-4], known as pacemaker cells (PC) and primarily located in the sinoatrial node (SAN)[4,5], and the correct transmission of their electrical pulse to the rest of the 10 billion cells total population of adult hearts[1,3] through the cardiac conduction system[4].

The former is accountable for maintaining an adequate heart rate, and the proper functioning of the latter is mandatory to achieve an orchestrated coordination between the contraction of the atria and ventricles and, thus, to provide an optimal cardiac output[6]. Cardiac arrhythmias which disrupt this wiring system, collectively known as bradyarrhythmias, lead to an inappropriately low heart rate and may evoke several symptoms including palpitations, fatigue, shortness of breath, activity intolerance or even hemodynamic collapse, syncope and sudden death[4,6-9].

Given that bradyarrhythmias are potentially debilitating and life-threatening, intensive research activity focused for decades on finding the best suited treatment and, eventually, the first prototypes of cardiac pacemakers were developed[7,8,10-17]. Since, cardiac implantable electronic devices (CIED) have underwent remarkable progress over the following 60 years with the aim of lessening their limitations[4,7,10,11,14-16,18,19].

Starting for right ventricle pacing-only devices, a minimum heart rate was guaranteed with the earliest models, and the advent of dual chamber pacemakers permitted the normal staging of atrial and ventricular contractions by generating the proper synchronized atrioventricular delay and, thus, improved cardiac output in these patients[7,20,21]. Quite a bit later, biventricular electronic pacing (otherwise known as cardiac resynchronization therapy, CRT) emerged to solve ventricular dyssynchrony derived from chronic right ventricular pacing and provided an additional treatment for some cases of congestive heart failure[4,7,14,20-22].

Over time, many other remarkable enhancements have been achieved, such as integration of defibrillating features[14,20], (limited)[4,7,10] rate responsiveness[10,20], intrinsic rhythm sensing capabilities[4], multisite pacing[4], internet-based remote monitoring systems[10,15,23], size reduction or increased battery longevity[4,10,15]. Although this technology is costly, CIEDs are considered a reliable, safe and effective therapy[6,7,15] with an expanding role in the management of cardiovascular diseases[14], are amongst the most successful implantable medical devices and have become the state-of-the-art prevention and treatment for cardiac rhythm disturbances[4,13,18,24,25].

The expanse in cardiac device technology has been matching parallel increasing implant rates over the past decades both in a global scale and in Europe[13,14,26-31] attributed to the ageing of the population and the improvement of diagnostic methods, which have broadened CIEDs' clinical indications[14,17,27]. At the present time, it is estimated that, worldwide, around 7 million people live with a CIED[21] and more than 700.000 have them implanted annually[9,15,16,32]. In Europe, the average implantation rate in 18 countries was reported to be 965 units per million inhabitants in 2016[33] and, only in Spain, a total of 39.217 devices were installed the same year, which implied a 1.6% increase compared with 2015 reports[33].

Downsides of current cardiac electronic devices and troubleshooting proposals

However, quintessential as the current pacemaker landscape may seem, the basic system paradigm of an extravascular subcutaneous case containing a battery-powered pulse generator and connected to one or more leads that traverse the venous system to reach the myocardium of one or more cardiac chambers[10,12,14,17,21] has not changed much in decades, and many issues arising from conventional pacing (and its proposed alternatives) still remain to be solved.

To begin with, pacemaker therapy is associated to significant peri- and/or post-procedural complications of varied nature[7,15], which implies the need for periodical checking or replacements[4,6]. Some of these complications are related to the device implantation procedure and are mostly anticipated prior the intervention, albeit they are far from negligible in many cases.

These include traumatic injuries[10,14-17,19,34-37], infection[4,5,10,14,15,19,34,37,38], thrombosis[10,17,34,36] and peripheral embolisation[10,39], cardiac perforation[10,15,17,19,34], lead disorders[4,10,11,15,17,19], tricuspid valve regurgitation[10,11,15,17,21,23,37], perioperative myocardial infarction[17] and death[17], amongst others.

Even further, the paced heart is inherently abnormal, and it develops pathological changes and ensuing clinical manifestations over time[8] such as heart failure[10,21,40,41], ischemic heart disease[25,41], a higher susceptibility to arrhythmias[10,21,40,42,43], and an increased mortality[10,21,40,44,45], amongst others. For the same reason, the baseline electrocardiogram (ECG) is aberrant in CIED carriers[46-50], which together with the fact that implanted patients may present cardiac troponin T or I elevation[51], can dangerously challenge acute myocardial infarction diagnosis.

Finally, the sole presence and functioning of these devices poses risks during surgical procedures[10,22,31,52,53] and certain treatments[13,31,53-55], causes electromagnetic interferences in many daily[54,56-58] and medical settings (of which the most relevant is Magnetic Resonance Imaging, MRI)[10,31,54,59-62] and rises concerns about cybersecurity[12,63-66] and ethical issues[12].

For each one of these hindrances many solutions have been proposed, which have contributed to CIEDs evolution so as their innovation and refinement have reached historical maximums[20]; however, none of them has been pivotal and electronic pacemakers remain acting as a palliative prosthesis with serious setbacks instead of a decisive cure. An exhaustive list of CIEDs' drawbacks and proposed solutions will be found in annex 1.

Insights into pacemaker cells intricacies

Aiming to address this issue, over the last few years there has been a growing interest in recreating the native pacemaker of the heart (SAN)[7,67] with entirely biological materials in which we know as biological pacemakers[6,11,20,68]; we will discuss the matter thereafter, but to work towards this goal, it is foundational to understand the intricacies of natural PC first[69].

What allows for PC automaticity is a stable *diastolic depolarization*[4,5,9,20] or "*prepotential*"[69], which rhythmically drives PC to threshold during phase 4 of each action potential (AP) until triggering the following[4,5,20]. Diastolic depolarization, in turn, is provided by two oscillatory interlinked mechanisms: the "membrane clock" and the "calcium clock"[2,4,5].

The membrane clock oscillator is located in the plasma membrane (PM) and relies on an inward Na^+ current (funny or pacemaker current, I_f)[4,6,8,20,67-69] generated through HCN[*] channels[2,6,8,9] (mostly HCN4)[4,9,69], which are activated during cell hyperpolarization[6,20,69] and deactivated on depolarization[20,69] and bind the β-adrenergic second-messenger cAMP to provide PC with autonomic responsiveness[8,20,69]. The generated Na^+ current activates voltage-induced inward T-type and L-type calcium channel currents (ICa^{2+}), which also contribute to diastolic depolarization[4,9,69].

The internally driven calcium clock facilitates diastolic depolarization by rhythmically releasing calcium from the sarcoplasmic reticulum (*SR*)[2,4], which induces an additional inward Na^+ current through the Na^+-Ca^{2+} exchanger (NCX)[4,9,20] of the PM[2]. Synergistical inward currents through HCN and NCX, together with a paucity of inward rectifier K current (I_{k1})[20,69] and outward currents during diastole[69], suffice to drive the PM to the threshold of activation of ICa, which boots the PC's AP upstroke phase[4,20,69].

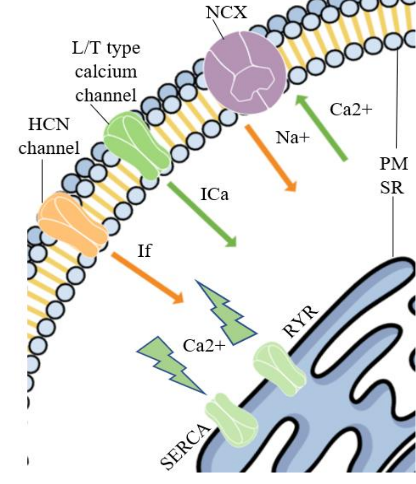

Fig 1: Membrane and Calcium clock. Created with Mind the Graph.

Other hallmarks of PC are their expression of low conductance connexins (Cx45 and Cx30.2) and a low abundance of assembled gap junctions, which provides the poor intercellular coupling[69,70] needed to overcome the current-to-load mismatch[70] between the small spontaneously beating area and the much bigger surrounding myocardium[69] and, thus, propagate the stimulus. This is relevant, as it implies that biological pacemakers may have to accomplish not only electrical but also architectural standards to perform robust pacing[9,69].

[*] *HCN: Hyperpolarization-activated cyclic nucleotide gated ion channels*[6,8,9]

Steps towards regenerative therapies: Biological pacemakers

So far, approaches to generate biological pacemakers have focused in providing quiescent non-pacemaker cells with automaticity, either by genetic engineering involving ionic channels, by adding adult or embryonic stem cell-derived pacemaker cells syncytia to the heart or by hybrid approaches in which cells were used as carriers for targeted gene therapies. A detailed list of the proposed schemes and its downsides will be found in annex 2.

None of the named strategies has hitherto demonstrated an optimal performance of biological units, which was quite foreseeable: As noted, the performance of PC is the consequence of an intricate interaction of multiple molecules, so a faithful replication of their behavior might require replicating the whole PC gene expression program instead of mirroring a single property.

On that basis arose the idea of cardiomyocyte reprogramming, which consists in reactivating developmental pathways to convert postnatal cardiac cells into PC by introducing transcription factors responsible for lineage commitment and maturation of nodal tissue[6,7]. This makes sense, as during early embryonic development, all cells of the heart tube beat spontaneously[4], and it is not until a later stage that the differential expression of clusters of transcription factors (TF)[†] ascertain whether a cell will activate the pacemaker gene program and become a PC or not[4,9,69].

In vivo cardiac reprogramming has already been proven feasible in various animal trials[1,70-72], but only one TF -Tbx18- has been shown able to successfully engineer phenotypic and functional PC from working cardiomyocytes alone[4,38]; however, further exploration is needed, as the obtained phenotypes were heterogeneous and pacing activity waned after two weeks[1,7,11,71].

HYPOTHESIS

H1: Tbx18-driven cardiomyocyte reprogramming into PC suffices to generate an effective and lasting biological pacemaker which is suitable to alleviate bradyarrhythmias.

[†] *Main TF involved in SAN's development: Tbx3, Tbx5, Tbx18, SHOX2, Isl1[4].*

OBJECTIVES

Our main objective will be to find the most suited strategy to achieve cardiomyocyte reprogramming into a pacemaker lineage.

Specific objectives:

1- To generate Tbx18-enriched human Mesenchymal Stem Cell (hMSC)-derived exosomes[‡].

