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Generation of a tamoxifen inducible *Tnnt2*^{MerCreMer} knock-in mouse model for cardiac studies

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Summary

Tnnt2, encoding thin-filament sarcomeric protein cardiac troponin T, plays critical roles in heart development and function in mammals. To develop an inducible genetic deletion strategy in myocardial cells, we generated a new *Tnnt2*:*MerCreMer* (*Tnnt2*^{MerCreMer/+}) knock-in mouse. *Rosa26* reporter lines were used to examine the specificity and efficiency of the inducible Cre recombinase. We found that Cre was specifically and robustly expressed in the cardiomyocytes at embryonic and adult stages following tamoxifen induction. The knock-in allele on *Tnnt2* locus does not impact cardiac function. These results suggest that this new *Tnnt2*^{MerCreMer/+} mouse could be applied towards the temporal genetic deletion of genes of interests in cardiomyocytes with Cre-LoxP technology. The *Tnnt2*^{MerCreMer/+} mouse model also provides a useful tool to trace myocardial lineage during development and repair after cardiac injury.

Keywords

Tnnt2; *MerCreMer*; cardiomyocyte; tamoxifen; heart

Introduction

Cre recombinase from bacteriophage P1 recognizes specific 34-bp LoxP sequences and excises LoxP-flanked DNA at high efficiency (Branda and Dymecki, 2004; Sauer and Henderson, 1988). Conditional knockout with Cre-LoxP technology allows inactivation of genes in a tissue-specific manner, and has greatly advanced our knowledge on genes' function in heart development and disease (Huang *et al.*, 2012; Jiao *et al.*, 2006; Song *et al.*, 2007). To achieve myocardial specific deletion, a number of Cre deleter mouse lines have been created based on the myocardial genes including myosin light chain 2v (*MLC2v*), cardiac myosin heavy chain (*Myh6*) and cardiac Troponin T (*Tnnt2*) (Agah *et al.*, 1997;

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Chen *et al.*, 1998; Jiao *et al.*, 2003; Moses *et al.*, 2001). However, these Cre lines are not inducible and therefore cannot mediate a temporal genetic deletion at specific stages. Many genes are important for heart development and function at early embryonic and later postnatal stages (Bruneau *et al.*, 2001; Cai *et al.*, 2003; Kuo *et al.*, 1997; Lyons *et al.*, 1995), and inactivation of these genes with straight Cre lines may lead to early lethality, preventing further analyses of their roles at later stages. To overcome embryonic or neonatal lethality, an inducible system allowing temporal inactivation in cardiomyocytes has been developed using a tetracycline-controlled transactivator (tTA) driven by a rat MHC promoter (Fishman *et al.*, 1994), and a reverse tetracycline-controlled transactivator (rtTA) directed by a rat *Tnnt2* promoter (Wu *et al.*, 2010). The tetracycline-inducible system requires two independent types of transgenic alleles including transactivator *tTA* or *rtTA*, and tetracycline responsive promoter *TetO*. In this system, administration of tetracycline for induction is water drinking based, and the tTA or “Tet-off” system sometimes does not mediate instant Cre excision (Robbins, 2004).

The inducible Cre recombinases are fusion proteins consisting of Cre recombinase and a specific ligand-binding domain, and can be activated by an exogenous ligand such as tamoxifen (Brocard *et al.*, 1997; Indra *et al.*, 1999; Zhang *et al.*, 1996). Several inducible Cre recombinase mouse lines have been developed including Cre-ERT, Cre-ERT2 and MerCreMer (Brocard *et al.*, 1997; Indra *et al.*, 1999; Zhang *et al.*, 1996). Compared with other inducible Cre recombinases, the MerCreMer system has been demonstrated to be stringent (Verrou *et al.*, 1999). In this study, we report generation of a *MerCreMer* knock-in mouse line based on the *Tnnt2* locus in mice.

RESULTS AND DISCUSSION

Generation and characterization of the *Tnnt2*^{MerCreMer/+} knock-in allele

Cardiac troponin T (cTnT or *Tnnt2*) is the tropomyosin-binding subunit of troponin (Tn) complex in the cardiomyocytes, and it plays a critical role in regulating cardiac muscle contraction by anchoring two other Tn components, troponin I (TnI) and troponin C, to tropomyosin on thin filaments (Lombardi *et al.*, 2008; Nishii *et al.*, 2008). *Tnnt2* has been widely recognized as a marker for cardiomyocytes from early embryonic stages to adulthood (Koyanagi *et al.*, 2007; Sato *et al.*, 2004; Walsh *et al.*, 2010). Given this, we decided to make a new inducible Cre mouse line, *Tnnt2*^{MerCreMer/+}.

