

Cardiac fibrosis in mice expressing an inducible myocardial-specific Cre driver

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SUMMARY

Tamoxifen-inducible Cre-mediated manipulation of animal genomes has achieved wide acceptance over the last decade, with numerous important studies heavily relying on this technique. Recently, a number of groups have reported transient complications of using this protocol in the heart. In the present study we observed a previously unreported focal fibrosis and depressed left-ventricular function in tamoxifen-treated *aMHC-MerCreMer*-positive animals in a *Tβ4shRNAflox* × *aMHC-MerCreMer* cross at 6–7 weeks following standard tamoxifen treatment, regardless of the presence of the floxed transgene. The phenotype was reproduced by treating mice from the original *aMHC-MerCreMer* strain with tamoxifen. In the acute phase after tamoxifen treatment, cell infiltration into the myocardium was accompanied by increased expression of pro-inflammatory cytokines (*IL-1β*, *IL-6*, *TNFα*, *IFNγ*, *Ccl2*) and markers of hypertrophy (*ANF*, *BNP*, *Col3a1*). These observations highlight the requirement for including tamoxifen-treated *MerCreMer* littermate controls to avert misinterpretation of conditional mutant phenotypes. A survey of the field as well as the protocols presented here suggests that controlling the parameters of tamoxifen delivery is important in avoiding the chronic *MerCreMer*-mediated cardiac phenotype reported here.

INTRODUCTION

A currently popular mode of conditionally manipulated gene expression in the mouse involves Cre-recombinase-mediated excision of engineered genomic target sequences flanked by *loxP* sites (reviewed in Smedley et al., 2011). Controlling Cre expression with tissue- or cell-specific promoters and drug-inducible cassettes allows spatial and temporal restriction of these recombination events. In the heart, inducible Cre-mediated excision driven by the cardiac-specific α -myosin heavy chain (*aMHC*) promoter has been extensively used to drive cardiomyocyte-restricted recombination of floxed genes in animal models. In *aMHC-MerCreMer* mice, Cre recombinase is fused to two mutated estrogen receptor domains (*Mer*); binding to the estrogen analog tamoxifen shuttles the fusion protein from the cytoplasm to the nucleus (Zhang et al., 1996; Sohal et al., 2001; Nakamura et al., 2006). *aMHC-MerCreMer* mice have found wide application in conditional knockout/knockdown and lineage tracing experiments (Hsieh et al., 2007). However, recent reports have indicated that tamoxifen-priming of mice carrying the cardiomyocyte-specific *MerCreMer* cassette causes dose-dependent acute cardiac dysfunction such as decreased left-ventricular fractional shortening within the first week following tamoxifen exposure and mitochondrial aberrations until 14 days post-treatment (Hall et al., 2011). Transient effects were shown to include substantial changes in the transcriptome (Hougen et al., 2010), further driving current

efforts to achieve maximal recombination with a minimum of cardiac side effects, mainly by reducing the total dose of tamoxifen that is delivered (Andersson et al., 2010). Here, we undertake a systematic morphological and molecular evaluation of Cre-recombinase induction in the mouse heart, using standard reagents and protocols, and present a protocol that avoids these pathological responses. Our detailed characterization of pathological effects of Cre induction on cardiac morphology, physiology and function affords a comparative analysis of published results and highlights the importance of transgenic background when interpreting conditional mutant cardiac phenotypes.