   1.1 To overexpress Tbx18 in cultured hMSC.

   1.2 To enhance Tbx18 protein loading efficiency into newly generated exosomes.

   1.3 To modulate engineered exosomes to boost their uptake by cardiomyocytes.

2- To analyze the potential of Tbx18-enriched exosomes to transdifferentiate a human cardiomyocyte-like cell into a PC *in vitro.*

3- To evaluate the therapeutic benefit of an *in vivo* delivery of Tbx18-carrying exosomes to an experimental model of bradyarrhythmia.

MATERIALS AND METHODS

OBJECTIVE 1

1. HMSC isolation

HMSC will be isolated from pericardial adipose tissue samples generated during cardiac surgeries performed in Germans Trias I Pujol Hospital. To this end, we will need to be authorized by the institutional ethics committee (*Comité de Ética de la Investigación del Hospital Germans Trias I Pujol*), so a complete copy of this protocol (*which is in accordance with the ethical standards outlined in the Helsinki Declaration of 1975 as revised in 2013 and the Nuremberg Code of 1946*), the applicable authorization form[§] and the proposed patient's informed consent (IC) form (annex 3) will be submitted.

[‡] *Exosomes are highly stable nanosized bioactive extracellular vesicles secreted by most eukaryotic cells which have innate cell-binding capability, as they naturally perform paracrine regulation of targeted cells, and do not tend to induce immunological responses even after repeated allogenic administration[73]. HMSCs have been chosen as their donor cells because they are known to be highly proliferative and to present an increased exosome release.*

[§] *Documents to be fulfilled for this section will be found in: http://www.ceicgermanstrias.cat/REQUISITOS_PI-EPA_CAST.pdf*

Once the authorization is obtained, samples will be collected from those patients who have been appropriately informed of the procedure and have given (they or their representatives) their IC. Then, hMSC will be isolated as described previously[74,75]; briefly, adipose tissue will be mechanically disrupted and enzymatically digested with collagenase type II in MEM-α medium for 30 minutes at 37ºC. Cells and tissue will be then pelleted by centrifugation for 10 minutes, after which pellet will be incubated in NH_4Cl for 10 min at room temperature (RT) to lyse contaminating red blood cells. After resuspension in MEM-α medium, cells will be filtered through a 100-μm cell strainer and immunophenotyped by Flow Cytometry (FC) with antibodies against CD34, CD73, CD90, CD105 and CD166[**].

Isolated hMSC will be then transferred to a culture flask with serum-free media[††] and kept at 37ºC in a humified atmosphere of 4-6% CO_2. Medium will be changed every 2-3 days to remove nonadherent cells and, at >90% confluency, cells will be harvested for further passaging using trypsin-EDTA (0,05%).

2. Plasmids engineering

Recombinant plasmids will be used as vectors to deliver Tbx18 genetic load to hMSCs, but their content will be further tailored to boost the efficiency of Tbx18 protein loading into exosomes and minimize potential confounding factors' influence on final results.

Taking Yim et al trial protocol[76] as the starting point, we will integrate a reversible protein-protein interaction scheme which takes advantage of the natural light-dependent phosphorylation and bounding of the photoreceptor cryptochrome[‡‡] CRY2 to CIB1[§§] with the endogenous process of exosome (here EXPLOR[***]) biogenesis in donor cells.

[**] *Minimum criteria of hMSC include: Remain plastic-adherent in culture, expression of CD73, CD90, CD105 and CD166 and lack of expression of CD34, amongst others.*
[††] *Serum-free media is preferred as serum contains trillions of pre-formed exosomes per mL, which hampers the identification of new ones*[73]
[‡‡] *Cryptochromes are blue-light receptors controlling multiple aspects of plant growth and development; they have significant homology to photolyases, but instead of repairing DNA they transduce blue light energy into a signal recognizable by the cellular signaling machinery*[77].
[§§] *Calcium and Integrin Binding protein 1.*
[***] *EXPLOR: EXosome for Protein Loading via Optically Reversible protein-protein interactions*[76].

Firstly, a vector containing GFP[†††]-labeled CIBN[‡‡‡] and the exosomal membrane-associated protein tetraspanin CD9[§§§] will be generated. Genes encoding GFP-CIBN will be obtained from the pCIBN(deltaNLS)-pmGFP vector[80], which includes a CMV promoter for enhanced plasmid expression in mammalian cells and a neomycin / kanamycin resistance gene (*NeoR/KanR site*). CD9's cDNA (*GenBank BC011988.1*) will be obtained from Integrated DNA Technologies© (IDT) and included to the vector's multiple cloning site (MCS) through restriction cloning. The fusion protein resulting from the expression of this plasmid will allow for the creation of a temporary light dependent exosomal membrane anchorage point for proteins bound to CRY2.

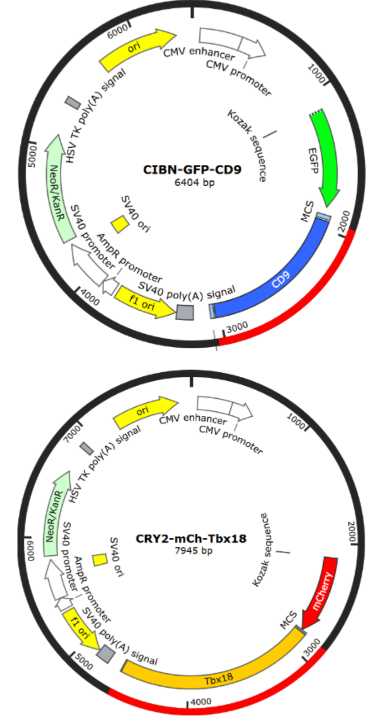

Fig 2: Visual depiction of plasmids composition. Created with SnapGene©.

A second plasmid will contain Tbx18 and mCherry-labeled CRY2. Similarly, Tbx18's cDNA (*GenBank: BC132715.1*) will be requested to IDT and inserted into the MCS of a plasmid containing mCherry-CRY2, a CMV promoter and a NeoR/KanR site[81] (see annex 4).

3. Plasmids transfection to hMSC

An initial cell transfection with CIBN-GFP-CD9 plasmids will be performed over the hMSC culture using the Effectene Transfection Reagent according to the manufacturer's instructions. After a 24h incubation, media will be replaced with a media containing the selection antibiotic G418 (Geneticin)[****] so as, over the next 3 to 7 days, cells which have incorporated the plasmid will be positively selected; this can be monitored using a fluorescence microscope. Once we have the clone of interest, a lower amount of G418 will be used for maintenance.

[†††] *Green Fluorescent Protein.*
[‡‡‡] *This is a truncated version of CIB1 CRY-interacting protein (aa 1-170), which has a shorter sequence and has been shown sufficient to maintain light-dependent specificity on the interaction with CRY2*[78]
[§§§] *Tetraspanins (CD9, CD63, CD81) are proteins involved in many biological processes that imply cell adhesion, motility, invasion or membrane fusion; they are abundant on endocytic membranes and have been widely used as exosomal markers*[79].
[****] *G418, also known as G418 sulfate and Geneticin, is an aminoglycoside antibiotic which blocks polypeptide synthesis in both prokaryotic and eukaryotic cells, which results in cell death. Resistance against this antibiotic is conferred by the neomycin resistance gene*[82].

This first step will allow for a proper anchorage of CIBN to cells' PM, after which Tbx18-carrying plasmids will be delivered likewise. Immediately after their transfection, a light-emitting diode will be activated to establish regular light cycles consisting of 1 minute of 460nm blue light illumination with a power of 20 to 50μW cm^{-2} followed by 1 minute of darkness[††††]. Under these conditions, newly synthesized CRY2-containing proteins will be bound to exosome membrane-assembled CIBN fusion proteins, thus ensuring their proper loading into the developing exosomes through attachment to their membrane. If desired, it would be possible to verify light-induced colocalization of proteins through a cryoimmunogold electron microscope.

After incubation for 48-72h, exosomes genesis and release will be expected to have finished, so the illumination source will be removed to allow for target proteins detaching from CIBN and subsequent release into the intraluminal space of the vesicle; this will take place in approximately 10 minutes[76] and will ensure an efficient delivery to receptor cells' cytosol.

HMSC may also be cultured in fiber cell culture systems or bioreactors (*ie: Fiber-Cell® Systems Hollow Fiber*), which allow for a high-density cell growth and a massive production of exosomes (*they may increase their concentration by 10-100x*); this would be particularly interesting when considering clinical scalability.

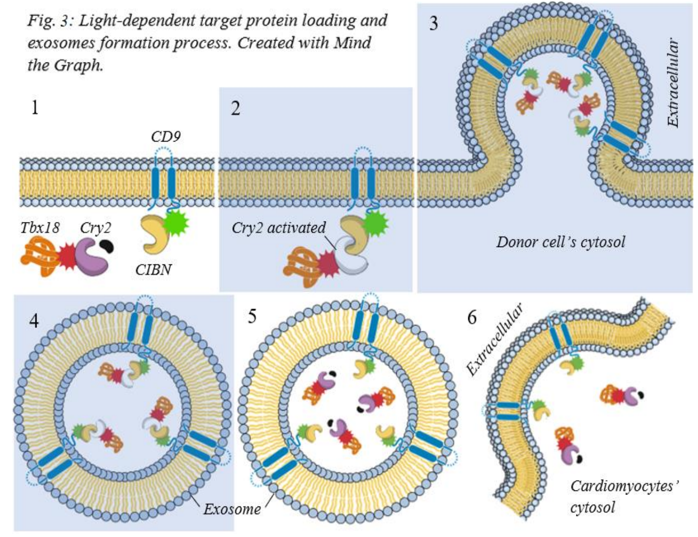

Fig. 3: Light-dependent target protein loading and exosomes formation process. Created with Mind the Graph.

4. Exosomes isolation and characterization

Exosomes will be isolated from the culture medium as previously described[83]: Firstly, supernatant will be collected and sequentially centrifuged to exclude cells and cell debris and the resulting medium will be ultrafiltrated to obtain a concentrated conditioned medium (CCM).

[††††] *This light regime has been determined the most efficient with regard of protein loading into EXPLORs in Yim et al trial, and the immediacy of its implantation is necessary to avoid nuclear migration of the transcription factor and, hence, to increase its exosomal uptake*[76].

The CCM will be subsequently diluted with saline and loaded onto gravity-eluted columns to separate its components by Size-Exclusion Chromatography (SEC)[‡‡‡‡]; around 21 fractions of the eluate will be obtained after loading, of which numbers 4 to 8 will be harvested as they will be expected to contain our exosomes. The absorbance of these sections will be read at 580 nm[§§§§] with a spectrophotometer to determine tbx18-containing fusion proteins concentration and, subsequently, exosomes presence in each SEC fraction will be confirmed by FC according to their content in tetraspanins (such as CD9), its size distribution determined by Nanoparticle Tracking Analysis (NTA) and its characterization by Cryoelectron Microscopy (CEM).