To generate this animal, *MerCreMer-FRT-Neo-FRT* cassette was inserted into the start codon of *Tnnt2* through gene targeting (Figure 1A). Long range PCR was performed to screen the targeted ES cells with two pairs of primers (P1/P2 and P3/P4) to detect recombination at 5' and 3' arms. 5.5-kb and 4.7-kb DNA fragments were amplified when homologous recombination occurs at 5' and 3' ends, respectively (Figure 1B). They were further confirmed by DNA sequencing. Targeted ES cells were expanded and injected into the fertilized mouse eggs to generate chimeras. Offspring from the chimeric mice were analyzed by tail PCR. The *Neo* cassette flanked by two *FRT* sites was removed by crossing *Tnnt2*^{MerCreMer/+} mice with Flippase deleter mice (Figure 1A) (Farley *et al.*, 2000).

***Tnnt2*^{MerCreMer/+} mediates robust and specific recombination in cardiomyocytes after tamoxifen administration**

To determine recombination efficiency mediated by *Tnnt2*^{MerCreMer/+} allele, the mice were crossed to *R26R*^{lacZ/lacZ} reporter mice (Soriano, 1999) which express β -galactosidase when the floxed stop cassette is removed. We first performed whole-mount X-gal staining on *Tnnt2*^{MerCreMer/+}; *R26R*^{lacZ/+} doubly heterozygous hearts without tamoxifen treatment at adult stage (3 months old). Very few (less than 3) β -galactosidase expressing cells were detected in the whole heart, indicating very minimal leakage of Cre excision without tamoxifen induction (Supplementary Figure 1).

We next injected tamoxifen intraperitoneally into the pregnant mice at embryonic day (E) 8.5, 10.5, 11.5, 14.5 or 16.5 for two consecutive days (24 hour interval, 0.05 mg/g body weight/day). Embryos or hearts were collected 48 hours later at E10.5, 12.5, 13.5, 16.5 and P0, respectively. With X-gal staining, we detected robust and uniform staining in the hearts, suggesting tamoxifen treatment was sufficient to initiate Cre-mediated recombination driven by *Tnnt2* at embryonic stages (Figure 2A-E). We also detected robust β -galactosidase expression in the postnatal heart at P30 and P60 after three times of tamoxifen injections (Figure 2F,G, 0.1 mg/g body weight/day). Further transverse sections of the hearts showed that X-gal staining was confined to the atrial and ventricular wall as well as the septum (notched arrows in Figure 2C-G), but was not in the aorta and pulmonary artery (arrowheads in Figure 2D1/E1/F1/G1), or valves (unnotched arrows in Figure 2D4/E4/F4/G4). These suggest that the *Tnnt2*^{MerCreMer/+} mouse line efficiently introduces recombination in the heart from embryonic stage to adulthood.

We then applied *R26R*^{tdTomato/tdTomato} mice (Madisen *et al.*, 2010) to examine cell types with recombination after tamoxifen induction. Consistent with the X-gal staining, we found tdTomato was also robustly expressed in the heart at E16.5, P0, P30 and P60 after 24 hours of tamoxifen induction (Figure 3A). Further immunostaining with antibody against *Tnnt2* (a marker for cardiomyocytes), α SMA (a marker for vascular smooth muscle cells) and PECAM (a marker for vascular endothelial and endocardial cells) (Hungerford *et al.*, 1996; Mack and Owens, 1999) showed that tdTomato is co-localized with anti-*Tnnt2* (notched arrows in Figure 3B-E), but not co-expressed with anti- α SMA or anti-PECAM in the coronary smooth muscle or endothelial/endocardial cells (unnotched arrows in Figure 3 F-I) from embryonic (E16.5) to adult stages (P60). tdTomato was not detected in the epicardial layer (arrowheads in Figure 3 F4). These observations suggested that *Tnnt2*^{MerCreMer/+} allele introduces specific recombination in the cardiomyocytes.