RESULTS

In previous studies of inducible Cre-mediated mouse mutagenesis, tamoxifen administered by intra-peritoneal (IP) injection ranged from 40 mg/kg to 560 mg/kg body weight (Nakai et al., 2007; Wang et al., 2009; Stokke et al., 2010) delivered over periods of 1–14 days. In the current study, we induced *MerCreMer*-mediated excision of an upstream inhibitory sequence by using a standard injection protocol of one injection per day of 20 mg tamoxifen/kg body weight (100 mg/kg total) for 5 days to drive expression of an shRNA specifically in cardiomyocytes (Sohal et al., 2001) to knock down cardiac thymosin β 4 (*Tβ4*) in adult mice. Echocardiography measurements at 6–7 weeks after tamoxifen injection of mice harboring an *aMHC-MerCreMer* transgene, with or without the *Tβ4shRNAflox* target allele, indicated decreased cardiac function in a significant portion of injected mice [*Tβ4shRNAflox/MerCreMer* % ejection fraction (EF) 55.90±6.29, % fractional shortening (FS) 25.35±3.57; *aMHC-MerCreMer* %EF 55.52±6.58, %FS 24.86±3.93, compared with control-injected *Tβ4shRNAflox/MerCreMer* %EF 62.36±2.76, %FS 29.49±1.93; Fig. 1A]. Histological analysis revealed that ~60% (*n*=17/28) of tamoxifen-treated *aMHC-MerCreMer*-positive animals presented significant cardiac fibrosis regardless of the presence of the floxed target gene (Fig. 1C,D). Fibrosis was patchy and present in the left ventricle, septum and right ventricle, and was not observed in *Tβ4shRNAflox* or wild-type (wt)

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TRANSLATIONAL IMPACT

Clinical issue

Cardiac fibrosis, which is characterized by excessive proliferation of cardiac fibroblasts and reduced contractility of the heart, is a hallmark of cardiomyocyte death during congenital or acquired heart disease. The detrimental effects of fibrosis on organ architecture and function can eventually lead to cardiac failure. Mouse models of human heart disease frequently involve the use of a genetic 'switch' that induces Cre recombinase activity in specific cardiac cells, thereby modifying expression of the gene under study in the living heart. However, the Cre driver itself might be a source of cardiac fibrosis, underscoring the need to refine the genetic manipulation and more clearly define any associated cardiac phenotype.

Results

Here, the authors systematically characterized the effects of Cre recombinase induction on cardiac morphology, physiology and function in mice. Tamoxifen was used to induce MerCreMer-mediated excision of cardiomyocyte-specific floxed cassettes, in line with standard protocols. Histological analysis revealed that ~60-100% of tamoxifen-treated Cre-driver mice displayed cardiac fibrosis (with variation in penetrance in different genetic backgrounds) independent of the presence of the floxed target gene. Moreover, echocardiography revealed significant depression of cardiac function in this group. The authors also reported elevated expression of pro-inflammatory cytokines following tamoxifen treatment of Cre-driver mice. In contrast to standard multiple-injection protocols, cardiac function was not perturbed in Cre-driver mice treated with a single tamoxifen injection to induce efficient recombination of the target gene.

Implications and future directions

The present study provides evidence for long-term pathological damage and compromised heart function caused by MerCreMer recombinase induction in heart tissue. These findings are consistent with prior reports of the negative impacts of tamoxifen exposure on cardiac function; however, previous studies suggested that the effects are transient. In addition, the group showed that differences in the genetic background can contribute to the observed variation in phenotypic penetrance. Thus, the adverse long-term side effects of Cre recombinase induction in the heart must be carefully monitored in mouse models of cardiac disease involving gene switching. The authors show that a single injection protocol can overcome the toxic effects associated with the method, and recommend the use of this or other delivery methods to minimize MerCreMer-mediated cardiac damage and thereby optimize the use of these mice as models for cardiac disease.

littermates ($n=5$) or in control-treated *Tβ4shRNAflox/MerCreMer* mice ($n=10$), all of which showed normal cardiac morphology (Fig. 1B).

