Video Article

Generation of Murine Cardiac Pacemaker Cell Aggregates Based on ES-Cell-Programming in Combination with Myh6-Promoter-Selection

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Abstract

Treatment of the “sick sinus syndrome” is based on artificial pacemakers. These bear hazards such as battery failure and infections. Moreover, they lack hormone responsiveness and the overall procedure is cost-intensive. “Biological pacemakers” generated from PSCs may become an alternative, yet the typical content of pacemaker cells in Embryoid Bodies (EBs) is extremely low. The described protocol combines “forward programming” of murine PSCs *via* the sinus node inducer TBX3 with Myh6-promoter based antibiotic selection. This yields cardiomyocyte aggregates consistent of >80% physiologically functional pacemaker cells. These “induced-sinoatrial-bodies” (“iSABs”) are spontaneously contracting at yet unreached frequencies (400-500 bpm) corresponding to nodal cells isolated from mouse hearts and are able to pace murine myocardium *ex vivo*. Using the described protocol highly pure sinus nodal single cells can be generated which *e.g.* can be used for *in vitro* drug testing. Furthermore, the iSABs generated according to this protocol may become a crucial step towards heart tissue engineering.

Video Link

The video component of this article can be found at http://www.jove.com/video/52465/

Introduction

The term “sick sinus syndrome” summarizes multiple diseases leading to the deterioration of the cardiac pacemaker system. It comprises pathological, symptomatic sinus bradycardia, sinoatrial block, sinus arrest as well as the tachycardia-bradycardia syndrome. Thereby, a “sick sinus syndrome” is often accompanied by general cardiac diseases such as an ischemic heart disease, cardiomyopathies or myocarditis. At present, therapeutic approaches are based on the implantation of electrical pacemakers. However, this goes along with a number of risks such as infections and battery failure. Overall, the incidence of complications is still very high in patients having implanted an artificial pacemaker. Furthermore, as opposed to the endogenous pacemaker, these devices do not respond to hormone stimulation.

A future alternative may rely on the availability of “biological pacemakers” for which PSCs could serve as a suitable cellular source and which would also be highly valuable for *in vitro* drug testing. Yet, a major problem lies in the very rare appearance of sinus nodal cells within embryoid bodies (EBs) - this typically does not exceed ~0.5%[1].

Previously, it was shown that “forward programming” towards specific cardiomyocyte subtypes is feasible via overexpression of distinct early cardiovascular transcription factors such as Mesoderm-specific-posterior 1 (MesP1) and NK2 transcription factor related, locus 5 (Nkx2.5)[2,3]. For normal size and function of the sinoatrial node (SAN), the T-box transcription factor Tbx3 is crucial, which has been shown to initiate the pacemaker gene program and to control differentiation of the SAN[4]. While this enhanced the appearance of functional pacemaker cells, the content still did not exceed ~40% within the entire cardiomyocytic cell population.

Therefore, an additional Myh6-promoter based antibiotic selection step[5] was introduced by us. This ultimately leads to yet unobserved cardiomyocyte aggregates (“induced sino-atrial bodies; “iSABs”) which exhibit highly increased beating frequencies (>400 bpm) *in vitro*, for the first time approximating those of a murine heart and comparable to in vitro cultivated sinus nodal cells isolated from a murine heart[6]. Under Isoprenaline administration even beating frequencies of 550 bpm are achieved. Notably, iSABs consist of over 80% functional nodal cells as evident from extensive physiological analyses[7]. Recently, several approaches to generate sinus nodal cells using direct reprogramming[19], surface markers[14] or pharmacological treatment with small molecules[16,17] were described. Yet, none of these methods led to such a high purity of pacemaker cells and beating frequencies close to the murine heart as observed in iSABs.

Moreover, in an *ex vivo* model of cultivated adult mouse ventricular slices which have lost their spontaneous beating activity, the iSABs are capable of integrating into the slice tissue, thereby remaining spontaneously active and robustly pacing the heart slices to contractions[7]. A detailed protocol for the generation of these iSABs is described in this paper.