5. Exosomal cardiomyocyte specificity assignment

Given that there is growing evidence that a surface targeting moiety must be present for an efficient exosome delivery[85], cardiomyocyte specificity will be attained by the so-called "copper-catalized azide-alkyne cycloaddition", which allows for the direct attachment of a desired ligand to exosomes surfaces via covalent bonds through azide-alkyne bioconjugation[79]. In our case, that ligand will be the primary cardiomyocyte specific peptide (PCM)[86,87], as it has shown to be the highest selectivity ligand for both *in vitro* and *in vivo* cardiomyocytes[86].

Firstly, exosomes will be functionalized with terminal alkyne groups through the crosslinking of primary amines of their surfaces with 4-Pentynoic Acid and carbodiimide (EDC) activation[85]. At the same time, the PCM will need to be redesigned to allow site-specific incorporation of the azide-bearing unnatural aminoacid azidohomoalanine (AHA)[88], and the resulting peptide will be requested to Biomatik©.

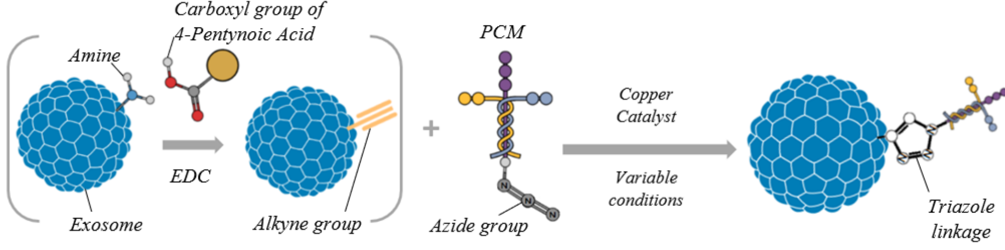

Fig 4: Azide-alkyne click reaction. Created with Mind the Graph.

‡‡‡‡ *SEC is the preferred technique for exosomes isolation because of its ability to keep them intact through the whole process[84].*
§§§§ *This value is included within the absorption spectrum of mCherry (determined by Chroma Spectra Viewer©).*

Once both alkyne-exosomes and azide-PCM are obtained, the click reaction will take place as follows[89]: reactants will be placed in a medium containing copper sulfate ($CuSO_4$), ascorbic acid and THPTA[*****] and allowed to react at RT with occasional pipet mixing. Within 24 hours, a triazole covalent linkage will be expected to be established between reactants[90], providing exosomes with stable cardiomyocyte specificity.

OBJECTIVE 2

1. Study design

Human induced Pluripotent Stem Cell (iPSC)-derived cardiomyocytes will be incubated in two cell culture trays with tbx18-enriched exosomes or without them for the experimental and the control group respectively. Data on their electrophysiological performance will be collected for 4 weeks, after which cells will be harvested for survival, genetic and phenotypical analysis.

2. IPSCs' cardiac differentiation and culture purification

Human iPSCs will be purchased from Sigma Aldrich© and treated as already described for cardiomyocyte differentiation[91]; briefly, cells will be maintained on hESC-qualified Matrigel coated plates in mTESR1 medium until 90% confluency, after which cardiac differentiation will be induced by Bone Morphogenetic Protein 4 (BMP4) and CHIR99021 in RPMI-1640 medium containing B27, glutamine and L-Ascorbic acid.

After 24h, cells will be kept in the same basal medium with CHIR for 18-36h and in RPMI basal medium with B27 without insulin for the next 24 hours. Then, medium will be replaced with a similar basal medium with IWR1 WNT inhibitor for 5 days and, afterwards, cells will be kept in a medium with B27 and insulin for 4 to 5 days more. Medium will be then replaced with cardiac enrichment medium (RPMI 1640 without glucose) and 4mM sodium L-lactate during 4 to 5 days more to purify cell population, after which medium will be switched back to basal medium with RMPI, B27 and glutamine.

[*****] *Tris-(hydroxypropyl)triazolylmehylamine; It is an accelerating ligand for Cu-mediated alkyne-azide click reactions[90].*

3. IPSC-derived cardiomyocytes culture and treatment administration

IPSCs-derived cardiomyocytes will be transferred to two plates containing fibronectin-coated polyacrylamide hydrogels with physiological elasticity (55 kPa) and 2,5-dioxopyrrolidin-1-yl6-acrylamidohexanoate[†††††] -both of which will be synthetized as previously described[92]-[‡‡‡‡‡] and cultured in Cardiomyocyte Maintenance Medium. Every two days, half the medium will be re-placed with the same volume of fresh medium, and, after 7-10 days in culture, cells will be ex-pected to beat spontaneously and will be ready for treatment.

Exosomes containing Tbx18 will be then added to the first culture (experimental group), which will be left to incubate for 24-48h. Then, cells will be washed once with saline and cells' nucleus will be dyed with 4,969-diamidino-2-phenylindole for 5 min to enable the observation of GFP and mCherry-labeled exosomes uptake by cardiomyocytes through fluorescence microscopy.

4. Evaluation methods

Electrophysiological analysis on cells' ionic currents, PM potential and depolarization rate will be performed weekly with standard microelectrode whole-cell patch-clamp techniques, and once the follow-up period is finished, cell survival will be assessed by TUNEL[§§§§§].

To evaluate cells phenotypical characterization, Pentacromic staining will be performed and their morphology, area, length and myofibrillar organization will be determined with ImageJ.

Finally, changes in the expression levels of genes with differential expression in PC and wor-king cardiomyocytes will be assessed through Western Blot analysis; once the membrane is lo-aded, it will be incubated with primary antibodies against HCN4, Cx45, Kir2.1 and sarcomeric α-actinin overnight at 4ºC, followed by 1-hour incubation with peroxidase-conjugated secon-dary antibodies. Protein quantification will be then determined by chemiluminescence.

[†††††] *This substance immobilizes matrix proteins to the surface of the PAA gel and allows for cardiomyocytes extended culture*[92].

[‡‡‡‡‡] *Neither suspension-culture nor adherence-culture on traditional cell culture plastic allows for an extended culture of cardiomyocytes due to massive mechanical stress and loss of sarcomere integrity. The used matrix resembles the elasticity of native heart tissue*[92].

§§§§§ Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick End Labeling.

OBJECTIVE 3

1. Preliminary considerations

According to the prevailing legislation about Animal Welfare Protection in Scientific Research, it will be necessary to obtain an Animal Experimentation Permit from the Generalitat de Catalunya. With this purpose we will request a report from the institutional animal experimentation ethics committee (*Comité de Ética en Investigación Animal del Instituto de Investigación en Ciencias de la Salud Germans Trias I Pujol*) and attach it to the official Authorization Form along with the Project's and the Procedure's Descriptive Memories and the Non-Technical Project Summary to be subsequently submitted[******].

2. Study design

Minimally invasive percutaneous delivery of Tbx18-carrying exosomes will be performed to a porcine model of SAN dysfunction. With this aim, 16 wild type adult Landrace pigs will be randomly distributed in two groups of 8 animals each and equal gender representation following a computer-generated random allocation sequence. The sample size has been estimated under the frequentist framework to achieve a power of 0.8, an α risk = 0.05 and considering a minimal relevant difference (δ) of 12 bpm, expected variances of the experimental and the control group responses (σ_1 and σ_2) of 9 and 6,25 respectively and a mortality rate of 16,67%[††††††].

All animals will undergo a native sinoatrial node cryoablation and will have a back-up electronic pacemaker implanted. Then, Tbx18-enriched exosomes will be delivered only to animals in group 1 (experimental), while animals in group 2 (control) will not receive other treatment.

The endpoint of the study will be the completion of a 4-week follow-up, after which animals will need to be euthanized to perform further histological studies.

[******] *All official forms will be found in: http://canalempresa.gencat.cat/ca/integraciodepartamentaltramit/tramit/PerTemes/Projectes-dexperimentacio-amb-animals-autoritzacions-i-comunicacions?moda=1*

[††††††] *δ, σ_1 and σ_2 have been estimated taking the results of a previous similar trial[71] as a reference; mortality in the same assay was reported to be 2/12=0.167, as noted in[38] → Calculations: $N = [(1,96+0,84)^2*(9+6,25)^2]/12^2 = 12,66$ → 12,66/0,8333 = 15,2.*

3. Porcine model of bradyarrhythmia and treatment administration

Once experimental animals have arrived at the animal facilities (*Centro de Experimentación Animal de la Fundación de Investigación en Ciencias de la Salud Germans Trias I Pujol*), they will be left to spend a 7 days acclimation period prior to their incorporation into the study. During this period and the whole following protocol, water and food will be supplied *ad libitum.*

At the appropriate time, all animals will undergo the same anesthetic protocol after 12h of fasting: Before sedation, an intravenous (IV) bolus of lidocaine (*1 mg/kg*) will be delivered followed by a continuous infusion (*1 mg/kg/h*), which will be maintained until 1 hour after sedation. Pigs will be then sedated with an intramuscular administration of ketamine (*20 mg/kg*), diazepam (*0.25 mg/kg*) and atropine (*0.25 mg/kg*) and anesthetic induction will be performed through IV propofol bolus administration (*4 mg/kg*) followed by orotracheal intubation and maintenance on 2% sevoflurane. An IV infusion of ketorolac (*0.15 mg/kg/h*) and tramadol (*0.5 mg/kg/h*) will be applied as intraoperative analgesia and a baseline 12-lead electrocardiogram (ECG) will be recorded.

All the following surgical procedures will be performed under ECG monitoring, pulse oximetry, arterial pressure and corporal temperature measurements. Firstly, by cannulating the right internal jugular vein, a single-chamber electronic pacemaker will be placed into the right ventricular apex of each animal to ensure a back-up therapy is available in the event of a failure of the biological unit. The device will be programmed in demand pacing mode at a rate of 50 bpm, so as it will only perform a stimulus if an alternative fastest impulse has not been produced.

Subsequently, a mapping catheter will be advanced into the right atrium by percutaneous catheterization of the femoral vein to conduct an electrophysiological study and determine the exact location of the native SAN; then, node cryoablation will be performed until complete dependence on the electronic unit (heart rate (HR) of 50 bpm with wide left-bundle-branch-like QRS) is verified.

Finally, through a deflectable injection catheter, a saline solution containing the engineered exosomes will be delivered intramyocardially to the right side of the interatrial septum (muscular part) only of animals included in group 1. Afterwards, catheters will be removed, and compressive hemostasis will be applied for 30-45 minutes on the surgical wounds.

