Fluorescent gene reporters in human pluripotent stem cells : as model for studying human heart development and cardiomyocyte differentiation

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CHAPTER 6

A Novel Approach for the Pure Isolation of Ventricular Cardiomyocytes from human Embryonic Stem Cells: a dual MYL2-T2A-mCherry NKX2-5-eGFP Fluorescent Reporter Line

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ABSTRACT

In recent years, differentiation of human pluripotent stem cells (PSCs) to the cardiac lineage has improved considerably leading to mixtures of cardiomyocyte subtypes. This allowed researchers to study human cardiac development and differentiation, and to obtain cardiomyocytes (CMs) for pre-clinical applications, such as drug discovery, safety pharmacology, disease modelling and cell-based therapies for cardiac repair. However, in order to increase the predictability and the robustness of stem cell based assays and follow-up experiments, it is essential to obtain pure populations of CM subtypes. Here, we describe the generation of a MYL2-T2A-mCherry-NKX2-5-eGFP hESC reporter line in order to study and steer *in vitro* cardiac differentiation to a ventricular cardiomyocyte subtype. MYL2 encodes for ventricular myosin light chain-2 (MLC-2v) and is specifically expressed in ventricular cardiomyocytes. During cardiac differentiation, MYL2 only becomes markedly expressed after 1 month of differentiation. Fluorescent labelling of cells expressing MYL2 will allow us to isolate ventricular cardiomyocytes and study their molecular and electrophysiological characteristics. Moreover, this may contribute to the identification of regulators that are important for the formation of the ventricular-specific-lineage. In this chapter, we focus on the technical challenges that we faced upon the generation of this fluorescent reporter line and our preliminary results on the identification of MYL2-T2A-mCherry expressing cardiomyocytes during cardiac differentiation.

INTRODUCTION

The heart is one of the first organs formed in the developing human embryo, indicating its vital importance in supplying oxygen and nutrients to the developing organism and at the later adult stage[1]. Formation of the developing heart is complex and requires a balanced interplay between specific molecular signalling pathways and transcription factors under tight temporal and spatial control. Congenital heart disease encompasses abnormalities in the developing heart due to mutations in important regulators of heart development, but may also be caused by prenatal environmental influences[2]. It is important to understand how these underlying defects result in the onset of congenital heart disease, which requires a complete understanding of the developing human heart. Acquired heart diseases, such as myocardial infarction, mostly result in a massive loss of ventricular cardiomyocytes (CMs) due to an ischemic event, leading to decreased cardiac function[1]. As cardiac disease is still a leading cause of morbidity and mortality worldwide, there is an urgent need for a better understanding of its onset and progression, and in the end, better treatment options[3, 4]. The adult human heart has no or limited regenerative capacity, and is therefore unable to restore damaged cardiac cells due to ischemia or cardiomyopathies, resulting in progressive heart failure[5]. Replacement of damaged heart tissue with viable hPSC-derived cardiomyocytes may be a possible therapeutic strategy. In particular, the possibility to generate human iPSC-derived cardiomyocytes created the opportunity to use cardiomyocytes for autologous transplantation, as opposed to the ethically sensitive use of human embryonic stem cells (hESCs). Optimized hPSC-CM protocols have been developed and result in 50-95% CM differentiation efficiencies[6]. Moreover, the use of hPSC-CMs offers an exclusive physiologically relevant human model system for drug discovery and pre-clinical development strategies. However, current cardiac differentiation protocols result in heterogeneous pools of CMs. Although these mixed cultures predominantly consist of ventricular-like cells, there is a significant percentage of other cardiomyocyte subtypes, such as atrial-like and nodal-like cells[7]. For high reliability in predictive pharmacological compound screenings, pure populations of cardiac subtypes are necessary. In particular, ventricular cardiomyocytes are of interest, since in the majority of cases, genetic cardiac disorders and cardiac injury affect ventricular cardiomyocytes.