Protocol

1. Recommendations Before Starting

1. Do not use PSCs contaminated with mycoplasma because they will not differentiate properly into sinus node cells. Test for mycoplasma contamination before starting the protocol. Do this using a PCR kit for rapid, highly sensitive detection of mycoplasmas and follow the manufacturer`s protocol.
2. For each Petri dish (step 2.3.4), coat one 10 cm^2 cell culture dish with sterile 7 ml 0.1 % gelatin from cold water fish skin for 1 hr at 37 °C. Remove the gelatin and let the dish dry under sterile conditions in a sterile bench.
3. Before you can start the differentiation protocol you need a double transfected stable mouse ES cell line clone containing the following features: i) Constitutive over-expression of TBX3 using a mammalian expression vector. Ii) The G418 resistance gene under the control of the Myh6-promoter[5].
4. The undifferentiated PSCs cells should be co-cultivated with irradiated feeder cells under standard conditions as described [1].

2. Differentiation Procedure

1. Two Days before Differentiation - Remove PSCs from feeder cells.
    1. Aspirate the medium, wash the cells with 10 ml phosphate buffered saline PBS, add 7 ml Collagenase IV solution and incubate the cells for 10 min at 37 °C. Place a sterile 40 µm Filter in a 50 ml tube.
    2. Carefully rinse the PSC colonies (avoid to remove the feeder cells) by pipetting the collagenase solution up and down 5 times.
    3. Transfer the cell suspension to a 40 µm filter, rinse the filter three times with 8 ml PBS. Turn around the filter and place it upside down in a petri dish. Remove the PSC cell colonies by pipetting 10 ml PBS to the bottom of the filter.
    4. Transfer the cell suspension to a 15 ml tube and centrifuge for 5 min at 300 x g.
    5. Remove the PBS, suspend the cells in 1 ml Accutase and incubate at 37 °C for 5 min.
    6. Add 10 ml PBS, mix the cell solution by pipetting it up and down 5 times and centrifuge for 5 min at 300 x g.
    7. Remove the PBS, suspend the cells in 10 ml cultivation medium and determine the cell number.
    8. Seed 15,000 cells/ cm^2 on a 75 cm^2 filter flask and cultivate them for 2 days at 37 °C, 5% CO2. After 2 days the flask should be 50-70% confluent.

2. Day 0 - Start of Differentiation
    1. Remove the medium and wash the cells with 10 ml PBS.
    2. Aspirate the PBS, add 2 ml Accutase and incubate the cells at 37 °C for 5 min. Place a sterile 40 µm Filter in a 50 ml tube.
    3. Add 10 ml PBS, transfer the cell suspension to the 40 µm filter, rinse the filter via addition of 10 ml PBS and centrifuge the flow-through for 5 min at 300 x g.
    4. Suspend the cells in 10 ml differentiation medium and determine the cell number.
    5. Dilute the cell suspension with differentiation medium to a final concentration of 20,000 cells/ml.
    6. Pipette 20 ml water and 5 ml hanging drop (HD) solution in a quadratic Petri dish to avoid drying-out of the HDs.
       NOTE: For each 24 well plate containing iSABs (see section 2.8.6.4/2.8.7) start with 16 Petri dishes.
    7. Pipette up to 50 ml cell suspension in a tray .
    8. Turn around the lid of the petri dish. Place 180 HDs each containing 20 µl (400 cells/HD) cell suspension onto the lid using a 12 channel pipette.
    9. Carefully turn around the lid and place it onto the Petri dish.
       NOTE: The speed of turning around the lid is very important. If the lid is turned around too slow or too fast, the surface tension of the cell suspension is not high enough to retain the hanging drops on the lid. Before trying to produce HDs for first time practice turning around the lid with 20 µl drops of medium.
    10. Cultivate the cells for 2 days at 37 °C, 5% CO_2 to let them form EBs.
        NOTE: Place one petri dish filled with water on the top of a stack of 5 Petri dishes. Otherwise the HD in the upper petri dish will dry.

3. Day 2
    1. Carefully turn around the lid of the quadratic Petri dish and transfer the EBs derived from two Petri dishes (360 EBs) to a 50 ml tube.
    2. Wait 10 min to let the EBs settle at the bottom of the tube (Do not centrifuge the EBs).
    3. Aspirate as much medium as possible, suspend the EBs in 10 ml differentiation medium and transfer the suspension to a 10 cm Petri dish.

4. Day 2-6 Suspension culture
    1. Cultivate the EBs in suspension for 4 days at 37 °C, 5% CO_2, change medium after 2 days.
       NOTE: Even though the petri dish is not coated (as opposed to a cell culture dish) the EBs often attach to the surface. Control the petri dishes for attached EBs every day and if necessary detach the EBs from the surface by gentle pipetting. Optional: Shake the petri dishes continuously during the suspension culture period. Be careful with the speed of the shaker. The EBs should neither accumulate in the middle nor float to the border of the Petri dishes.