Before finishing the intervention, subcutaneous telemetry transmitters will be implanted in the left thoracic region of all animals to allow serial electrocardiogram and eventual 24-hour Holter recordings and pacemaker interrogations. Once the procedure has been completed, intramuscular Tularomicine (*2.5 mg/kg*) will be administered as antibiotic therapy and animals will be taken back to their facilities. After, and for 3 days, all animals will receive Carprofen (*2mg/kg*) on a daily basis as postoperative analgesia.

4. Evaluation methods

4.1 Clinical evaluation and monitoring

During the 4 weeks follow-up period, all animals will undergo daily examinations by a veterinary for signs of surgical wound infection, weight loss or other distress manifestations, congestive heart failure (fatigue and/or activity abnormalities, weight gain, edemas, dyspnea, etc.), or any other clinical entity derived from the malfunction of the biological unit, such as syncope.

4.2 Electrophysiological data analyses

Both experimental and control animal groups will undergo ECG examinations on alternate days to monitor their heart rate and QRS complexes width: Pigs' normal resting HR range from 70 to 120 bpm[93], so successfully reprogrammed cardiomyocytes will be expected to display pacemaker activity at a rate included in this interval. Due to the strategical location of the biological and the electronic units, the morphology of QRS complexes will translate the generation site of the primary impulse: Narrow QRS complexes (*<120 ms*) at frequencies over 70 bpm will be attributed to the adequate performance of biological units, whilst narrow QRS complexes at lower rates (*50-70 bpm*) will be interpreted as a junctional escape rhythm (*from the AV node*) and wide

QRS complexes (*>120 ms*) at 50 bpm and, possibly, retrograde conduction, will translate an electronic unit-rescue rhythm, both of the latter due to a failure of the biological pacemaker.

Electronic pacemaker interrogations will be performed weekly (days 7, 14, 21 and 28) to ascertain the proportion of electronically paced beats, which would be desirable to be under 10-15%. In addition, and just as often, 24-hour Holter recordings will be performed to evaluate the treatment's arrhythmogenic potential.

Additionally, and given that one of the major downsides of electronic pacemakers is their non-existent or deficient (in novel devices) autonomic responsiveness, this feature will be tested on biological units: Under ECG monitoring to detect potential inducible tachycardias, isoproterenol and methacholine administration will be conducted once every two weeks (days 14 and 28) for rhythm stimulation and suppression testing, respectively.

Finally, the last day of the follow-up period, after the anesthetic protocol previously described and before euthanasia, the generation site of the primary impulse will be determined through an electrophysiological study to reliably distinguish between a biological unit or SAN-driven rhythm (in case of SAN recovery) and a fast junctional scape rhythm.

4.3 Transfected cells identification and vector biodistribution quantification

After euthanasia, the heart, lungs, liver, spleen, kidney and brain of all animals will be extracted to perform further evaluations.

Given that the GFP-labeled CIBN-CD9 protein is included in the exosomal membranes and our nanocarriers are expected to deliver their content by membrane fusion with target cells, the recombinant protein will likely be included in receptor cells PM; thus, GFP fluorescence detection will be used both for identifying transfected cells at the injection site and for detecting the vectors' presence in other organs. Nonetheless, some of the routinely employed tissue preparation protocols inactivate the fluorescence of fluorophores, and those which preserve it may compromise tissue morphology[94].

To avoid this inconvenience, we will follow Nakagawa et al[94] protocol for tissue samples preparation, as it allows for a clear visualization of the natural fluorescence of reporter proteins while maintaining excellent tissue morphology. Briefly, comparably sized samples of endocardial tissue at the injection site, left ventricular epicardium, lungs, liver, spleen, kidney and brain will be harvested, all of which will be fixed and dehydrated with ethanol and then cleared with xylenes. After warming them at RT, they will be permeated with paraffin and cut with a microtome in sections of 5µm thick, and the resulting portions will be dried, deparaffinized and washed using xylenes, ethanol and Tris-buffered saline. After 35 hours, samples will be ready both for fluorescence reading with a UV microscope and for subsequent histological studies.

4.4 Morphometric and immunohistochemical analysis

Firstly, on tissue sections from the injection site and to detect the presence of local inflammation, anti pig-CD3 and CD25 primary antibodies will be applied to determine the presence of lymphocytes and activated lymphocytes, respectively. Afterwards, a 1-hour incubation with peroxidase-conjugated secondary antibodies will be performed, and samples will be then analyzed for immunoreactivity by confocal microscopy.

Cells phenotypical characterization and differential gene expression on tissue sections from the injection site containing GFP-labeled cells will be assessed as noted for *in vitro* cultures.

STATISTICAL ANALYSIS

To determine whether parametric hypothesis testing methods are suitable for our data groups, we will perform the Saphiro-Wilk test for normality assessment of the variables distributions, as it is the preferred normality test when working with small sample sizes (<50). However, with such a limited number of observations it is unlikely that the test detects non-normality, so the hypothesis testing methods for each variable will be as follows[‡‡‡‡‡‡]:

[‡‡‡‡‡‡] *If non-normality: Mann-Whitney test for unpaired and Wilcoxon test for paired quantitative variables, and Fisher test for categoric.*

Variable	Type	Test
% activation of I_f, I_k, I_{Ca}, I_{Na} and I_{NCX} currents (*Patch-clamp*)	Categoric	Chi-squared test
Basal heart/cells rate	Quantitative	Unpaired t-test
Heart rate - Isoproterenol (before/after)	Quantitative	Paired t-test
Heart rate - Methacholine (before/after)	Quantitative	Paired t-test
Paced beats (proportion)	Categoric	Chi-squared test
Arrhythmic events (Animal model)	Quantitative	Unpaired t-test
Targeted gene expression (*aggregation of the results of independent tests for each gene*)	Quantitative	Unpaired t-test

The independent variable will be Tbx18 delivery. Data will be processed with the statistical analysis package IMB SPSS and an intention-to-treat approach and will be represented as mean ± standard error of the mean. P values <0.05 will be considered statistically significant.

CHRONOLOGY

The whole study may be completed within around 8 months without considering the time needed for legal procedures. The complete chronological scheme will be found in annex 5.

EXPECTED RESULTS

Considering the results of previous animal trials applying a similar strategy[1,71], it is expected that treated cardiomyocytes undergo a complete phenotypic switchover and that they exhibit robust pacing activity in an autonomic-responsive manner and at a rate sufficiently fast to override the junctional rhythm just as the native SAN naturally would.

Further, both features are expected to endure over the follow-up period and variability in cellular effects to be reduced because of the proposed strategies for protein loading efficiency enhancement and targeted treatment delivery refinement.

COSTS ESTIMATION

The estimated overall costs for material, reactants and surgical procedures has been deemed to be 112.065€; an indicative breakdown of expenditure is given in annex 6.

ETHICAL CONCERNS

The proposed study design was tailored to match the regulations that focus on the “three Rs” framework to ensure an ethical use of experimental animals, the goals of which are *replacement* of animals with nonanimal methods when possible, *reduction* in the number of animals used and *refinement* of experimental techniques to reduce animal pain and suffering[95,96].

Many *in vitro* and/or *in vivo* trials have already proven the feasibility of all procedures listed in our protocol, so further advancement on the matter at hand requires the involvement of experimental animals. As the present project intend to move a step forward clinical translation of biological pacemakers, experimentation with an *in vivo* model as close as possible to human is crucial at this point, and the porcine heart distinguishes itself by the similarity of its anatomy and physiology with human’s. For the reasons stated, there is not a better alternative method than the proposed one for this trial, so the *replacement* principle could not be properly met.

When considering the number of animals to include in our protocol, the sample size was calculated to be the minimum necessary to ensure a reasonable power (80%) and the usage of additional animals for biodistribution assays was avoided by implanting a method by which all post-mortem studies could be performed with the same samples. All this aims to match the *reduction* foundation.

Finally, efforts have been specially directed towards *refining* our methods to ensure animal welfare and minimize pain or suffering. All subjects will have the time to adapt to their new environment and will be kept in a social context. Anesthetic and surgical procedures have been adapted to reduce distress and pain at a minimum and postoperative monitoring of all animals will be performed daily by expert veterinaries to provide the appropriate care. In addition, cardiomyocyte targeting ability of exosomes is expected to importantly reduce the vector’s biodistribution and potential undesired side effects.

Importantly, since our protocol includes a surgical allocation of catheters and electronic devices under general anesthesia, albeit with low expected postinterventional levels of pain or distress, the overall severity of the procedure would be defined as "moderate"[97]. This makes necessary to develop an animal supervision protocol to allow for the assessment of animal suffering and to formulate corrective measures criteria[98]; our proposed protocol will be found in annex 7.

STUDY LIMITATIONS

The first shortcoming we need to assume is the ineluctable inter-animal genetic variation, which might importantly dictate the effect that Tbx18 exerts over each animal's cells and modify the outcomes of the study. Also related to individual idiosyncrasy is the probability of native SAN's activity reinstatement after ablation, which has been deemed of strong likelihood[99] and may trigger uncontrollable arrhythmias due to competitive pacing with the biological unit[4].

The local puncture for exosomes delivery might also lead to arrhythmogenic outcomes by inducing a local inflammatory response due to traumatic damage and, further, it poses the risk of atrial wall perforation. Animal death might ensue from all weak points noted so far and, hence, a considerable risk of attrition bias has been foreseen but only partially addressed by considering the expected mortality rate in the calculation of the sample size and by implementing an intention-to-treat analysis of data.

An especially important limitation to consider is our inability to control or determine the amount of cardiomyocytes which will receive the treatment, which is central, as the reprogramming strategy should produce enough PC to maintain the required proportion with the surrounding myocardium to perform robust pacing activity[7,9,67]; besides, cell reprogramming will be homogeneous with our approach, while the native SAN is heterogeneous in a gradient fashion from the center to the periphery in terms of cell size, ionic channels, current densities and electrical coupling[100], which implies that, even if we achieve permanent cell reprogramming, the performance of our biological pacemaker might be deficient.

FUTURE PERSPECTIVES

There is controversy about the role of some transcription factors regarding adult cardiomyocyte reprogramming to a PC, but it is generally accepted that the interplay of different genes is necessary for PCs differentiation during embryonic development[2,4,5,69,101,102]. Hence, if Tbx18 alone-based approaches were not to be successful, a reasonable alternative would be inducing a combinatorial overexpression of various genes involved in SAN's development.

