Journal of Molecular and Cellular Cardiology 97 (2016) 278–285

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

A series of robust genetic indicators for definitive identification of cardiomyocytes

Jianyun Yan [a,1], Lu Zhang [a,1], Nishat Sultana [a,1], Jae Gyun Oh [b], Bingruo Wu [c], Roger J. Hajjar [b], Bin Zhou [c], Chen-Leng Cai [a,*]

[a] Department of Developmental and Regenerative Biology, The Black Family Stem Cell Institute, and The Mindich Child Health and Development Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
[b] Department of Medicine, Cardiovascular Research Center, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA
[c] Departments of Genetics, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461, USA

ARTICLE INFO

Article history:
Received 17 May 2016
Accepted 2 June 2016
Available online 3 June 2016

Keywords:
Heart regeneration
Heart development
Myocardial reporter
Tnnt2
Myh6

ABSTRACT

Definitively identifying the cell type of newly generated cells in the heart and defining their origins are central questions in cardiac regenerative medicine. Currently, it is challenging to ascertain the myocardial identity and to track myocardial progeny during heart development and disease due to lack of proper genetic tools. This may lead to many misinterpretations of the findings in cardiac regenerative biology. In this study, we developed a set of novel mouse models by inserting double reporter genes nlacZ/H2B-GFP, mGFP/H2B-mCherry into the start codon of *Tnnt2* and *Myh6*. nlacZ (nuclear lacZ) and mGFP (membrane GFP) are flanked by two LoxP sites in these animals. We found that the reporter genes faithfully recapitulated *Tnnt2* and *Myh6* cardiac expression from embryonic stage and adulthood. The reporter mice provide unprecedented robustness and fidelity for visualizing and tracing cardiomyocytes with nuclear or cell membrane localization signals. These animal models offer superior genetic tools to meet a critical need in studies of heart development, cardiac stem cell biology and cardiac regenerative medicine.

1. Introduction

Heart failure with myocardial loss and dysfunction is the main cause of morbidity and mortality in the world [1,2]. The transplantation of cardiac stem cells (CSCs) [3,4] or induced pluripotent stem cells (iPSCs) [5], the stimulation of the resident CSCs [6,7], the direct reprogramming of committed cells to cardiomyocytes [8,9], and the stimulation of cell cycle of the existing cardiomyocytes [10–12] are among the main strategies that may provide effective treatments in patients with heart failure. In studying these therapies with animal models, one prominent obstacle is to precisely conclude the identities of the newly generated cardiomyocytes upon treatment. Direct labeling cardiomyocytes has been an immediate, critical need in cardiac regenerative medicine.

Tnnt2 (*Troponin T Type 2, cardiac Troponin T/cTnT*) and *Myh6* (*Myosin Heavy Chain 6, α-MHC*) encode sarcomeric proteins specifically expressed in the cardiomyocytes but not in the non-cardiomyocyte populations (endothelium, smooth muscles, and fibroblasts) in the mammalian heart [13,14]. *Tnnt2* expression begins early, soon after the cardiac mesodermal precursors differentiate into myocardial cells during development, at embryonic day (E) 7.5 in mice [15]. *Myh6* myocardial expression begins slightly later (E8.0) but is very specific in the myocardium during gestation, and the level is elevated after birth [14]. In this study, we generated a set of mouse models by knocking-in reporter genes *lacZ*, *GFP* and *mCherry* on *Tnnt2* and *Myh6* loci to directly visualize cardiomyocytes during heart formation. We found these genetic reporters provide unprecedented robustness and fidelity for visualizing and tracing cardiomyocytes with nuclear or cell membrane location signals. These animals offer superior genetic tools and will have important applications in cardiac studies in the future, especially in determining mechanisms of heart development, repair and regeneration.

2. Methods

2.1. Mouse models

Tnnt2$^{nlacZ\text{-}H2B\text{-}GFP/+}$, *Tnnt2*$^{mGFP\text{-}H2B\text{-}mCherry/+}$, *Myh6*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ and *Myh6*$^{mGFP\text{-}H2B\text{-}mCherry/+}$ mouse lines were generated by gene targeting. Vectors contained the reporter cassette *LoxP-nlacZ-4XpolyA-LoxP-H2B-*

* Corresponding author at: Department of Developmental and Regenerative Biology, Icahn School of Medicine at Mount Sinai, 1 Gustave L. Levy Pl, New York, NY 10029, USA.
E-mail address: chenleng.cai@mssm.edu (C.-L. Cai).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.yjmcc.2016.06.003
0022-2828/