The generation of fluorescent pluripotent stem cell reporter lines offer an excellent opportunity to study the derivation of specific cellular subtypes[8, 9]. In order to obtain pure populations of ventricular CMs, here, we generated a MYL2 (MLC2V) fluorescent reporter in the previously described NKX2-5-eGFP hESC line[9]. *In vivo*, MLC2V expression is restricted to the ventricular segment of the developing human heart, followed by expressed in the entire ventricular compartment in the adult heart[10]. Previous studies have described the generation of MYL2 reporter lines in mouse ESCs, reporting a specific MYL2 promoter element or endogenous MYL2 expression[11, 12]. Here, we reported endogenous MYL2 expression in hESCs through a fusion strategy, making use of a cleaving 2A peptide sequence which is positioned in frame between MYL2 and the coding sequence of mCherry[13]. 2A-mediated cleavage is a universal phenomenon in all eukaryotic cells. However, it has not been observed in prokaryotic cells[14]. Transcriptional activity of the MYL2 transcript leads to production of a single messenger RNA encoding both proteins, MYL2 and mCherry. Here, we will discuss our technical approach for the generation of a MYL2-fluorescent reporter line, preliminary results, and possible future strategies for obtaining pure ventricular CM populations.

MATERIAL AND METHODS

Generation of the MYL2 targeting construct

To generate the *MYL2* targeting vector, bacterial artificial chromosome RP11-587H5, containing the MYL2 sequence (Source Bioscience, Lifesciences, Nottingham, UK), was modified by recombineering (Gene Bridges, Heidelberg, Germany) **(Fig. 1)**. An R6k plasmid was generated through cloning, containing the T2A sequence[15], followed by mCherry (in-frame), and a neomycin selection cassette, flanked by LoxP recombination sites. 20ug of the complete cassette of interest was linearized by the two enzymes PvuI and EcorV, restricting an upstream PvuI site, and a downstream EcoRV site **(Fig. 1a)**. Recombineering

was performed using the Red/ET recombination technology (Genebridges). Here, we introduced the Red/ET plasmid (pSC101-BAD-gbaA-tet) into E. Coli containing BAC RP11-587H5 through electroporation. Genes from plasmid pRed/ET promote base precise exchange of DNA sequences flanked by homology arms. The exonuclease Redα and the DNA annealing protein Redβ catalyze the *in vivo* reaction. Further, two primers were designed, containing 40bp that were homologous to the ends of the linearized R6K cassette, and containing 50bp homologous to the region of the MYL2 BAC upstream and downstream of the MYL2 stopcodon (**Fig 1b**). Addition of 10% L-arabinose (to a final concentration of 0.1%) for induction of RedET genes, followed by electroporation of the linearized R6k cassette and both primers into the MYL2 BAC containing E. Coli, resulted in quadruple recombination of the BAC. Oligonucleotide primers oligo1 and oligo2 were used for recombineering (**Table 1**). Recombineered BACs were screened by PCR; 7 out of 40 clones displayed a correct product size (17.5%)(**Fig. 1c**). For clone 3, PCR products were generated for the 3' homology arm (HA) and 5' HA recombined regions, and sent for sequencing (100% correct). The modified MYL2 locus, including 5' and 3' homology arms, were subsequently subcloned into a MYL2 targeting vector. For this, a PCR (Phusion, Thermo Scientific) was performed on a linearized minimal vector (digested by XhoI and HindIII), containing a Diphtheria Toxin A (DTA) sequence for negative selection, an ampicillin resistance cassette, and an origin of replication (ORI) sequence (**Fig. 2a**). Oligos that are used to generate this PCR product contain extra 50bp sequences that are homologous to the BAC sequence that are upstream (5' HA, 8.8kb) and downstream (3' HA, 2.3kb), so that regions in between these homologous regions are subcloned into a final target construct, containing the PCR product as backbone (**Fig. 2a, Table 1**). For subcloning, the pRed/ET plasmid was re-introduced into the E.Coli, containing modified BAC clone 3, and activated by L-arabinose prior to electroporation of the PCR product of the minimal vector. The newly subcloned minimal vectors were then isolated and screened by PCR; 80% of the clones was positive (**Fig. 2b**). Three clones were further verified using Bgl1 and XbaI digestions (**Fig. 2c**). Clone 3.9 displayed the correct sequence and was further used for MYL2 targeting.