***Tnnt2*^{MerCreMer/+} mice have normal cardiac structure and function**

Tnnt2^{MerCreMer/+} is a heterozygous null allele for *Tnnt2* and homozygous null animals (*Tnnt2*^{MerCreMer/MerCreMer}) cannot survive beyond E10.5 with heart development defects. To assess the potential derangements in cardiac structure or function with heterozygous deletion of *Tnnt2*, male and female *Tnnt2*^{MerCreMer/+} mice were subjected to transthoracic echocardiography (n=11 for both wild type and *Tnnt2*^{MerCreMer/+} mice). We found *Tnnt2*^{MerCreMer/+} mice had normal left ventricular wall thickness, internal dimensions, and function when compared to the wild type littermate controls at 2–4 months old

(Supplementary Table 1), and no difference were found between male and female animals (data not shown). Representative M-mode images from *Tnnt2^{MerCreMer/+}* and wild type mice are shown in Figure 4 A. These results are in agreement with previous reports demonstrating normal cardiac indices in mice haploinsufficient for *Tnnt2* (Ahmad *et al.*, 2008; Nishii *et al.*, 2008).

Several reports showed that the myocardial inducible Cre mouse model *aMHC-MerCreMer* (Sohal *et al.*, 2001) had DNA damage with heart failure and even death at moderate to high dosage of tamoxifen administration (Bersell *et al.*, 2013; Buerger *et al.*, 2006; Hall *et al.*, 2011; Hougen *et al.*, 2010; Koitabashi *et al.*, 2009; Lexow *et al.*, 2013; Molkenin and Robbins, 2009). Specifically, three days/doses of tamoxifen administration at 0.03–0.09 mg /g body weight/day to the adult *aMHC-MerCreMer* mice resulted in 10–50% mortality within 1 week. The surviving animals displayed significant myocardial fibrosis and cardiac dysfunction within one month. High level Cre expression on *aMHC-MerCreMer* mice also leads to myocardial apoptosis within two weeks after injection (Bersell *et al.*, 2013; Lexow *et al.*, 2013). As a comparison, we evaluated cardiac tissue health, structure, and function on *Tnnt2^{MerCreMer/+}* mice (n=4, 2–3 months old) with wild type littermates (n=5, 2–3 months old) after three doses of tamoxifen at 0.1 mg/g body weight/day. We found that *Tnnt2^{MerCreMer/+}* mice did not display significant changes in myocardial apoptosis, fibrosis, wall thickness or cardiac function using transthoracic echocardiography within 1–5 weeks after tamoxifen administration (Figures 4 and 5, and Supplementary Table 1), suggesting Cre expression in *Tnnt2^{MerCreMer/+}* mice did not result in cardiotoxic effects with the dosage used in our study. The difference between *aMHC-MerCreMer* and *Tnnt2^{MerCreMer/+}* animals in response to tamoxifen might be due to the number of *MerCreMer* copies in their genome: *aMHC-MerCreMer* is a transgenic line that may contain multiple copies of *MerCreMer* in each myocardial cell, whereas *Tnnt2^{MerCreMer/+}* is a knock-in line where only one copy of *MerCreMer* is present in each myocardial cell. Therefore, Cre expression and nuclear translocation in the cardiomyocytes of *aMHC-MerCreMer* mice might be much higher than that of *Tnnt2^{MerCreMer/+}* animals with moderate to high dosage of tamoxifen.

In summary, we generated a new inducible *Tnnt2^{MerCreMer/+}* knock-in mouse model that allows temporal genetic deletion of genes of interest in the cardiomyocytes from embryonic stages to adulthood, providing a valuable tool to dissect functions of genes in cardiomyocytes at specific developmental time points. The *Tnnt2^{MerCreMer/+}* mice with *R26R* reporter alleles are also very useful genetic tool to trace myocardial progeny during heart development as well as regeneration after cardiac injury.

MATERIALS AND METHODS

Generation of the *Tnnt2^{MerCreMer/+}* knock-in mouse line

Tnnt2^{MerCreMer/+} knock-in mice were generated by inserting *MerCreMer-FRT-Neo-FRT* cassette into the start codon of *Tnnt2* locus, with disruption of endogenous ATG. Long range PCR was applied to screen the targeted ES cells using Expand Long Range dNTPack kit (Roche Cat # 04829042001) following the manufacturer's instructions. PCR program consisted of an initial denaturation of 2 min at 92°C, 42 cycles of reactions (92 °C for 10 s; 60 °C for 15 s, 68 °C for 4 min), and a final elongation of 10 min at 68 °C. The primers used

for long range PCR were: P1: CAGTCCCTGTTTCAGAGGTAAGACA; P2: CTCTTCTTCTTGGGCATGGTCTGC; P3: GCGAGCACGTACTCGGATGGAAGC; P4: GTGACAGGACATCAAGACTCACTG. PCR fragments from the targeted ES cells were confirmed by DNA sequencing. Positive ES cells carrying the recombinant allele were injected into blastocysts to generate chimera mice. *Tnnt2*^{MerCreMer-Neo/+} mice were obtained by crossing male chimeras with Black Swiss female mice. The animal model is available to the research community upon request.