GFP-polyA-FRT-Neo-FRT or *LoxP-mGFP-4XpolyA-LoxP-H2B-mCherry-polyA-FRT-Neo-FRT* are flanked by a 5′ homologous arm and a 3′ homologous arm. Linearized vectors were transfected into mouse 129/SvJ ES cells by electroporation. Long-range PCR was used to identify the targeted ES cells using the Expand Long Range dNTPack Kit (Roche, USA). The two pairs of primers (P1 + P2, P3 + P4) used for PCR were as follows:

P1: CAGTCCCTGTTCAGAGGTAAGACA (*Tnnt2*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ and *Tnnt2*$^{mGFP\text{-}H2B\text{-}mCherry/+}$); GGTGTAGGAACTGACCAGTGACTT (*Myh6*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ and *Myh6*$^{mGFP\text{-}H2B\text{-}mCherry/+}$); P2: ATTCGCGTCTGGCC TTCCTGTAGC (*Tnnt2*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ and *Myh6*$^{nlacZ\text{-}H2B\text{-}GFP/+}$); GACACGCTGA ACTTGTGGCCGTTT (*Tnnt2*$^{mGFP\text{-}H2B\text{-}mCherry/+}$ and *Myh6*$^{mGFP\text{-}H2B\text{-}mCherry/+}$); P3: GCGAGCACGTACTCGGATGGAAGC; P4: GTGACAGGACATCAAGACTC ACTG. (*Tnnt2*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ and *Tnnt2*$^{mGFP\text{-}H2B\text{-}mCherry/+}$); AAGAGGGAGCAA CGAGGTTGGACT (*Myh6*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ and *Myh6*$^{mGFP\text{-}H2B\text{-}mCherry/+}$).

Primers P1 and P4 were external to the homologous arms. P2 and P3 were located in the reporter cassettes (Supplementary Figs. 1 and 2). PCR products were further confirmed by DNA sequencing. The targeted ES cells were microinjected into blastocysts to generate chimeric mice. F1 germline transmission mice were obtained by crossing the chimeric male mice with Black Swiss female mice. The *Neo* cassette flanked by two *FRT* sites was removed by crossing F1 mice with *FLPe* deleter mice [16]. Genotypes were determined by tail PCR.

All animal experiments were conducted in accordance with the Guide for the Use and Care of Laboratory Animals with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Icahn School of Medicine at Mount Sinai.

2.2. Echocardiography

Transthoracic echocardiography of *Tnnt2*$^{nlacZ\text{-}H2B\text{-}GFP/+}$, *Tnnt2*$^{mGFP\text{-}H2B\text{-}mCherry/+}$, *Myh6*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ and *Myh6*$^{mGFP\text{-}H2B\text{-}mCherry/+}$ animals (n = 3–5 for each line, P60–120) was performed using the Vevo 2100 high-resolution ultrasound imaging system (VisualSonics, Canada) with methods described previously [17,18].

2.3. X-gal staining

X-gal staining was performed to examine β-galactosidase encoded by *lacZ*. For whole-mount staining, embryos or hearts were harvested and dissected in ice-cold phosphate-buffered saline (PBS), followed by fixation in 4% paraformaldehyde/PBS for 30 min on ice. The fixed samples were washed twice with PBS and incubated in X-gal solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ and 1 mg/ml X-gal overnight at room temperature. For cryosectioning, the hearts were placed in 30% sucrose/PBS solution overnight at 4 °C and then embedded in Optimal Cutting Temperature (OCT) compound (Tissue-Tek) on dry ice. All samples were sectioned to 10 μm thickness (coronal). Sections were stained in X-gal solution at 37 °C overnight.

2.4. Immunofluorescence

Frozen hearts were sectioned to a thickness of 6 μm (coronal). After being washed with PBS, the cryosections were blocked with 10% goat normal serum in PBS for 30 min at room temperature, followed by incubation with the primary antibody mouse anti-Troponin T (1:200, Thermo scientific) or rabbit anti-Nkx2.5 (1:50, Abcam) for 1 h at room temperature. The cryosections were rinsed in PBS and then incubated with Alexa Fluor 594-conjugated secondary antibodies (1:500; Invitrogen) for 45 min at room temperature and counterstained with DAPI. The sections were visualized and photographed under a Leica fluorescence microscope.