Generation of the MYL2-T2A-mCherry Reporter Line

The final targeting vector comprised a 8.8-kb 5_homology arm, T2A-mCherry, loxP-flanked G418 resistance cassette, a 2.3-kb 3_homology arm, and a Diphtheria Toxin A (DTA) sequence for negative selection (**Fig. 2a**). The targeting vector was linearized by PvuI digestion and 20 µg was electroporated into $10x10^6$ NKX2-5-eGFP reporter hESCs (HES3-NKX2-5eGFP/w) [9] as described previously[16]. Homologous recombination will result in replacement of the MYL2 STOP codon, localized in exon 7. Targeted clones were identified by PCR using oligonucleotides specific for mCherry (MYL2_screen_F1: CCCCCTGAACCTGAAACATA) and a region immediately 3' of the targeting vector (MYL2_screen_R1: GCATCTCTGTATGTCCCTGTG) (**Fig. 3a,b**). After identification of correctly targeted clones, five hESC clones were maintained as single cell cultures, enzymatically passaged, and transfected with a Cre recombinase construct using lipofectamin (Invitrogen) for removal of the neomycin cassette. 24 Hours after transfection, cells were cultured in the presence of puromycin (1ug/mL) for 36 hours. After puromycin selection, single cells were sorted based on SSEA-4 expression, screened for neomycin excision, and further cultured as single cells (**Fig. 3a,c**).

By the use of a T2A cleavage peptide to report MYL2 gene activity, endogenous gene expression remains normal. Here, we replaced the MYL2 stopcodon with the T2A and mCherry sequences (in-frame), and a selection cassette, which is excised after targeting to prevent interference with transcriptional activity. Upon MYL2 activation, one RNA transcript is generated, followed by ribosomal translation, where T2A is cleaved through ribosomal skipping, resulting in the presence of two separate proteins MYL2 and mCherry (**Fig. 3e**).

Flow Cytometry

For analysis on MYL2-T2A-mCherry and NKX2-5-eGFP expression, cells were dissociated using 1x or 10x TrypLe Select (Life Technologies). After dissociation, cells were resuspended in FACS Wash buffer (PBS, 2% FCS (Gibco), 2mM EDTA) and filtered through a 40µm cell strainer (Falcon). Cells were analyzed using a Miltenyi VYB Flow Cytometer using a 488 nm and a 561 nm laser.

Single Cell Sorting:

For single cell sorting, hESCs were enzymatically passaged using 1x TrypLE Select (Life Technologies). After dissociation, cells were resuspended in FACS sorting buffer (PBS with 0.5% BSA and 2.5mM EDTA) and filtered through a 40µm cell strainer (Falcon). Cells were sorted using a BD ARIA III flow cytometer into 96 wells flat bottom low attachment plates. Live cells were gated on the basis of side scatter, forward scatter and propidium iodide exclusion. Flow cytometric gates were set using control cells labelled with the appropriate isotype control antibody.

Cardiac differentiation

MYL2-mCherry/w-NKX2-5-eGFP/w hESCs were cultured on mouse embryonic fibroblasts in 2.85 cm2 organ dishes (Falcon), in 1 mL hESC medium (DMEM F12, Non Essential Amino Acids, Knock Out Serum (Gibco by Life Technologies); bFGF (Miltenyi Biotec)) and were mechanically passaged. One or two days before differentiation, cells were passaged and seeded in a 12-well tissue culture dish coated with Matrigel (BD Biosciences; growth factor reduced, phenol red–free), in 1 mL of hESC medium per well. On the first day of differentiation, day 0, hESC medium was replaced by low insulin serum-free medium BPEL (BSA, polyvinyl alcohol, essential lipids, as previously described[9] containing the cytokines BMP4 (30 ng ml−1, R&D Systems) and Activin A (30 ngml−1, Miltenyi Biotec), and Chir99021 (1.5uM, AxonMedchem). At day 3 of differentiation, the medium was refreshed with BPEL containing 5uM Xav939 (R&D). On day 7 of differentiation, the medium was replaced with BPEL without growth factors.