Because high levels of Cre are reportedly cytotoxic, we analyzed Cre copy numbers in fibrotic and normal mice (Buerger et al., 2006). Significant fibrosis was observed in both homozygous and heterozygous *αMHC-MerCreMer* mice (12/19 heterozygotes and 5/8 homozygotes affected). To address the possibility of strain-specific effects of MerCreMer expression in our crosses (129Ola/C57Bl6/J mixed background), we examined 8-week-old heterozygous *αMHC-MerCreMer* on C57Bl6/J background 1 month following tamoxifen treatment. We observed a significant depression of cardiac function by echocardiography (%EF 51.04 ± 2.10 , %FS 23.40 ± 2.00), compared with wt mice (%EF 62.55 ± 3.00 , %FS 29.35 ± 2.17), untreated *αMHC-MerCreMer* mice of various ages (8 weeks %EF 60.21 ± 3.64 , %FS 28.36 ± 1.44 , 7-8 months %EF 59.27 ± 1.34 , %FS 27.07 ± 1.35) and tamoxifen-treated *MerCreMer*^{-/-} littermates (%EF 59.31 ± 2.50 , %FS 27.55 ± 2.37 ; Fig. 2A), as well as interstitial and perivascular cardiac fibrosis (5/5;

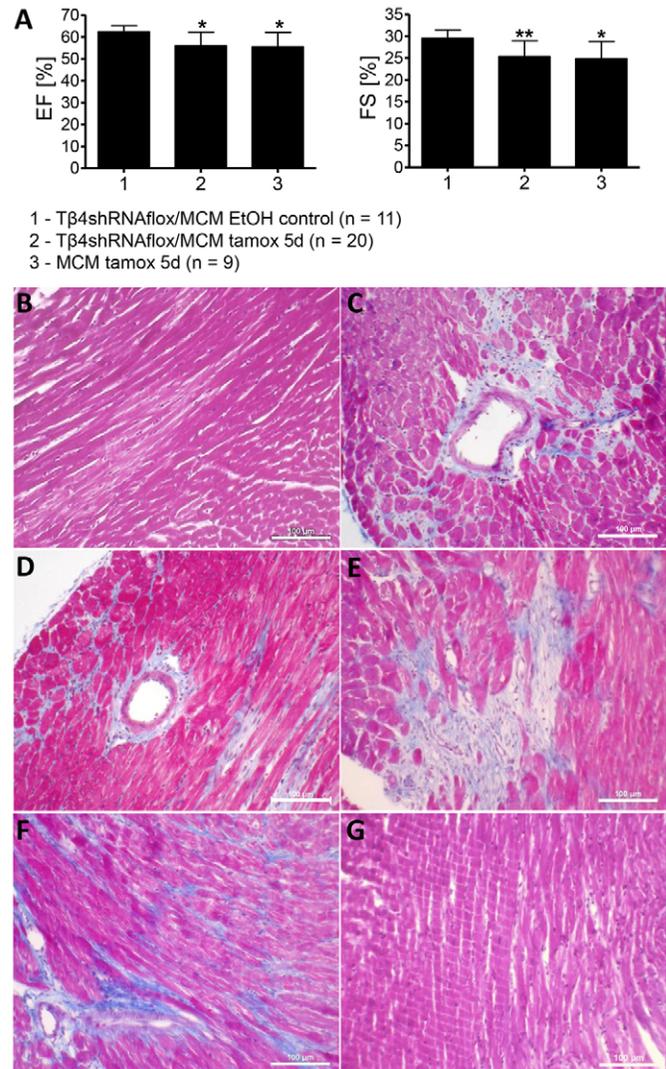


Fig. 1. Echocardiography analysis of mouse hearts 6-7 weeks after completing 5 days of tamoxifen injections (20 mg/kg body weight IP daily). (A) Ejection fraction (EF) measurements were recorded in 2D-mode and fractional shortening (FS) in M-mode. Control-injected (EtOH) *Tβ4shRNAflox/MerCreMer* (MCM) double transgenic mice served as controls. * $P < 0.05$, ** $P < 0.01$. (B) Normal cardiac tissue in tamoxifen-treated wild-type and *Tβ4shRNAflox* animals and control-treated *Tβ4shRNAflox/MerCreMer* mice. (C,D) Representative images of the extensive cardiac fibrosis and cardiomyocyte replacement observed in ~60% of tamoxifen-treated *Tβ4shRNAflox/MerCreMer* (C) and *αMHC-MerCreMer* (D) littermates. (E,F) Interstitial (E) and perivascular (F) fibrosis in the original *αMHC-MerCreMer* strain after 5 days tamoxifen treatment and 1 month of rest. (G) Normal cardiac morphology observed in *αMHC-MerCreMer* mice following a single injection of tamoxifen (40 mg/kg body weight).