Concerning the delivery method, the injection-based system could be replaced with the employment of exosomes-carrying solid scaffolds with endocardial adhesion ability and reduced potential dislodgement and thrombogenicity. This would avoid the risk of atrial perforation and would define the treated area to tailor the faux SAN's size to match the required proportion.

Either way, biological pacemakers are still in their early stages of development, and even if they succeeded in clinical translation, their application would require understanding spatial, structural and functional determinants of each patient's arrhythmia and identifying vulnerable parameters whose modification would suppress it without adverse events for the patient[68].

Certainly, much remains to be done, but, in the meanwhile, we expect that our hopefully more efficient and with low adverse and off-target effects gene transfer strategy play a part in biological pacemakers' progress towards gaining a clinical application.

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ANNEXES

ANNEX 1 – Electronic pacemakers' drawbacks and troubleshooting

Implantation procedure-related complications (<1 – 6%[10,17] (up to 10%)[16])		*SOLUTION PROPOSALS*
SHORT-TERM Mostly mechanical[17], heavily skill and technique dependent[17,36] (8-12%)[10,11,15,17,32,34]	Cardiac perforation[10,15,17,19,34] (0,3-0,6%)[34] → Pericarditis, refractory pericarditic pain, pericardial effusion, cardiac tamponade[14], unacceptable pacing/sensing[10,17]	High-volume operators[17,27]; Reintervention[16]
	Traumatic (1-2,7%)[16]: Pneumothorax, hemo-thorax[10,14-17,19,34-37]	High-volume operators[17,27]; Contrast venography or ultrasound guidance[35]; Different vascular approach (jugular, cephalic)[35,36]
	Mistaken location of leads[10,17] → Thrombus formation[10,17,34,36] (0,5%)[34], embolization[10,39], may require reintervention[39]	High-volume operators[17,27]
	Lead displacement[10,15,16,17,19,32,34] (2,4-3,3%)[16]	Reintervention
	Pocket hematoma[10,14,17,34,37,103] (2-5%)[10,30] → 7-fold greater[30] risk of pocket infection[103], may require reintervention[17] or device extraction[15]	If related to drugs (risk 16%)[103], optimize therapy[17,103], avoid bridging therapy with heparin[103]; Elastic compression bandage[103]; Cauterization of all bleeding arteries, irrigation of the pocket, topical thrombin or absorbable hemostats[17]
	Infection (1,2%)[14,15,34]	Preprocedural antimicrobial prophylaxis and sterile precautions[17,27]; Antibacterial-coated device[17,27]; Antibiotic +/- extraction[38]
	Perioperative myocardial infarction, stroke, death[17]	
	Others: Arterial puncture[14], AV fistula, venous air embolism, thoracic duct/nerve injury, intraluminal dissection, puncture of the Aorta[36]	High-volume operators[17,27]
INTERMEDIATE TERM	Tricuspid valve (TV) regurgitation[4,10,11,15,17,21,23,37] (symptoms 5%)[16] → ↑ mortality[23]	If severe, device extraction + TV surgery[17,23]
	Device dislodgement[10,14,37]	Reintervention
	Upper extremity deep venous thrombosis & stenosis[4,14,16,19,23,37] (partial 21-25%[16,23], total 9-12%[23,104]) → Unilateral edema, vena cava syndrome[10,11,15,23,104], thromboembolism[10,21,23,104], increased mortality[104]	Limit number of leads if possible; Anticoagulation, thrombolysis, venoplasty (high recurrence); Surgery, stenting (more perdurable results)[23]
	Infection[10,37]	Preprocedural antibiotic and sterile precautions; Antibacterial-coated device[17,27]; Antibiotic +/- extraction[38]
	Local pain, discomfort[10]	Analgesia
	Hypertrophic scar, keloid → Itching, photosensitivity[10]	Monofilament suture, good surgical technique, avoid excessive tension on the suture[10]; Keloid: Intralesional steroid injection, silicone sheeting, laser phototherapy[10]
LONG-TERM (3-year and 5-year rates: 7,5%[105], 19,7%[15,34])	Lead-related[10,11,15,17,19] (most common and relevant, + in pediatric patients)[10,15,16,21,37]: • Lead erosion/ fracture[14,15,17,19,23,37] • Generator-lead interface abnormalities[14,15,17,19,23,37] • Insulation defects[11,15,17,19,37] • Twiddler's syndrome[15,17,37§§§§§§] • Exit block[10*******]	↓ nº of leads if possible[27]; Smaller and more durable and easily extractable leads[23]; Pre-marketing bench testing[23]; Addition of an outer copolymer, oxidation and abrasion-resistant materials or steroid-eluting insulation sheaths[104]; Corrosion-free or steroid-eluting lead tips[10,104]; LEADLESS PACEMAKERS[4,10,11,14,15,16,19,21,23,32,37,104]

§§§§§§ The Twiddler's syndrome consists in an axial rotation of the pacemaker's generator that stirs twisting of the electrodes, which, at turn, may fracture or dislodge the leads. This effect may be due to excessive size of generator's pocket, the weight and size of the generator, iterative left arm/pectoral region movements, obesity, active manipulation by the patient or unknown cause[106]

******** The exit block consists in a gradual rise of impedance and pacing thresholds without any identified damage; it is attributed to the development of scar tissue or the deposition of calcium hydroxyapatite crystals at the lead-myocardium interface[10].*

LONG-TERM (3-year and 5-year rates: 7,5%[105], 19,7%[15,34])	Replacement or extraction-related (1,4-5,6%)[34,104]: • Bleeding[11,17,21,23,30,104] • Infection[30] • Venous/cardiac perforation/tearing[11,12,17,21,23,104] • Traumatic tricuspid valve injury (3,5-19%)[21,23,104] • Death[11,17,21] (0-2,6%)[28,104]	Do not explant leads when replacing a CIED[12,104]; Countertraction, laser, radiofrequency or surgical-based extraction techniques[28]
	Pocket-related (0,7-2,4%)[32]: • Skin erosion and infection[105] • Chronic pain[19,23] • Limitation of arm/shoulder motion[19] or disability[23]	Analgesia / antibiotics / physiotherapy; Device extraction[23]
	Infection[10,19,37] (1,6-2,5%)[16,17,34] → Increased mortality[27] (up to 12-31% if endocarditis)[32]	Preprocedural antibiotic and sterile precautions, antibacterial-coated device[17,27]; ↓ nº of leads if possible[27]; Antibiotic +/- extraction[38] (mostly)
	Delayed-type hypersensitivity[105] → Related to damages to pacemaker components and complications[105]	Device extraction?
	Harmful effects of radiation (contrast venography) → Specially in obese patients[35,107], which, at turn, have an increased probability of receiving a CIED[107]	Ultrasound[36] or surface landmarks-guided[35] puncture; If contrast venography is used: Reduce fluoroscopy frame rate, avoid unnecessary cine loops, asymmetric collimation, image integration, low dose programs[107]

Impact of right ventricular pacing site	
• Pacemaker syndrome[108†††††††] • New-onset left ventricular systolic dysfunction[41] • Heart failure[10,21,40,41] • Persistent atrial fibrillation[21] • Ischemic heart disease[25] (as QRS increased)[41] • Increased mortality[10,21,40,44,45]	Non-apical stimulation of the right ventricle if using single-chamber devices[25]; Dual-chamber devices, specially by applying optimized timing algorhythms to diminish ventricular stimulation[10,108]; Biventricular pacing[21,40,41]

Challenging acute myocardial infarction dx	
• Secondary baseline T wave changes ("QRS-T appropriate discordance")[46-49,110] • ST segment QRS-discordant elevation or depression[46,49,110] • Elevation of cardiac troponins T or I[51] → Potential overtreatment or delayed revascularization[46]	Sgarbossa's original and Smith's modified criteria[46-50,110,111][Based in the "concordance" and "excessive discordance" principles as abnormality criteria[49,110]]

Dependency on batteries	
Require pulse generator exchanges[112] →Source of complications	Batteryless devices with intracorporeal energy-harvesting systems[4,10,15,21,112]; Solar light-powered devices[4,112]

Arrhythmogenic potential	
• Prolonged QT interval[40,42] and increased sensitivity to drug-induced QT lengthening[40] → Ventricular arrhythmias and cardiac death[40] • Increased ventricular ectopy and onsets of refractory ventricular arrhythmias[23] • Pacemaker mediated tachycardia (reentrant circuit-mediated, bicameral devices)[10] • Upper rate behavior and pacemaker Wenckebach (sensing alterations-mediated, bicameral devices with activated inhibition and tracking mode)[10,43]	Device extraction[23]; Application of a magnet[‡‡‡‡‡‡‡], pacemaker algorhythms adjustment[10]

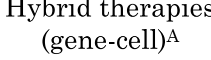
[†††††††] The pacemaker syndrome: Dyspnea, palpitations, malaise, and syncope, most often ascribed to the loss of atrioventricular synchrony, though recent data may also implicate left ventricular dysynchrony, both potentially caused by right ventricular pacing[109]
[‡‡‡‡‡‡‡] Application of a magnet over a pacemaker results in the onset of an asynchronous pacing mode, by which the device stops tracking intrinsic cardiac electrical activity and starts delivering impulses autonomously[10].