5. Day 6
    1. Transfer the EBs from one Petri dish to one gelatin coated 10 cm^2 cell culture dish.

6. Day 7-12
    1. The cells should start to beat after 8-12 days. Check for beating foci of the cells daily under a microscope. The cells start to form a layer.

2. Change medium daily.
   NOTE: After changing medium, the cells require 3-4 hr to recover and to start beating again.

7. Day 12-15 Selection of sinus nodal cells.
   1. Three days after the cells have started beating (around day 12-15), aspirate the medium and pipette 10 ml of fresh differentiation medium containing 400 µg/ ml G418 to the cells.

8. Four days after the beginning of the selection dissociate the cell layer.
   NOTE: It is possible that the cell layer is already detached from the surface.
   1. Remove the medium carefully without aspirating the cell layer.
   2. Add 10 ml of PBS and remove the PBS carefully without aspirating the cell layer.
   3. Add 10 ml of Collagenase IV solution, transfer the cells to a 50 ml tube and incubate the cells at 37 °C for 5 min.
   4. Vigorously mix the suspension by pipetting up and down 10 times and incubate the cells at 37 °C for 5 min. A suspension of small clusters should occur. If there are still huge parts of the layer left, then repeat step 2.8.4 two more times.
   5. Add 20 ml of PBS and centrifuge for 10 min at 300 x g.
   6. Aspirate the PBS carefully.
      NOTE: If iSABs are to be generated proceed with step 2.8.7. If iSAB derived sinus nodal single cells are to be generated go on with step 2.9.
   7. Suspend the cells in 12 ml differentiation medium containing 400 µg/ml G418 and seed them on 6 wells of gelatin coated 24 wells (2 ml/well).

9. Generation of single nodal cell.
   1. Suspend the cells from step 2.8.6 in 5 ml Accutase and incubate at 37 °C for 5 min.
   2. Vigorously mix the suspension by pipetting up and down 10 times and incubate the cells at 37 °C for 5 min.
   3. Add 20 ml of PBS and centrifuge for 5 min at 300 x g.
   4. Suspend the cells in 12 ml differentiation medium containing 400 µg/ml G418 and seed them on 6 wells of a gelatin coated 24 wells (2 ml/well).

10. Day 2 after dissociation, aspirate the medium and wash the cells three times with PBS. Add 1 ml differentiation medium/well.
11. Day 4-8 after dissociation, change Medium every 2nd day. On day 10 after Dissociation use iSABs or iSAB derived sinus nodal single cells are available for further analysis.

Representative Results

The described protocol allows generation of iSABs with a beating frequency of around 450 bpm from PSCs (shown in the movie) which is near to the mouse heart beating frequency. After dissociation of iSABs (step 2.8.8) the observed single cells show the typical shape of cells of the sinus node (spindle and spider cells) as shown in **Figure 1**. These cells highly express proteins which are known to be essential for the function of the sinus node like hyperpolarization-activated cyclic nucleotide-gated cation channel 4 (Hcn4), Connexin45 (Cx45), Connexin30.2 (Cx30.2) and Myh6 (**Figure 2A**-**C**). After treatment of iSAB derived cells with pharmaceuticals, the cells show the expected behavior: like in the sinus node, the funny channel blocker ZD7288 (**Figure 3A**) as well as the muscarinic receptor agonist carbachol (**Figure 3B**), both cause a significantly reduced beating frequency of iSAB derived cells. The β-adrenoreceptor agonist isoprenaline leads to an elevated beating frequency (**Figure 3C**).