Fig. 1E,F). Cardiac hypertrophy was not observed, with normal inner ventricular dimensions measured across all groups (Fig. 2B). Furthermore, relative heart or lung weights, secondary indicators of hypertrophy or heart failure, were unchanged (Fig. 2C).

To further investigate the cause of the phenotype, we analyzed histological sections of *αMHC-MerCreMer* hearts 3 days after tamoxifen treatment and identified focal cell infiltrates in the

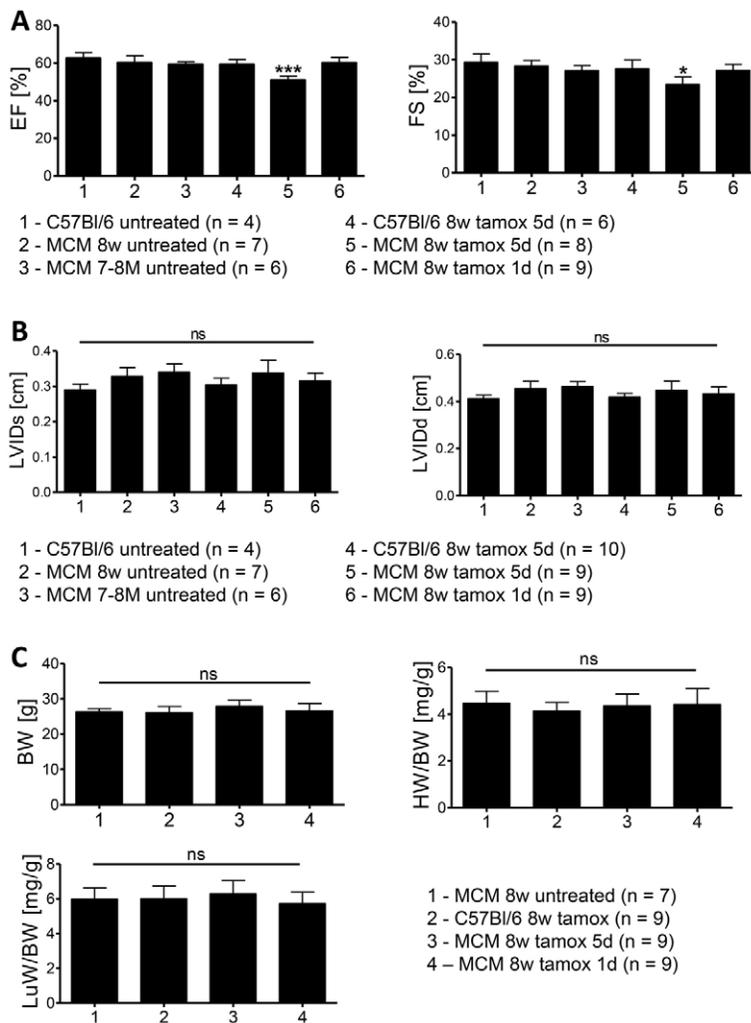


Fig. 2. Assessment of treating 8-week-old α MHC-MerCreMer mice with tamoxifen. (A) Echocardiography analysis of mouse hearts 1 month after completing 5 days of tamoxifen injections (20 mg/kg body weight IP daily) compared with untreated wild-type and α MHC-MerCreMer (MCM) animals of various ages, tamoxifen-treated α MHC-MerCreMer-negative (C57Bl/6) littermates and α MHC-MerCreMer animals treated with a single injection of 40 mg tamoxifen/kg body weight. Ejection fraction (EF) and fractional shortening (FS) measurements were recorded in 2D-mode and M-mode, respectively. * P <0.05, *** P <0.001. (B) Left-ventricular inner diameters during systole (LVIDs) and diastole (LVIDd) calculated from M-mode recordings. (C) Body weight (BW), relative heart weight (HW/BW) and relative lung weight (LuW/BW) recorded 1 month following tamoxifen or control treatment. Not significant (ns): P >0.05.