To enhance MYL2 expression, cardiomyocytes were dissociated at day 21 of differentiation with 10x TrypLe (Gibco) for 10 minutes at room temperature, and plated as single cells in Cardiomyocyte Medium (CM) (Pluriomics BV). After 7 days, medium was changed to commercially available maturation medium (MM), containing T3 hormone (Pluricyte medium, Pluriomics BV).

RESULTS

Generation of the Dual Cardiac Reporter hESC Line MYL2-T2A-mCherry-NKX2-5-eGFP

In order to isolate and characterize ventricular-specific cardiomyocytes, derived from hPSCs, we generated a MYL2 fluorescent reporter line in the previously generated NKX2-5-eGFP hESC line[9]. The MYL2 targeting construct was generated, linearized and electroporated into NKX2-5-eGFP hESC line (**Fig3a**). Homologous recombination resulted in the replacement of the MYL2 stopcodon with the T2A-mCherry sequences and a selection cassette (**Fig3a**). Correctly targeted clones were identified by PCR screening (**Fig 3a, b, c**). Five correctly targeted clones were obtained (18% targeting efficiency), transfected with Cre recombinase for excision of the neomycin-resistance cassette, and after selection of 36 hours, sorted as single cells. From these clones, one clone (8.1D1) that showed neomycin excision was selected for further experiments in this study (**Fig 3c**). A schematic overview of the newly generated dual reporter line is given in Figure 2a.

Ventricular Cardiomyocyte Derivation from hESCs

To study the presence of MYL2-mCherry expressing cells upon cardiac differentiation from hESCs, we analysed cardiac cells on mCherry and eGFP expression at different timepoints during differentiation (**Fig. 4a**). In order to increase expression of MYL2 in cardiomyocytes, cells were dissociated at day 21 of differentiation and further maintained in physiologically optimized CM medium (*Pluriomics, BV*). 7 Days after dissociation (day 28 since the start of differentiation), we analysed MYL2-T2A-mCherry expression by FACS. We observed that ~30% of cells expressed NKX2-5-eGFP levels at day 28 of differentiation. However, only about ~3% from the total differentiated cell population was expressing MYL2-T2A-mCherry, which was ±16% from total NKX2-5-eGFP expressing cardiac cells (**Fig. 4b**). It has been previously shown that MYL2 expression is only strongly increased after approximately one month of cardiac differentiation[12]. Therefore, we extended cardiomyocyte differentiation in the presence of maturation-promoting media (MM). At day 56 of differentiation, cells were analysed by FACS (**Fig. 4b**). When we cultured dissociated CMs in CM medium, or additionally in MM medium, we detected a decreased percentage of NKX2-5-eGFP expressing cells (~7-17%), which is most likely due to the proliferative capacity of non-cardiomyocytes that are present in these differentiation cultures. Nonetheless, we found a clear increase in the percentage of MYL2-T2A-mCherry positive cells in the NKX2-5-eGFP positive populations (~27-39%), indicating an increase in the proportion of ventricular cardiomyocytes in prolonged and optimized culture conditions (**Fig 4b**). In agreement, we could confirm the presence of double NKX2-5-eGFP+MYL2-T2A-mCherry+ cardiomyocytes by fluorescent microscopy, showing endogenous mCherry expression in day 67 dissociated cardiomyocytes (**Fig 4c**). Interestingly, when we measured NKX2-5-eGFP+MYL2-T2A-mCherry+ levels in undissociated cultures at day 56 of differentiation, which were continuously cultured in BPEL medium, we could almost not detect any MYL2-T2A-mCherry expressing cells (~0.2%) (**Fig. 4b**). Thus, either by dissociating cardiomyocytes, and thereby inducing mechanical stress, or by culturing in medium containing maturation-inducing-components, or both, may result in MYL2 upregulation, allowing detection and isolation of MYL2-T2A-mCherry positive cells.