MRI Electromagnetic interferences (EMI)	

(50-75% of CIED implanted patients will eventually need MRI!)[10,59,61]
• Torque effect → Paresthesia in the pocket[60], vibration, movement or dislodgement of the device[59,62]
• Induced electrical currents → Over or undersensing[59,60], triggering of fatal ventricular arrhythmias[10,54,60,62].
• Antenna effect (heating of lead tips through resonance phenomena)[10,59,54,60,62] → Thermal damage[31], edema, necrosis, fibrosis[10,59] → Device dysfunction[10,59,60]
• Reed switch activation → Unpredictable pacing behaviors[10,54,60], accelerated battery depletion[59]
• Electrical reset[54,62] → Default factory settings
• Others: Generator-site dyscomfort, atrial fibrillation or flutter onset[61,62], partial reset[61], MRI artifacts[10,54]
	- Off-label use of classic devices[60,62] → Previous device reprogramming[54,59], strict monitoring during procedure[10,54,59,60,62] and following device interrogation and settings restoring[10,64]
	- In-label use: MR-conditional devices[10,54,59,60,61]
	  ○ Software-based protection modes[54,59,60]
	  ○ Hall sensors[13,54,59,60]
	  ○ Hermetically sealed cases[13,59,60]
	  ○ Lead modifications to decrease risk of heating[10,59,60]

Other EMI	
Daily life and occupational sources of EMI (power lines, electric tools, entertainment electronics, security systems, equipment in work settings)[54,56,57] • Ventricular oversensing[54] • Current induction[54] • Asynchronous pacing[54] • Pacing rate changes[58] → Asystole, palpitations, dizziness, syncope[56]	Avoid close contact of electrical equipment with CIEDs[54]; Keep a distance from potential sources of EMI[56]; Bipolar instead of unipolar sensing[56]; Reprogramming devices to a lower sensitivity (specially in occupational exposure)[54,56]
Anesthesia and surgery-related hazards	
EMI with the electrocautery unit (ECU)[10,22,31] → Hypotension, tachy or bradycardia, infarction[53]	Bipolar or ultrasonic unit[10,31,53]; Application of a magnet or preoperatory programming to asynchronous mode[10,22,31,53]; >15cm distance between ECU and CIED generator/ leads[10,22,31,53]; <5s bursts, >5s between bursts, lowest feasible energy levels[10,22,53], continuous monitoring[22,31,53]
Anesthesia-related adverse outcomes Loss of capture → Bradycardia, hypotension, admission to the intensive care unit[52]	Avoid high doses of fentanyl or dexmedetomidine[31]; Application of a magnet[22]; Continuous monitoring[22,31,53]
Absence of reflex tachycardia in case of hypotension (asynchronous mode)[31]	Attention to fluid replacement[31]
Potential meddling with other therapies	
Radiotherapy • CIED attenuates beam profile and dose[13] • Pacing inhibition[13,54], pauses >10s[13] • CIED failure and reset[13,31,54,55] • Declining device battery voltage[55] • Induction of ventricular arrhythmias[54]	Preferably photon radiation or short-range brachytherapy[13]; Minimum 3-5cm distance between radiation field and CIED[13] → Surgical relocation[13,31,53]; Monitoring in each session[13]; Limiting beam energy and dose[13]; Device interrogation during and after radiation, controls after up to 6 months[13]
Extracorporeal shock wave lithotripsy: Interference with the pacing circuitry[31]	Asynchronous mode[31,53]; Distance between generator and lithotripsy beam[53]
Radiofrequency (RF) ablation: Asynchronous pacing, pacing inhibition, loss of capture, reset, run-away pacing[54]	Distance between generator and RF current path[53]
Cardioversion/defibrillation**:** Capacitive coupling (discharge at electrode-endocardium interface) → Transient/permanent alterations in CIEDs' function[22,31]	Pads at least 8-10cm away from the device[22,31], antero-posterior manner[31]
Cybersecurity concerns (remote monitoring)	
• Potential for interception, wrong delivery or loss of information[12,66] • Potential cyberattacks[66]: Identity theft[65], reputational damage[66], care interruption[65,66], harm to patients[65,66], litigation or financial consequences[66]	Promote greater awareness in the health care environment[66]; Firmware update with added layers of security[64]; Cybersecurity protection into future CIEDs manufacture[66]; Increased crosstalk between clinicians, technology professionals and manufacturers[66]
Ethical issues	
Multimillion dollar market around CIEDs – might have negative impact on patient care; Patients' rights regarding information about their health may be limited[12]	

ANNEX 2 – Biological pacemaker approaches and downsides

Approach	Proof-of-concept studies	Drawbacks
Functional reengineering (gene therapy)[A]	Overexpression of β2-adrenergic R or Adenylyl Cyclase 6[113]: Mice* and pig*	Persistent dependence on adrenoceptor stimulation for induction of function[114], requires a functional native biological pacemaker[115], risk of arrhythmogenesis[20,115]
	Inhibition of Ik1 (delivery of Kir2.1AAA)[116]: Mice**	Prolongation of action potential duration[4] and QT interval[20,115,117,118], potential arrhythmogenesis[4,20,115], lack of direct modulation by autonomic stimuli[114]
	Overexpression of native or mutated HCN channels: Dog*^, pig*, guinea pig***	Low heart rates[8,20,70,119,120] variable between studies[8,20], suboptimal response to autonomic modulation[114], potential unpredictable outcomes by heteromultimerization with endogenous channels[114], highly invasive delivery methods[119]
	Overexpression of HCN2 + Kir2.1AAA[121]: Pig"	Suboptimal sensitivity to autonomic modulation, action potential prolongation[114]
	Overexpression of HCN2 + SKM1[122]: Dog^	Large transgene[114], scarce data
	Overexpression of Adenylyl Cyclase 1 (AC1)[120,123]: Dog^, rat	Increased intracellular cAMP content[123], concerns about Ca^{2+} overload[114] and proarrhythmia[120]
	Overexpression of AC1 + HCN2[120]: Dog^	Excessively fast beating rates[120], abnormalities of QT interval (significantly shortened with the Matsunaga formula and prolonged with Bazett's)[120], auto-suppression of HCN2/AC1-induced rhythms[120], concerns with regard to potential Ca^{2+} overload[114], proarrhythmia[114]
Stem cell therapy[B]	Delivery of fetal cardiomyocytes[124]: Dog"	Immunogenicity issues and poor cell survival[114]
	Delivery of hESC-derived cardiomyocytes[125,126]: Guinea pig**, pig**	Ethical concerns[118], need for immunosupression[4,20,114], potential tumorigenesis[3,127,128] and cell dedifferentiation[20,67], heterogeneity of obtained cells[3,67,115,119], potential arrhythmogenesis[7,119,127,128], difficulty to obtain a sufficient number[128] and isolating appropriate cell lineages[9,67]
	Delivery of iPSCs-derived cardiomyocytes[129]: Dog**	Heterogeneity of obtained cells[4,114,119] - possible substrate for reentry-induced arrhythmias[119], concerns about potential migration or cell dedifferentiation[4], potential teratoma formation[115], requires weeks to months to develop[4]
	Adult/somatic stem cells-derived cardiomyocytes (cardiac[130,131], bone[102], adipose[132]): In vitro	Only tested in vitro[102,130,132], inconsistent and suboptimal results[102,130-132], potential migration[130], dedifferentiation, tumorigenesis or IS rejection, uncertainty about gap-junctional coupling stability, requires weeks[102,130-132]
Hybrid therapies (gene-cell)[A] 	Delivery of HCN2-expressing hMSCs (gap-junctional coupling)[133]: Dog**	Low heart rates[4,115], concerns about potential migration and further differentiation[4,8,20,67,114,115,119,128], moderate response to catecholamine infusion[114]
	Delivery of HCN1-expressing fibroblasts (formation of fibroblast-myocyte heterokaryons)[134]: Guinea pig**	Not responsive to cholinergic agonists[135], scarce data

 Somatic Reprogramming[B]	Overexpression of TBX3[70]: *Mice**	Heterogeneity of phenotypes obtained[70,114] attributed to high dose-sensitiveness and duration of the reprogramming process[114], failed to induce the complete pacemaker phenotype[2,70,101] and pacemaker activity[1,70,114] (absence of I_f)[70], potential dose-dependent cell death[70]
	Overexpression of TBX18[1,71]: *Guinea pig**, pig"*	Heterogeneity of phenotypes obtained[71,114] attributed to high dose-sensitiveness and duration of the reprogramming process[114], misgivings about its actual contribution to heart rate outcomes alone in large animal models[38]
	Activation of Notch signaling[72]: *In vitro*	Heterogeneity of phenotypes obtained, induced a Purkinje-like phenotype instead of a nodal-like[72]

* Atrium ** Ventricle ^His bundle "AV junction IS: Immune system

A: Functional Pacemaker B: Phenotypic and functional pacemaker

[Neonatal or adult cells → Transgenic models excluded] [Illustrations created with Mind the Graph]

PATIENT'S INFORMED CONSENT FORM

(Please take the time to read this form very carefully and to decide whether you wish to give consent).

A. TO THE PHYSICIAN MEMBER OF THE CARDIAC SURGERY DEPARTMENT

TYPE OF PROCEDURE: Extraction of small pieces of pericardial fatty tissue generated during the surgical intervention without exposing the patient to any additional risk or harm. The collected samples will be stored in strict confidence and eventually used for research on prevention and treatment of heart diseases.

i. I confirm that I have explained the nature of the procedure to be performed upon the patient named below. The explanation I have given is in my judgment suited to the understanding of the patient and/or the guardian of the patient.

Signed……………………………………………….

Medical Board Number……………………………...

B. TO THE PATIENT/GUARDIAN/RESPONSIBLE PERSON

i. If you do not understand the explanation of the procedure to be undergone, or if you require further information please ask your doctor.

ii. Please check that all information on the form is correct. If it is and you understand all the exposed, then sign the form.

I………………………………………………………………………………. with Identity Card nº…………………………….. hereby consent to undergo the proposed procedure to be performed upon myself. The nature and purpose of this procedure has been understandably explained to me and my doubts satisfactorily allayed by Mr/Mrs/Dr……………………… I comprehend that my participation is voluntary and that I am free to withdraw my consent at any time prior to the intervention.

Signed……………………………………………….

Badalona, on Date……………………………………………….

HOJA DE CONSENTIMIENTO INFORMADO DEL PACIENTE

(Por favor, tome su tiempo para leer este documento cuidadosamente y para decidir si desea conceder su consentimiento)

A. AL FACULTATIVO MIEMBRO DEL SERVICIO DE CIRUGÍA CARDÍACA

TIPO DE PROCEDIMIENTO: Extracción de pequeños fragmentos de tejido graso pericárdico generados durante la intervención quirúrgica sin exponer al paciente a ningún riesgo ni daño adicional. Las muestras recogidas serán almacenadas en la más estricta confidencialidad y eventualmente utilizadas para investigación con el fin de mejorar la prevención y el tratamiento de las enfermedades cardíacas.

i. Confirmo que he explicado la naturaleza del procedimiento a ser aplicado sobre el paciente indicado a continuación. La explicación proporcionada ha sido, a mi juicio, adecuada a la comprensión del paciente y/o del tutor del paciente.

Firmado……………………………………………….

Número de Colegiado…………………………………

B. AL PACIENTE/TUTOR/PERSONA RESPONSABLE

i. Si no entiende la explicación del procedimiento a realizar o requiere más información sobre el mismo, por favor consulte a su médico.

ii. Por favor, compruebe que toda la información en este documento es correcta. Si lo es, y entiende todo lo expuesto, firme en el lugar indicado.

Yo, ……………………………………………………………………………… con DNI nº………………………….. consiento ser sometido al procedimiento propuesto. La naturaleza y propósito de tal procedimiento me han sido explicados de manera comprensible y mis dudas resueltas por el Sr/Sra/Dr………………… Comprendo que mi participación es voluntaria y que soy libre de retirar mi consentimiento en cualquier momento previamente a la intervención.