Animal breeding and tamoxifen administration

Rosa26:lacZ (*R26R^{lacZ/lacZ}*) (Soriano, 1999) and *Rosa26:tdTomato* (*R26R^{tdTomato/tdTomato}*) (Madisen *et al.*, 2010) reporter mice were obtained from the Jackson Laboratory. *Tnnt2*^{MerCreMer/+} mice were bred with *Rosa26* reporter mice to generate double heterozygous animals (*Tnnt2*^{MerCreMer/+}; *R26R^{lacZ/+}* and *Tnnt2*^{MerCreMer/+}; *R26R^{tdTomato/+}*). Tamoxifen (Sigma-Aldrich) was dissolved in sesame oil at 10 mg/ml as stock solution. To induce Cre nuclear translocation, tamoxifen was administered to mice by intraperitoneal (IP) injection for two consecutive days (24 hours interval between each administration) at 0.05 mg/g body weight/day for embryonic stages, and 0.1 mg/g body weight/day for three consecutive days (or day 1, 3, 5) for adulthood. 24 hours later after the last injection, tissues were harvested for analysis. Mouse husbandry was carried out according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Icahn School of Medicine at Mount Sinai.

X-gal and Trichrome staining

Mouse embryos or hearts were fixed in 4% paraformaldehyde for 30 min on ice, washed twice with PBS and then stained with X-gal solution (5 mM Potassium Ferricyanide, 5 mM Potassium Ferrocyanide, 2 mM MgCl₂, 1 mg/ml X-gal) overnight at room temperature. The tissues were washed twice with PBS, followed by photography with a Leica stereomicroscope. To examine β -galactosidase activity inside the heart, frozen cardiac samples were cryosectioned into 10 μ m in thickness followed by X-gal staining at 37°C overnight and counterstained with eosin. Trichrome staining to determine cardiac fibrosis was performed with a Masson kit (Sigma-Aldrich, HT15) following the manufacturer's instructions on cryosections.

Immunofluorescence

Mouse hearts were fixed in 4% paraformaldehyde on ice for 30 min and rinsed with PBS. Tissues were soaked in 30% sucrose and then embedded in Optimal Cutting Temperature compound (Tissue-Tek) on dry ice. Frozen samples were cut into 6 μ m in thickness. Mouse anti-Troponin T (1:200, Thermo scientific), anti- α SMA (1:100, Sigma) and anti-PECAM (1:100, BD Biosciences) antibodies were used in this study. Alexa Flour 488 conjugated secondary antibodies (1:500, Invitrogen) were applied to detect primary antibodies.

Echocardiography and data analysis

Transthoracic echocardiography was performed using the Vevo 2100 high-resolution ultrasound imaging system with a real-time 30MHz linear array scanhead (MS400) at a

frame rate of 235fps, a focal length of 8 mm, and a 10x10-mm field of view (Visualsonics; Toronto, Canada). Mice were anesthetized with 2% isoflurane, and hair was removed from the chest using a depilatory cream (Nair; Church & Dwight Co, Inc; Princeton, NJ). Warmed ultrasound transmission gel was placed on the chest and used to obtain left ventricular endpoints of cardiac function. B-mode cardiac imaging was conducted on transverse (short axis) plane. The papillary muscles were used for the short axis imaging landmark. M-mode recordings of the left ventricle were also recorded at the short axis B-mode imaging plane to obtain left ventricular function and dimensions through the cardiac cycle. Heart rate was monitored and core body temperature was maintained at 37°C using a heated platform and a hair dryer throughout the procedure. Data analysis was performed on VisualSonics Vevo 2100 V1.5.0 software (Visualsonics; Toronto, Canada). The following parameters were measured using short axis M-mode: diastolic and systolic left ventricular internal diameter (LVID), anterior wall thickness (LVAW), and posterior wall thickness (LVPW). From these measurements, left ventricular ejection fraction (LVEF) and percent fractional shortening (LVFS) were calculated within the Vevo software. Mean and standard deviation were reported for each group. Endpoints were compared using Sidak 1-way ANOVA. $P < 0.05$ was considered statistically significant. All micro-ultrasound data analysis was conducted by 2 operators blinded to the experimental groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Generation of *Tnnt2*^{MerCreMer/+} knock-in mice

(A) Schematic representation of the targeting strategy. *MerCreMer-FRT-Neo-FRT* cassette was introduced into the *Tnnt2* genomic locus (6 bp upstream of the ATG). The *Neo* cassette is flanked by two *FRT* sites. *Tnnt2*^{MerCreMer-Neo/+} mice were generated from the positive ES cells. Flippase deleter mice were crossed to *Tnnt2*^{MerCreMer-Neo/+} mice to remove the *Neo* cassette. (B) PCR analysis of genomic DNA from the targeted ES cells. Two fragments (5.5-kb/5' arm and 4.7-kb/3' arm) were amplified by long range PCR using primers P1 (external to 5' arm) and P2 (in *MerCreMer* cassette), P3 (in *Neo* cassette) and P4 (external to 3' arm), respectively.