DISCUSSION

Here, we describe the generation of a MYL2-fluorescent reporter line for the identification of hESC-derived ventricular CMs, in order to allow isolation and to further study ventricular-specific CMs. We developed a targeting strategy in which one allele of MYL2 was genetically modified into a bicistronic site, by replacing the stop codon of MYL2 for the coding sequences for a T2A viral peptide and fluorescent protein mCherry. When transcription of MYL2 is activated, one single messenger RNA molecule is transcribed. Upon ribosomal translation, T2A is cleaved, resulting in the presence of the two separate proteins MYL2 and mCherry. Small peptide T2A is described to be more reliable and efficient than the currently used internal ribosomal entry site (IRES) and leads to the expression of multiple cistrons at equimolar levels[13, 17-19]. Moreover, the use of a multi-cistronic fusion reporter construct, can be superior to a knockin replacement strategy in which one allele of the gene of interest is replaced by the fluorescent reporter construct, resulting in haplo-insufficiency and thus differential expression levels of the gene.

Here, we show that upon cardiac differentiation, up to day 28, only low levels of MYL2 expressing cardiomyocytes could be detected. This is in alignment with previous cardiac differentiation studies in mESCs, where also only 0.5 - 1.2 % of MYL2+ expressing cells could be identified between day 10 and day 30 of differentiation[11, 12].

This is in significant contrast with the presence of high numbers of ventricular CMs in current cardiomyocyte cultures, based on electrophysiology data[20], indicating the preference to search for an alternative ventricular marker for the efficient isolation of early ventricular CMs during differentiation. Only one earlier study in hESCs reports the presence of 67-98% MYL2-eGFP positive cardiomyocytes, but this has not been repeated[21]. Here, they made use of a randomly integrated reporter construct, which expression could have been influenced by surrounding genomic regulatory elements.

Further, we show that either dissociation of cardiac monolayers into single cell cultures or culturing cardiomyocytes in thyroid hormone-based culture medium additionally enhances MYL2 levels. Thyroid hormone is described to be a critical regulator of cardiac growth and

development, both in fetal life and postnatally, and induces mature electrophysiological properties[12, 22-24]. In our study, it will be of further interest to isolate the double positive MYL2-T2A-mCherry-NKX2-5-eGFP cardiomyocytes, and study their electrophysiological and molecular properties, including genome wide analysis for the identification of ventricular-specific cell surface markers and important molecular regulators. The identification of a ventricular-specific cell surface marker, would allow ventricular CM isolation from any PSC-derived cardiac culture, without the need of genetic modifications. Here, we show that, although MYL2 could potentially be a proper marker for ventricular CM isolation, due to its low expression in early ventricular CMs, the identification of an additional early ventricular-specific marker would be a preference for the isolation of (early) ventricular CMs. Moreover, as MYL2 expression levels increase upon further maturation of our cardiomyocytes, it could be a potential maturation marker.