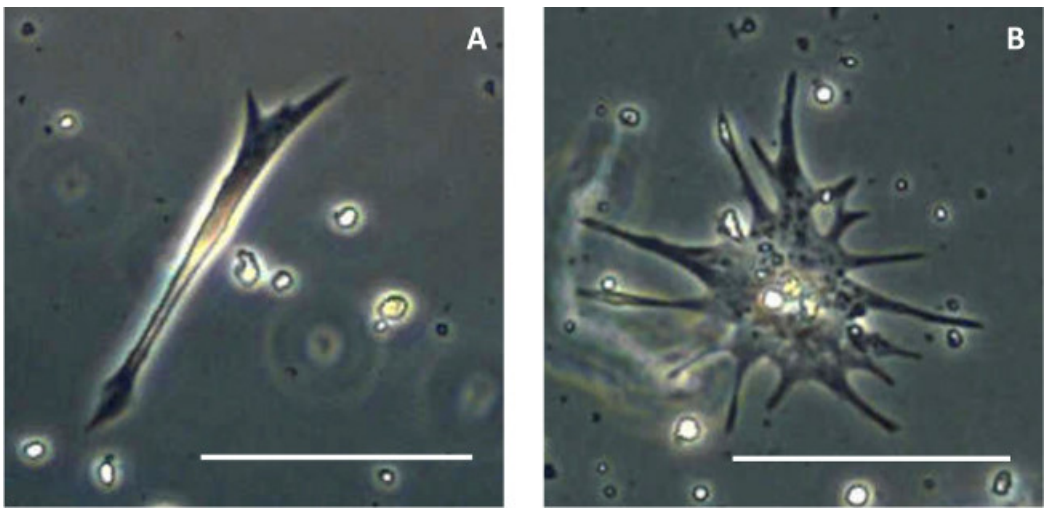

Figure 1: Cellular shape of spindle and spider cells Typical cellular shape of iSAB derived spindle **(A)** and spider **(B)** nodal cell. Scale bar 20 µm.

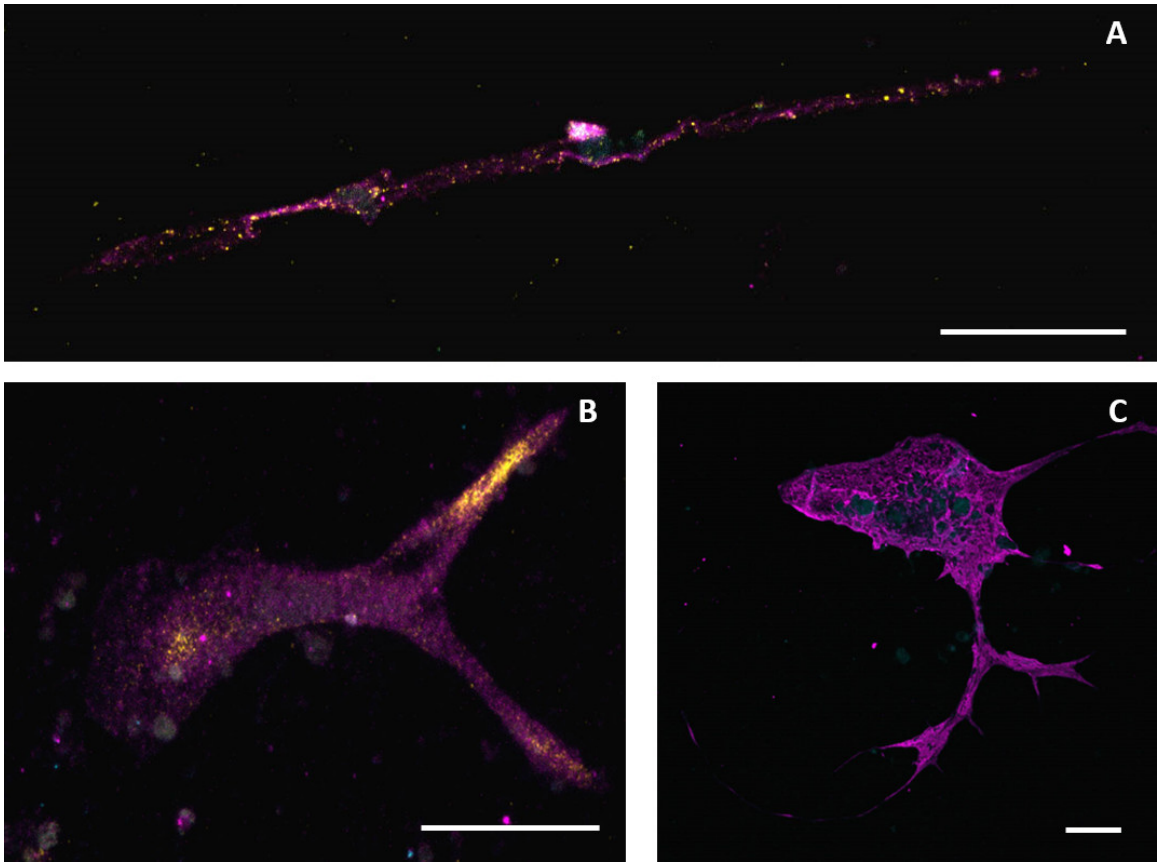

Figure 2: Expression of sinus node markers. (A) Expression of HCN4 (yellow) and Cx45 (magenta), **(B)** Expression of Cx45 (magenta) and Cx30.2 (yellow) and **(C)** Myh6 (magenta) in sinus node cells. Counterstaining of nuclei (turquois). Scale bar 20 µm.