myocardium (Fig. 3A,B). We also performed real-time PCR (rtPCR) analyses of cytokines 3 days following tamoxifen treatment to study the contribution of inflammation to the observed phenotype. Pro-inflammatory markers (*IL1 β* , *IL6*, *TNF α* , *TFN γ* and *Ccl2*) were significantly elevated in tamoxifen-treated animals compared with controls, as were markers of cardiac remodeling (*ANF*, *BNP* and *Col3A1*) (Fig. 3D). Interestingly, expression of the anti-inflammatory cytokines *IL-10* and *TGF β* was also induced at this time point.

Because Hougen et al. proposed the use of a single injection of tamoxifen (40 mg/kg) to avoid a transient Cre-mediated cardiomyopathy, we investigated the suitability of this protocol to prevent the chronic defects observed following the 5-day protocol (Hougen et al., 2010). We observed normal cardiac function (%EF 60.14 \pm 2.92, %FS 27.16 \pm 1.62) and cardiac morphology on the microscopic scale in 8-week-old α MHC-MerCreMer mice 1 month after the single injection protocol (n =9) (Fig. 2A; Fig. 1G). To determine whether a single injection would induce efficient Cre-mediated recombination, α MHC-MerCreMer mice were crossed with a tdTomato/Ai14 reporter line (Madisen et al., 2010) and the hearts of 8-week-old animals were analyzed 2 weeks after either a single tamoxifen injection (40 mg/kg) or the 5-day consecutive injections (20 mg/kg). As seen in Fig. 4, whereas control mice

injected with vehicle (EtOH) showed low levels of spontaneous Cre recombination that have been seen elsewhere (Kemp et al., 2004; Liu et al., 2010), all mice injected with tamoxifen showed uniform recombination in cardiomyocyte fibers, irrespective of the injection protocol. We conclude that a single injection of tamoxifen at 40 mg/kg effectively avoids the cardiomyopathological effects of prolonged drug exposure in α MHC-MerCreMer mice.

DISCUSSION

In this report we show that episodic tamoxifen-primed nuclear Cre recombinase accumulation in adult hearts causes irreversible damage to the myocardium, reflected in decreased left-ventricular function. Because a single injection of tamoxifen (40 mg/kg) induced efficient Cre-mediated recombination but did not result in cardiac fibrosis in α MHC-MerCreMer mice, the observed phenotype presumably depends either on the nuclear levels of MerCreMer protein or its duration in the nucleus, or a combination of both. This possibility is supported by the observation of normal cardiac function and morphology in aged untreated animals, indicating that cytoplasmic MerCreMer itself is not toxic. Tamoxifen alone was also shown to have no effect in any MerCreMer-negative background.