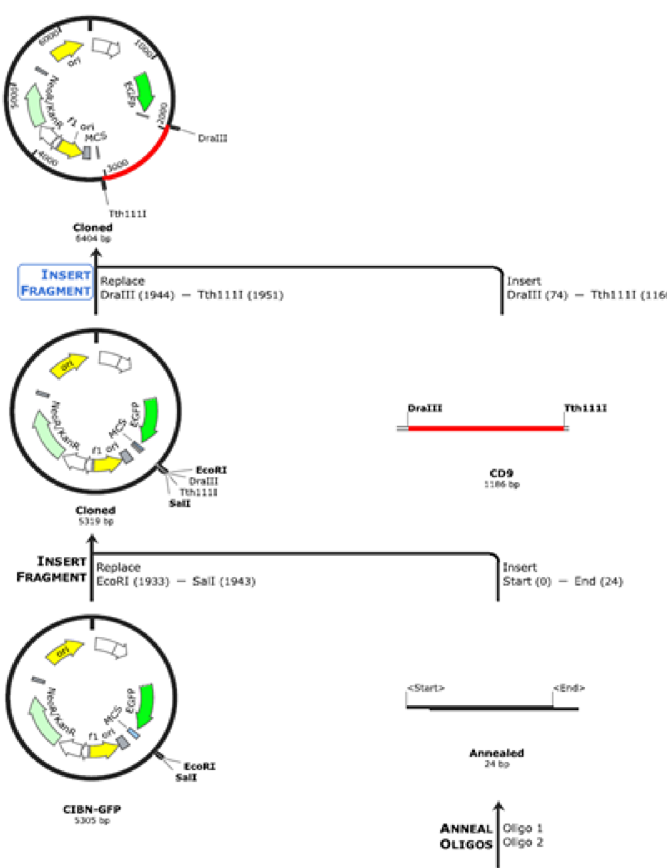
Firmado……………………………………………….

Badalona, en Fecha……………………………………………….

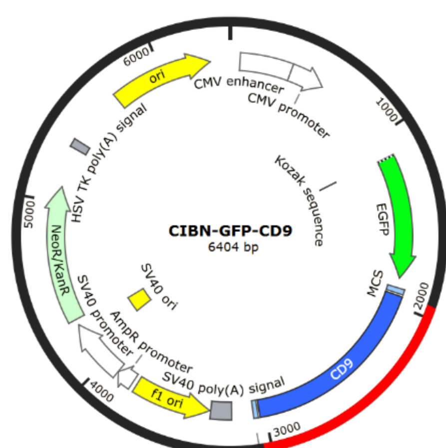
ANNEX 4 – Plasmids engineering

1. CIBN-GFP-CD9

To create this vector, the MCS of the pCMV-CIBN-GFP vector will have to be modified to include restriction sites for DraIII and Tth111I, which are needed to insert the CD9 sequence. To this end, we will design a set of overlapping oligonucleotide sequences containing the desired restriction sites[136] and additional bases at the ends that complement the overhangs generated when digesting pCMV-CIBN-GFP with restriction enzymes whose restriction sites are included in the vector's MCS, in this case EcoRI and SalI.



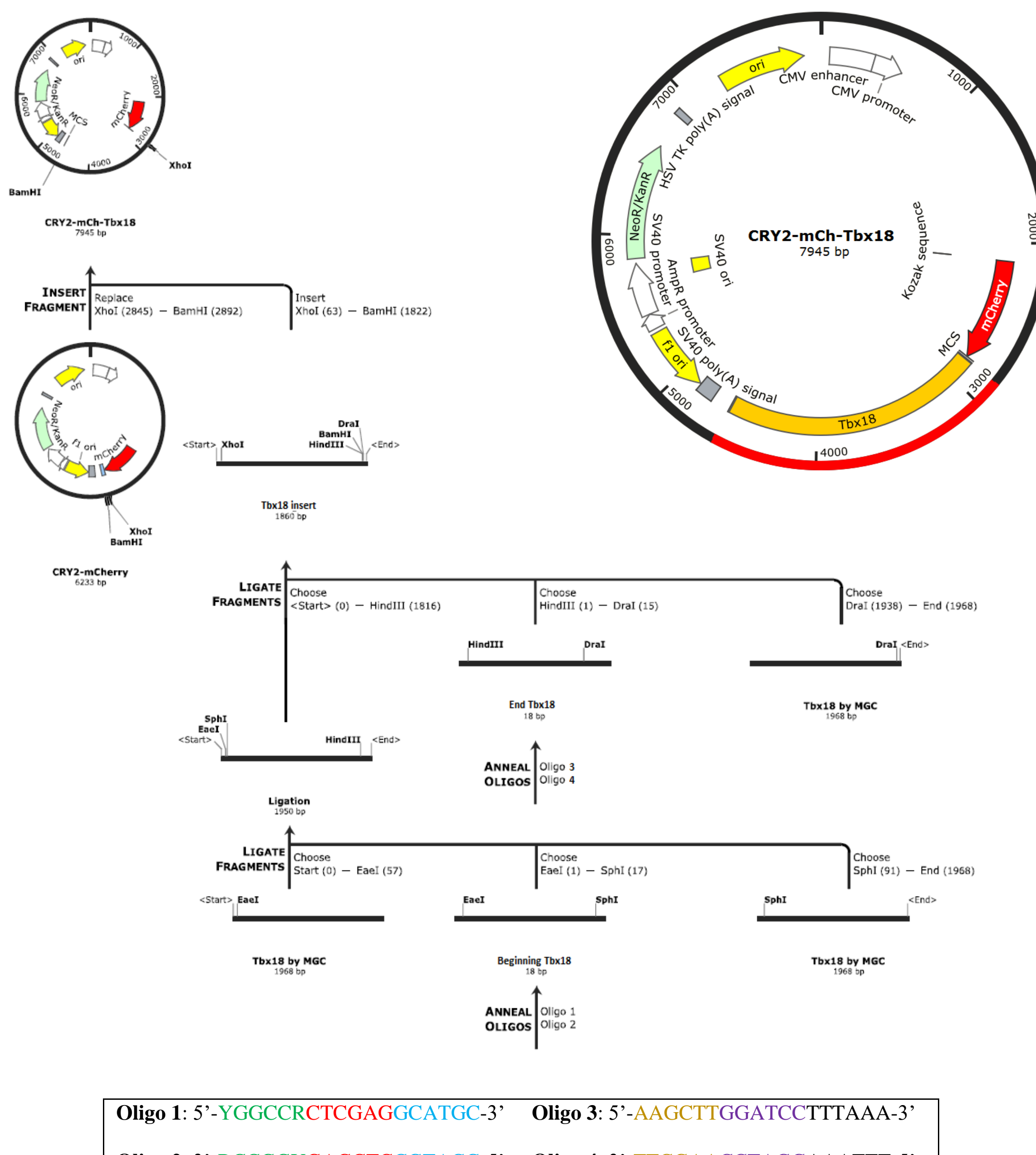
Once we anneal the designed oligonucleotide sequences, they will be easily inserted into the MCS of the backbone vector by applying a ligase. A second cycle of restriction cloning, in this case with DraIII and Tth111I, will allow for the successful insertion of CD9.



Oligo 1: 5'-AATTCCACNNNGTGGACNNNGTCG-3' **Oligo 2**: 3'-GGTGNNNCACCTGNNNCAGCAGCT-5' EcoRI DraIII Tth111I SalI
*Representation of restriction sites included in annealed overlapping oligonucleotide sequences used for CIBN-GFP-CD9 vector construction**

2. CRY2-mCherry-Tbx18

Likewise, restriction sites included in pCMV-CRY2-mCherry and Tbx18 do not match, which will be solved in a similar way. In this case, XhoI and BamHI restriction sites will need to be included in the Tbx18 sequence in a two-steps procedure, as represented below.

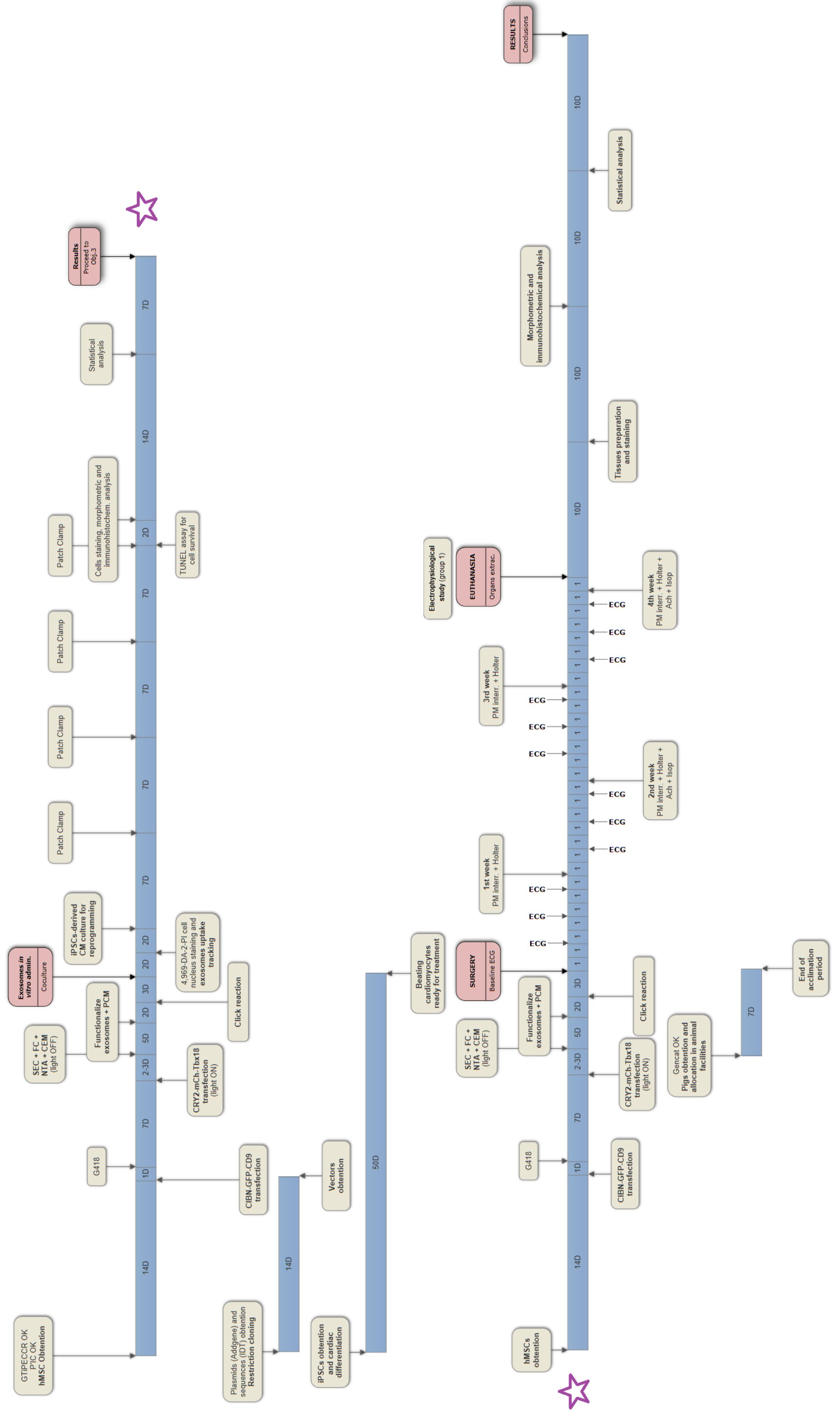


Oligo 1: 5'-YGGCCRCTCGAGGCATGC-3'	**Oligo 3**: 5'-AAGCTTGGATCCTTTAAA-3'
Oligo 2: 3'-RCCGGYGAGCTCCGTACG-5'	**Oligo 4**: 3'-TTCGAACCTAGGAAATTT-5'
EaeI XhoI SphI HindIII BamHI DraI	