FIGURES

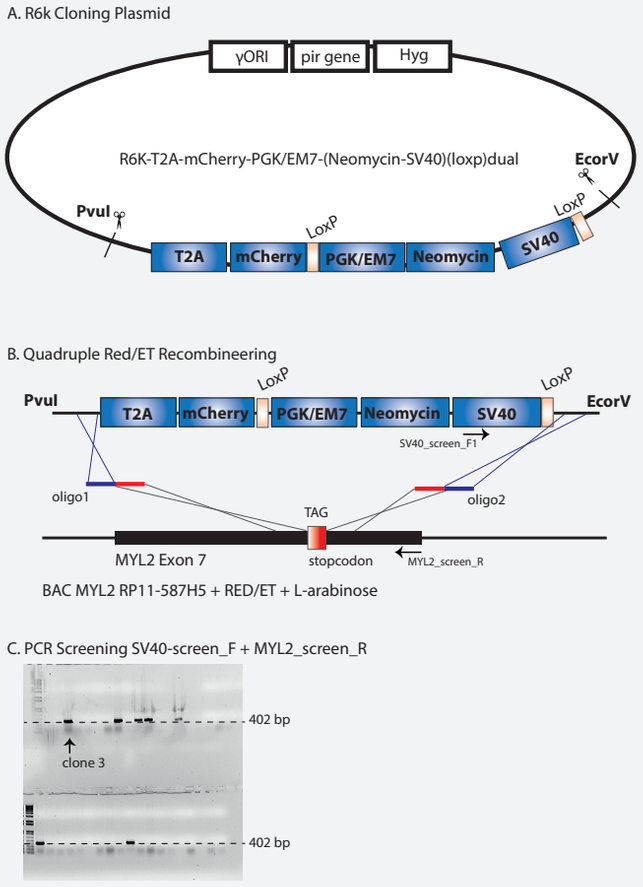

Figure 1. MYL2 BAC recombineering. **A:** An R6k cloning plasmid containing the T2A sequence, in-frame with the following mCherry sequence, and a eukaryote/bacterial selection cassette, was digested with PvuI and EcorV to obtain a linearized construct. **B:** A BAC, containing the MYL2 coding sequence, was modified through Red/ET quadruple recombineering (GeneBridges BV) T2A, mCherry, and the selection cassette replaced the TAG stopcodon sequence of MYL2, located in its last exon 7. **C:** PCR screening on BAC clones to identify for correctly modified BAC.

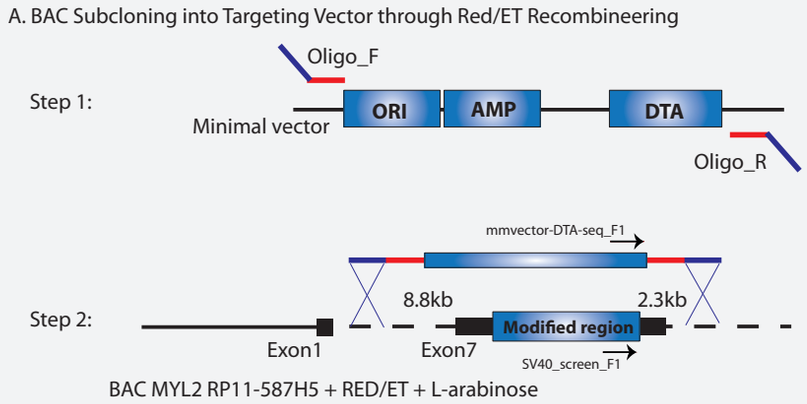

Figure 2. BAC subcloning into targeting vector. **A:** Step 1: A minimal vector containing an ampicillin-resistance cassette, and a DTA sequence for negative selection, was digested into a linearized construct. A PCR construct was generated, using oligos with 50 bp long arms (purple) that were homologous to regions in the modified MYL2 BAC, 8.8kb upstream of the modified region (Oligo_F), and 2.3kb downstream (Oligo_R). Step 2: The MYL2 modified region in the BAC was subcloned into the PCR construct through the homologous regions between BAC and PCR construct ends (purple). **B:** A PCR was performed to screen for correctly recombineered subclones. **C:** Integrity of a selection of subclones was checked by digestion with either BglI or XbaI. Clone 3.9 was used for further MYL2 genomic targeting in hESCs.