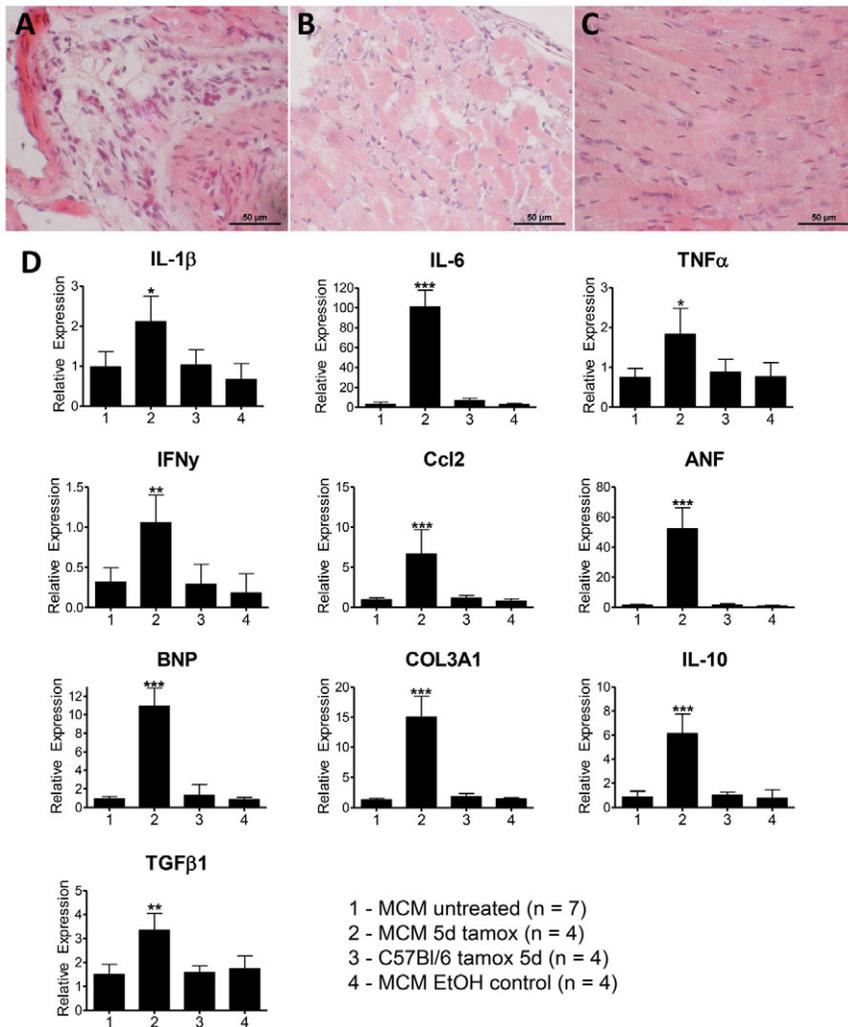


Fig. 3. Inflammatory response in α MHC-MerCreMer mice 3 days after 5 daily tamoxifen injections (20 mg/kg body weight). (A-C) H&E staining reveals cell infiltrates in severely affected areas (A,B) and normal morphology in healthy regions (C) in cardiac sections of tamoxifen-treated α MHC-MerCreMer mice. (D) Real-time PCR analysis of whole heart RNA extracts following 5 days of tamoxifen (20 mg/kg body weight IP daily) or control injections and 3 days of rest. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The toxicity of nuclear Cre could potentially stem from promiscuous specific genomic recombination at cryptic *loxP* sites on the genome (Loonstra et al., 2001; Semprini et al., 2007), in which case chromosomal aberration from DNA fragmentation could be responsible for cell death and subsequent replacement of cardiac myocytes by fibroblasts, resulting in the observed phenotypes. The observed cell infiltration and upregulation of various cytokines involved in acute inflammation in the tamoxifen-treated α MHC-MerCreMer group reported here is a further indication of the negative impact of excessive active MerCreMer in cardiac tissue. Although this response could be secondary to excessive tissue damage or necrosis, it is a further factor potentially compromising experimental interpretation, as inflammatory response and cell death constitute primary readouts in cardiac regeneration research. This also applies for markers of cardiac hypertrophy (*ANF*, *BNP*, *Col3a1*), frequently used as indicators of hypertrophy in cardiac-specific conditional knockout mice. Elevated pro-hypertrophic markers in the α MHC-MerCreMer model have been reported previously, although higher doses of tamoxifen were used in these studies (40 mg/kg daily for 4 days, 160 mg total) (Andersson et al., 2010). The fact that *IL-10* and *TGF β 1* mRNA levels are increased after 3 days is likely a negative feedback response and indicates the

end of the acute inflammatory reaction following MerCreMer-mediated insult.