3. Results

3.1. *Tnnt2*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ and *Myh6*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ double reporters specifically mark all the cardiomyocytes

We first generated *Tnnt2*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ and *Myh6*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ knock-in mouse models by inserting a *LoxP-nlacZ-4XpolyA-LoxP-H2B-GFP-polyA-FRT-Neo-FRT* cassette into the start codons of *Tnnt2* and *Myh6* (Supplementary Fig. 1A and Supplementary Fig. 2A). Nuclear *lacZ* (*nlacZ*) with *4XpolyA* stop signals (*nlacZ-4XpolyA*) is flanked by two *LoxP* sites, and *H2B-GFP* is an enhanced *GFP* fused with the human histone *H2B* gene [19] at the N-terminus. Both nlacZ and H2B-GFP expression are restricted to the nuclei under the control of *Tnnt2* or *Myh6* full regulatory elements (without deletion genomic sequence). *Tnnt2*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ and *Myh6*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ hearts (and also the hearts of *Tnnt2*$^{mGFP\text{-}H2B\text{-}mCherry/+}$ and *Myh6*$^{mGFP\text{-}H2B\text{-}mCherry/+}$ mice described below) develop normally and functional abnormalities were not detected with echocardiography analysis (data not shown, n = 3–5/line, postnatal day (P) 60–120).

We tested the reporter genes by X-gal staining of *Tnnt2*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ and *Myh6*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ mice. Whole mount staining showed strong lacZ expression in the hearts of *Tnnt2*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ mice at embryonic day (E) 7.5, 8.5, 12.5, 16.5, neonatal P0 and postnatal P60 (Fig. 1A), but not in the aorta or pulmonary artery (Fig. 1A, asterisks). Further staining of cardiac sections revealed evenly distributed lacZ expression in all four chambers as well as the atrial and ventricular septa at all embryonic stages (Fig. 1B). No ectopic expression was observed in the valves or the coronary vessels (Fig. 1B, arrows). Similarly, lacZ expression in *Myh6*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ mice was detected in the myocardium (Fig. 1D), but not in the aorta, the pulmonary artery (Fig. 1D, asterisks), or the valves (Fig. 1E). Notably, lacZ expression in the atria of *Myh6*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ hearts was much stronger than that in the ventricles during gestation, and the difference became less perceptible at the adult stage (Fig. 1D6). In both *Tnnt2*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ and *Myh6*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ hearts, H2B-GFP expression is completely prevented and undetectable, thereby confirming a strong stop signal in the *4XpolyA* cassette (Supplementary Fig. 3).

To determine whether the knock-in reporter genes driven by the *Tnnt2* and *Myh6* loci are restricted to all the cardiomyocytes, *Tnnt2*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ and *Myh6*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ mice were crossed with *Protamine-Cre* mice [20] to generate *Tnnt2*$^{H2B\text{-}GFP/+}$ and *Myh6*$^{H2B\text{-}GFP/+}$ animals (Supplementary Fig. 1A and Supplementary Fig. 2A). *Tnnt2*$^{H2B\text{-}GFP/+}$ hearts were stained using antibodies against Nkx2.5 and *Tnnt2*. We found the H2B-GFP signals are fully co-localized with Nkx2.5 and *Tnnt2* antibody staining from the embryonic stages (E13.5) to adulthood (Figs. 1C and 2A–D). Compared to *Tnnt2*$^{H2B\text{-}GFP/+}$ mice, which exhibit almost uniform GFP expression in all four chambers, *Myh6*$^{H2B\text{-}GFP/+}$ mice exhibit stronger GFP fluorescence in the atria than in the ventricles (Fig. 2E), and higher GFP expression in the trabecular myocardium than in the compact myocardium of the ventricles at embryonic stages (Fig. 2F1). All GFP-expressing cells in *Myh6*$^{H2B\text{-}GFP/+}$ hearts were Nkx2.5-positive and *Tnnt2*-positive cells (Figs. 1F and 2F, G). We further isolated single cardiac cells from adult *Tnnt2*$^{nlacZ\text{-}H2B\text{-}GFP/+}$, *Myh6*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ and *Tnnt2*$^{H2B\text{-}GFP/+}$ hearts (P60–90), respectively. Nuclear lacZ and GFP expression was exclusively present in the cardiomyocyte population (Fig. 3A–C). These observations demonstrate that the *Tnnt2*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ and *Myh6*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ reporter alleles specifically label all the myocardial cells in the heart.

3.2. *Tnnt2*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ and *Myh6*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ mice are ideal genetic tools to determine myocardial lineage

We next attempted to determine whether *Tnnt2*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ and *Myh6*$^{nlacZ\text{-}H2B\text{-}GFP/+}$ mice are suitable for tracing myocardial progeny derived from cardiac progenitor or stem cells. The *Mef2c*$^{AHF\text{-}Cre}$ transgenic allele delineates anterior heart field (AHF) or second heart field (SHF) cardiac precursors [21]. *Mef2c*$^{AHF\text{-}Cre}$ mice were crossed with