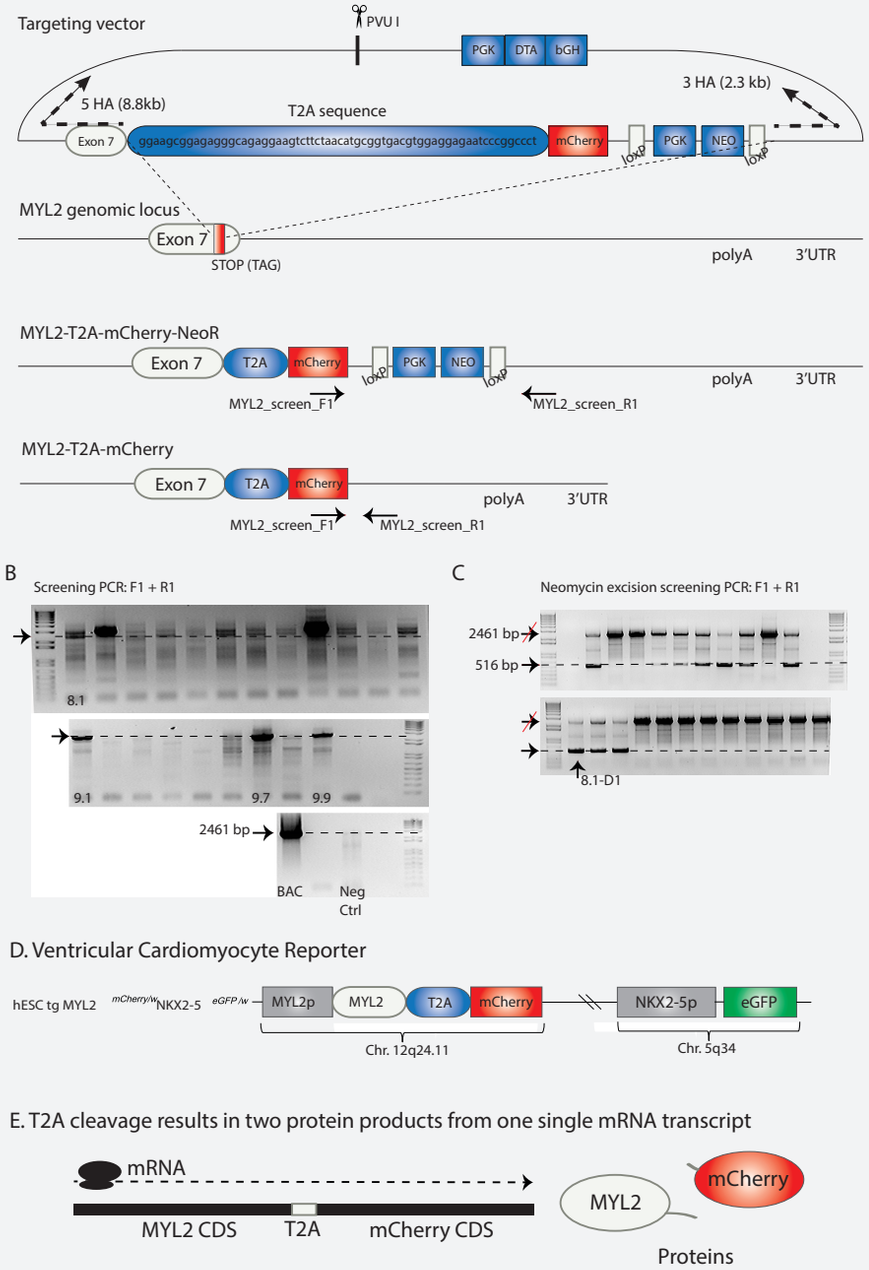

Figure 3. MYL2 targeting in NKX2-5$^{eGFP/w}$ hESCs. A: The MYL2 targeting construct was designed to replace the MYL2 stopcodon, located in exon 7. Prior to targeting, the construct was linearized through PvuI digestion, and electroporated into NKX2-5eGFP/w hESCs[9]. Targeted cells were selected through their resistance to G418, which was added to the culture medium after electroporation (Oligos used: MYL2_screen_F1 + MYL2_screen_R1). Cre-mediated excision of the selection cassette (through the presence of two distal loxP sites) resulted in the final MYL2-T2A-mCherry reporter line. **B:** PCR screening of selected clones. Positive control was the modified MYL2 BAC, showing a 2461 bp band. Negative control was the unmodified NKX2-5$^{eGFP/w}$ hESCs line. **C:** PCR screening on neomycin cassette excision, which would result in a 516 bp band, and the absence of the 2461 bp band. **D:** Overview of the finally generated dual cardiac reporter line. **E:** Schematic drawing of the ribosomal translation of one mRNA transcript containing both MYL2 CDS and mCherry CDS. Cleavage of the T2A sequence during ribosomal translation results in two separate proteins.