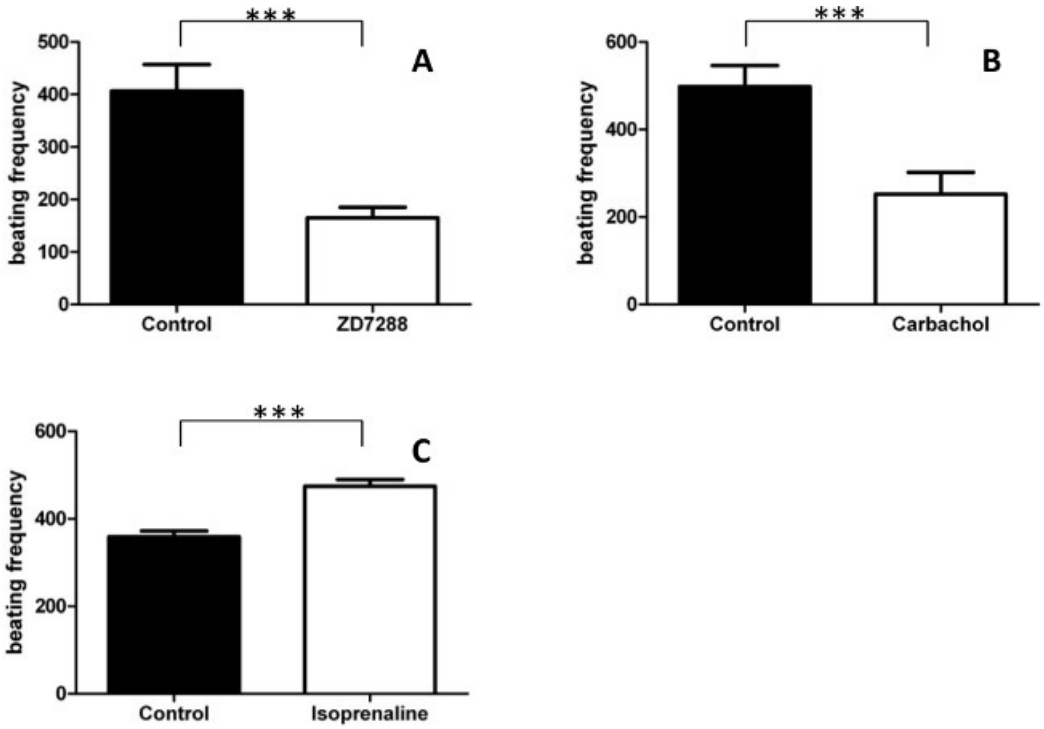

Figure 3: Pharmacological treatmentof iSABs Representative beating frequency of iSABs before (control) and after treatment with ZD7288 **(A)**, Carbachol **(B)** and Isoprenaline **(C)**. The data represent eight independent iSABs and are presented as mean ± SD. *** p <0.001