Figure 2. *Tnnt2^{MerCreMer/+}* drives efficient Cre recombination in hearts
Tnnt2^{MerCreMer/+} mice were crossed to *R26R^{lacZ/lacZ}* mice to trace *Tnnt2* progeny (*Tnnt2^{MerCreMer/+};R26R^{lacZ/+}*). X-gal staining shows β -galactosidase expression in the embryonic and postnatal hearts at E10.5-P60 after tamoxifen induction. Notched arrows indicate positive staining (cardiomyocytes). Arrowheads in D1/E1/F1/G1 indicate pulmonary artery. Unnotched arrows in D4/E4/F4/G4 indicate valves. C2/D3/E3/F3/G3 are high magnification images for C1/D2/E2/F2/G2 in areas labelled by asterisks. RA, right

atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; PA, pulmonary artery; VS, ventricular septum.

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Figure 3. *Tnnt2^{MerCreMer/+}* directs specific recombination in cardiomyocytes
 tdTomato expression in *Tnnt2^{MerCreMer/+};R26R^{tdTomato/+}* hearts after tamoxifen induction. Images in A1-4 show whole-mount views of hearts at E16.5, P0, P30 and P60, respectively. B-I are transverse sections of hearts with co-immunostaining with antibodies to *Tnnt2* (B-E), α SMA (F,G) and PECAM (H,I). tdTomato-expressing cells are cardiomyocytes (co-expressed with *Tnnt2*, B-E), and are not coronary smooth muscle cells (not co-localized with α SMA, F,G) or endocardial/endothelial cells (not co-localized with PECAM, H,I). Notched arrows in B-E indicate *Tnnt2* antibody staining. Unnotched arrows in F,G indicate α SMA

antibody staining. Unnotched arrows in H/I indicate PECAM antibody staining in endocardial/endothelial cells. Arrowheads in F4 indicate epicardial layer. B4, C4, D4, E4, F4 and G4 are overlay images for B1/2/3, C1/2/3, D1/2/3, E1/2/3, F1/2/3 and G1/2/3, respectively. H1/I1 are PECAM staining and H2/I2 are merged images of DAPI/tdTomato/PECAM at P60.

Figure 4. Normal left ventricular function and dimensions after Cre induction in *Tnnt2^{MerCreMer/+}* mice

Tamoxifen was administered at 0.1 mg/g body weight/day for three days. **(A)** Representative M-mode echocardiography images of *Tnnt2^{MerCreMer/+}* and wild type controls five weeks after tamoxifen treatment. **(B)** Cre induction does not result in any cardiac function or structural changes between genotypes or treatment groups. TMX, Tamoxifen; EF, ejection fraction; LVID, left ventricular internal diameter; LVAW, left ventricular anterior wall; LVPW, left ventricular posterior wall. All values were plotted as mean ± STDEV.

Figure 5. Tamoxifen induction does not cause myocardial apoptosis or fibrosis in *Tnnt2^{MerCreMer/+}* mice

TUNEL and trichrome staining were performed on *R26R^{tdTomato/+}* and *Tnnt2^{MerCreMer/+};R26R^{tdTomato/+}* hearts after tamoxifen administration. (**A,B**) A few apoptotic cells were observed on both *R26R^{tdTomato/+}* and *Tnnt2^{MerCreMer/+};R26R^{tdTomato/+}* hearts (arrows) after one week (P67). (**C,D**) The injection leads to sufficient excision on *Tnnt2^{MerCreMer/+};R26R^{tdTomato/+}* hearts, but not on *R26R^{tdTomato/+}* hearts when *Tnnt2^{MerCreMer/+}* is not present. (**E-H**) Cardiac fibrosis was not detected by trichrome

staining on $R26R^{tdTomato/+}$ and $Tnni2^{MerCreMer/+};R26R^{tdTomato/+}$ hearts after one (E,F) and five weeks (G,H, P95) of injection.

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