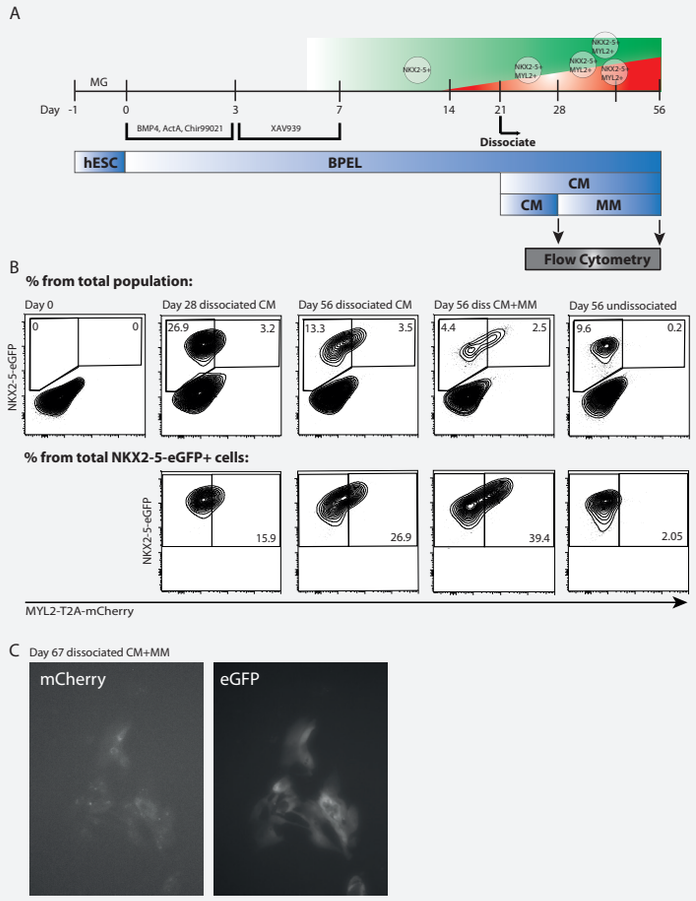

Figure 4. Directing hESC differentiation towards ventricular cardiomyocytes. A: Overview of cardiac monolayer differentiation protocol. hESCs were plated on matrigel, one day prior to induction with BPEL containing BMP4, Activin-A, and GSK3-inhibitor Chir99021. After three days, growth factors were replaced with BPEL containing Wnt signalling inhibitor Xav939. This was replaced with plain BPEL for further continuous culture. At day 21 of differentiation, cells were dissociated and further cultured.

BAC Quadruple Recombineering	
Oligo1	TGGACTACAAGAACCTGGTGCACATCATCACCCACGGAGAAGAGAAGGACggaagcggagagggcagaggaagtcttctaacatgcggtg
Oligo2	CAGGGACCACTCTGCAAAGACGAGCCCAGGGCGCAGCAGCGAGCCCCCTCGATGGATGCAGAGTGTGCGTAGACTATGAACGTACTTAAC
BAC screening	
SV40-screen_F	CCCCCTGAACCTGAAACATA
MYL2_screen_R1	GCAAAGAAGATGGAGGTGGA
Subcloning	
Oligo_F1	CTGTCAAGTGGATACTATTATTATCATCCCCATTTTACAGGAGAGGCAACGTCGACGCTCTCCTGAGTAGGACAAATC
Oligo_R1	cagagttggtccaggccgagagcctcataccgggctcctgtggtcagagtcgacTCCGCCTCAGAAGCCATAGA
Subcloning screening	
SV40-screen_F	CCCCCTGAACCTGAAACATA
mmvector-DTA-seq_F1	CGATCTCTTTGTGAAGGAACC
MYL2 Targeting Screening	
MYL2_screen_F1	CCCCGTAATGCAGAAGAAGA
MYL2_screen_R1	GCAAAGAAGATGGAGGTGGA
MYL2 Neomycin Excision Screening	
MYL2_screen_F1	CCCCGTAATGCAGAAGAAGA
MYL2_screen_R1	GCAAAGAAGATGGAGGTGGA

Table 1. Oligo sequences

Genes	Forward primer	Reverse primer
hARP	CACCATTGAAATCCTGAGTGATGT	TGACCAGCCCAAAGGAGAAG
MYL2	GATGTTCGCCGCCTTCCCCC	GCAGCGAGCCCCCTCCTAGT
MYH6	GCTGGCCCTTCAACTACAGA	CTTCTCCACCTTAGCCCTGG
MYH7	GAGGACAAGGTCAACACCCT	CGCACCTTCTTCTCTTGCTC

Table 2. Quantitative PCR primers for human genes

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