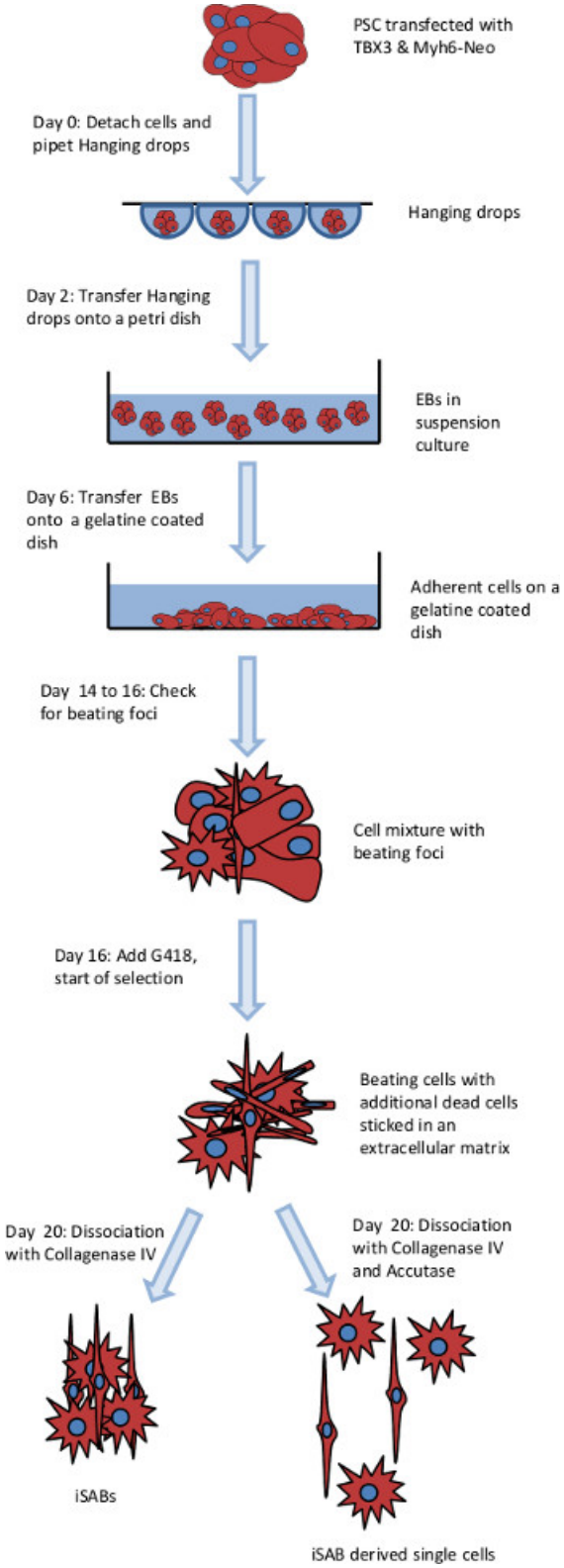

Figure 4: Schematic of the differentiation protocol. Simplified cartoon reflecting the experimental flow-chart for iSAB generation.

Movie: Beating of iSABs Beating of a typical induced sinoatrial body (iSAB) after dissociation. Please click here to view this video.

Discussion

The ability to produce stem cell derived cardiac pacemaker cells may allow reconstitution of the proper cardiac rhythm in the sense of "biological pacemakers". Likewise, drug testing in vitro will benefit from their availability. PSCs can give rise to any cell type of the mammalian body including cardiomyocytes with pacemaker cell properties[8,9,10,11,12,13]. However, typically the cell populations within "Embryoid Bodies" are highly heterogeneous, which inevitably leads to the requirement of reliable selection and isolation strategies – this applies in particular to the very rare cell type of cardiac nodal cells. Numerous approaches based on exogenous and endogenous surface markers[14], on pharmacological administration of small molecules[15,16,17] and on direct reprogramming strategies[18,19] have been followed. In addition, non-stem-cell-based approaches aimed at emulating biological pacemakers via manipulation of terminal effector molecules underlying sarcolemmal electrophysiology instead of de novo generating fully functional nodal cells[20,21].

Yet, neither of these came up with the necessary high spontaneous contraction frequencies and cellular purity. In contrast, our protocol leads to cells that do not only exhibit electrical oscillations but also the subtle electrophysiological and calcium signaling characteristics as well as distinctive morphological features of endogenous pacemaker cells[7]. This technology may become an important prerequisite for alternatives to electronic pacing devices.

Although this protocol was optimized during its development, there are still some steps that may cause problems: The starting point for the selection of iSAB is critical of the protocol. Beating of the cells is an indicator for αMHC-Expression in differentiating cells. Myh6-Expression goes along with expression of the G418 resistance gene. Starting the selection too early will extinguish a lot of the cells which potentially would differentiate into cardiomyocytes and will therefore decrease the overall yield of sinus nodal cells. Another critical step is the dissociation of the cell layer (step 2.8). The reason for this is that the cells form a robust extracellular matrix and a strong force is necessary to dissociate the cell layer. If necessary, step 2.8.4 should be repeated till small clusters of cells have developed from the cell layer. Avoid direct dissociation of the cell layer by *e.g.* trypsin because it turned out to be of very low efficiency.

For future clinical applications, the following obstacles still need to be addressed: transference of the approach to the generation of human iSABs which may become a crucial step towards future cell therapy and *in vitro* drug-testing. Also, the technique is still based on stable genetic modifications of PSCs and therefore will need to be modified before it can be applied to patients.

Disclosures

The authors have nothing to disclose.

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