*Representation of restriction sites included in annealed overlapping oligonucleotide sequences used for CRY2-mCherry-Tbx18 vector construction**

* *All oligonucleotide sequences will be requested to IDT.*

ANNEX 5 - Chronological scheme



ANNEX 6 – Breakdown of expenditure (materials, reagents and surgical procedures)

hMSC OBTENTION AND MAINTENANCE	
Collagenase type II *(Gibco by Life technologies, #17101-015)*	295€
Minimum Essential Medium (MEM) - alpha modification *(Sigma Aldrich, #M4526)*	40,50€
StemPro® MSC serum-free media (*Thermo Fisher Scientific, #A1033201*)	370€
Trypsin/EDTA 0.05% Solution (*Gibco, #25300054*)	34,19€
PLASMIDS CONSTRUCTION	
pCIBN(deltaNLS)-pmGFP plasmid x2 (*gift from Chandra Tucker, addgene plasmid #26867*)	105,2€
CD9's cDNA, 1186 bp (*IDT product*)	190€
Tbx18's cDNA, 1968 bp (*IDT product*)	290€
pCMV-CRY2-mCherry plasmid (*gift from Won Do Heo, addgene plasmid # 58368*)	105,2€
Annealed oligos for CIBN-GFP-CD9 vector, 24 bp (4,8€) x2 (*IDT product*)	9,6€
Annealed oligos for CRY2-mCherry-Tbx18 vector, 18 bp (*3,6€*) x 4 (*IDT product*)	14,4€
HMSCs TRANSFECTION AND SELECTION	
Effectene Transfection Reagent (*Qiagen, #301425*)	274,4€
G418 1G liquid @ 100mg/ml (*Invivogen #ant-gn-5*)	89,46€
AgroMax Total Blue Solo Strip LED Grow Light Bar (115,73€) + High Yield 2 Outlet Digital Timer w/ Battery (16,16€) (*HTG Supply*)	131,89€
Anti CD34 antibody (ab) (*ThermoFisher Scientific #11-0349-42*)	178€
Anti CD73 ab (*ThermoFisher Scientific #11-0739-42*)	175€
Anti CD90 ab (*ThermoFisher Scientific # 11-0909-42*)	191€
Anti CD105 ab (*ThermoFisher Scientific # 12-1057-42*)	199€
Anti CD166 ab (*ThermoFisher Scientific # 12-1668-42*)	238€
CARBODIIMIDE CROSSLINKER CHEMISTRY AND PCM PEPTIDE FUNCTIONALIZATION	
EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) (*ThermoFisher Scientific # 22980*)	157€
4-Pentynoic acid 95% (*Sigma Aldrich, # 232211*)	40€
Sulfo-NHS (N-hydroxysulfosuccinimide) (*ThermoFisher Scientific #24510*)	301€
Click-IT™ AHA (L-Azidohomoalanine) (*ThermoFisher Scientific #C10102*)	257€
PCM peptide >95% purified, 21 aa (*Biomatik product*)	260€
CLICK CHEMISTRY CATALYSTS AND LIGANDS	
Phosphate buffered saline, bioultra solution 1L (*Sigma Aldrich, #79378*):	210€
Copper sulfate ($CuSO_4$) (*Sigma Aldrich #451657*)	76€
Tris-(hydroxypropyl)triazolylmehylamine (THPTA) (*Sigma Aldrich #762342*)	64€
Sodium L-ascorbate crystalline (*Sigma Aldrich # A7631*)	48€
Aminoguanidine hydrochloride (*Sigma Aldrich # 396494*)	46,30€
IPSCs' CARDIAC DIFFERENTIATION AND PURIFICATION	
iPSC epithelial-1 (*Sigma Aldrich, # IPSC0028*)	1.455€
Matrigel coated plates, hESC qualified x2 (*Corning, #354277*)	648€
mTESR1 medium (*Stem Cell Technologies, #85850*)	299€
BMP4 (*Sigma Aldrich, #SRP3016*)	241,50€

Item	Cost
CHIR99021 (*Sigma Aldrich, #SML1046*)	346€
RPMI1640 medium (*ThermoFisher Scientific, #21875109*)	124€
B27 supplement (*ThermoFisher Scientific, #17504044*)	97,25€
B27 supplement minus insulin (*ThermoFisher Scientific, #A1895601*)	89,50€
RPMI 1640 medium, no glucose (*ThermoFisher Scientific, #11879020*)	53,75€
IWR1 WNT inhibitor (*Sigma Aldrich, #I0161*)	320€
Sodium L-lactate (*Sigma Aldrich, #71718*)	65,80€
IN VITRO CULTURE AND STAINING OF iPSCs-DERIVED CARDIOMYOCYTES	
Cardiomyocyte Maintenance Medium (*Axol Bioscience, #ax2530-500*)	254€
15% Acrylamide solution (*SRH-Life Science, #AB79391-01*)	43€
N,N'-Methylenebisacrylamide powder (*Sigma Aldrich, #M7279*)	58€
Sodium Phosphate Monobasic (*Sigma Aldrich, #S8282*)	178€
Potassium Persulfate (*Sigma Aldrich, #216224*)	39,50€
TEMED (*Sigma Aldrich, #GE17-1312-01*)	47,90€
Poly-tetrafluoroethylene (*Sigma Aldrich, #430943*)	220€
Hepes pH 8,5 (*Avantor, #7365-45-9*)	90,55€
Human fibronectin (*Sigma Aldrich, #F1056*)	223€
4,969-diamidino-2-phenylindole dihydrochloride (*Sigma Aldrich, #D8417*)	113€
ANIMALS	
35-40 Kg adult Landrace pigs (363€ each) x 16	5808€
Maintenance (18,15€/pig/day) for 35 days (635,25€/pig) x 16 pigs	10.164€
Surgery (121€/pig) x 16 pigs	1936€
Initial electrophysiological study (1000€) x 16	16.000€
Final electrophysiological study (1000€) x 8	8.000€
Electronic pacemaker (1000€) x 16	16.000€
SAMPLES RENDERING AND ANALYSIS	
Russell-Movat pentachrome kit (*American MasterTech #KTRMP*)	303,59€
Anti CD3 ab (*Genway Biotech #GWB-12CD5F)*	306,22€
Anti CD25 ab (*ThermoFisher Scientific #MA5-16753*)	427€
Anti HCN4 ab *(ThermoFisher Scientific #MA3-903)*	393€
Anti Cx45 ab *(ThermoFisher Scientific #41-5800)*	331€
Anti Kir2.1 ab *(Rockland Immunochemicals, #200-301-F87)*	331,93€
Anti sarcomeric alfa actinin ab *(ThermoFisher Scientific #MA1-22863)*	412€
Goat anti-mouse IgG secondary ab Alexa fluor plus 488 x3 *(ThermoFisher Scientific #A32723)*	855€
RIPA lysis and extraction Buffer (*ThermoFisher Scientific # 89900*)	92,50€
Halt™ Protease Inhibitor Cocktail (100X) (*ThermoFisher Scientific #78430*)	193€
APO-BrdU™ TUNEL Assay Kit (*ThermoFisher Scientific #A23210*)	516€
Patch-Clamp Technology	40.632

TOTAL EXPENSES 112.065€

ANNEX 7 - Animal suffering assessment

Suffering assessment score

Weight loss	No loss of weight	0
	Weight loss below 10%	1
	Weight loss between 10 and 20%	2
	Weight loss above 20%; the animal is not drinking or eating	3
Appearance	Normal	0
	Fur in bad condition	1
	Fur in bad condition and/or presence of ocular or nasal secretions	2
	Abnormal posture	3
Spontaneous behavior	Normal	0
	Inability to move normally	1
	Inability to reach food/water or isolation from other animals	2
	Tendency to hide/retreat, no response to stimuli	3
Behavior in response to handling	Normal	0
	Slight changes	1
	Moderate changes	2
	Aggressive or comatose	3
Clinical parameters	Normal	0
	Hypothermia, fever, isolated arrhythmia	1
	Cardiac failure signs, pulmonary edema, repeated arrhythmia	2
	Cardiogenic shock, ascites >10% of weight, recurrent arrhythmias	3

0 to 4 points	Observation
5 to 9 points	Supervise carefully; consider analgesics and/or other treatment*
10 to 14 points	Intense suffering; provide analgesics and/or other treatment* and consider euthanasia
15 to 20 points	Euthanasia^

*Envisaged corrective measures depending on animal condition:

- *Pulmonary edema*: Furosemide.
- *Arrhythmia*: Amiodarone.
- *Pain*: Anti-inflammatory drugs.
- *Maintained hypotension*: IV saline solution.
- *V. fibrillation*: External defibrillation.
- *Hematomas at puncture sites*: Antithrombotic cream
- *Cardiogenic Shock*: IV saline solution and high-flow oxygen therapy. If non-response, euthanasia.

Score-independent endpoint criteria

- Comatose state.
- Evidence of severe heart failure.
- Reiterated and/or refractory arrhythmias.
- Any other non-treatable extreme suffering.

^When the follow-up period has been completed or when animal suffering has been deemed intense and unmanageable, euthanasia will be humanely performed by anesthetic overdose with Pentobarbital after previous sedation.