Our findings are consistent with recent studies reporting negative impacts of Cre and/or tamoxifen administration on animal physiology (Bersell et al., 2013). A survey of the literature revealed a number of recent studies investigating phenotypes of α MHC-MerCreMer cross-bred animals without including tamoxifen-treated α MHC-MerCreMer single-genotype controls. Among the reported phenotypes were decreased cardiac function, cardiac fibrosis, and increased expression of *ANF* and *BNP*. In light of our results these observations would have potentially been confounded by a Cre-mediated cardiomyopathy. Through personal communications with the authors of studies in which tamoxifen-treated α MHC-MerCreMer mice were not included, we found that, indeed, in most cases the control mice had been monitored, but were excluded from the published experiments owing to the absence of any remarkable phenotype. Differences in genetic background could be a contributing factor to the observed variation in phenotypic penetrance, which is supported by the fact that we observed fibrosis in 60% of *T β 4shRNA^{flox}/MerCreMer* mice (*129Ola/C57Bl6/J*) and in 100% of α MHC-MerCreMer mice (*C57Bl6/J*), following tamoxifen treatment.

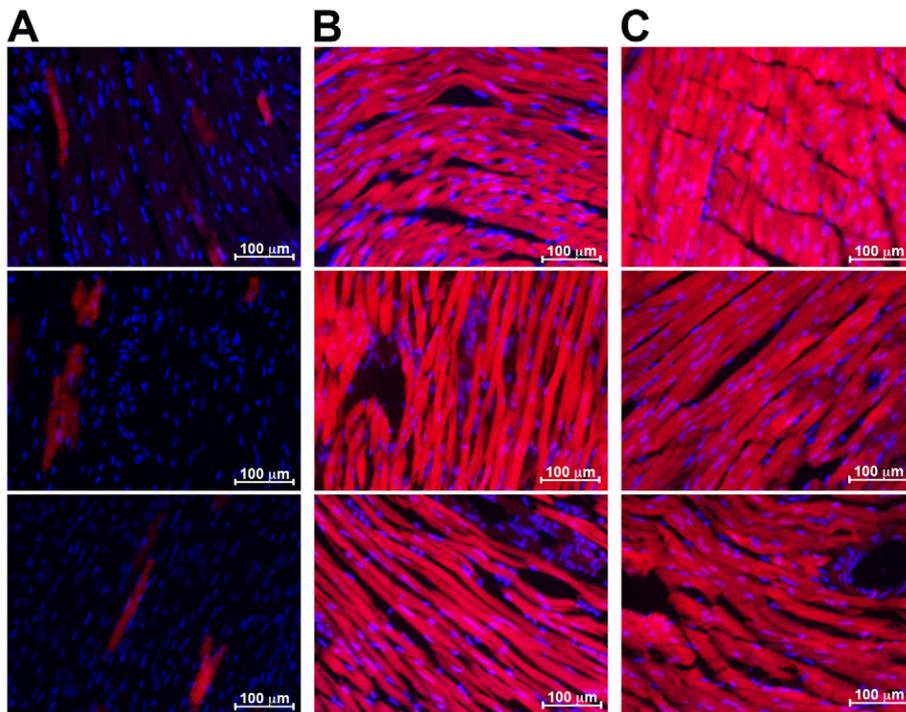


Fig. 4. A single tamoxifen injection is sufficient to induce robust α MHC-MerCreMer recombination. 8-week-old α MHC-MerCreMer \times *tdTomato* mice were injected with (A) control (EtOH), (B) a single dose of tamoxifen (40 mg/kg body weight) or (C) 5 daily tamoxifen injections (20 mg/kg body weight) and analyzed 2 weeks after injection. DAPI (blue) and *tdTomato* (red) staining shows background recombination in various regions of control heart tissue (A), and complete recombination in cardiomyocyte fibers but not in other cell types of induced mice regardless of injection protocol (B,C). A separate mouse was analyzed for each panel of B and C.

The transient effects of tamoxifen exposure on cardiac function have been well documented, and methods have been proposed to reduce these effects (Hougen et al., 2010). In addition to the injection protocol, the form of tamoxifen used might constitute a parameter contributing to diverse outcomes. As reported previously by Kiermayer et al., tamoxifen citrate seems to be equally as effective and a potentially safer alternative to tamoxifen or its active metabolite 4-hydroxytamoxifen, but the oral delivery proposed is sensitive to diet refusal by the animals, resulting in insufficient recombination, weight loss and starvation-related phenotypes (Kiermayer et al., 2007; Andersson et al., 2009) (and our unpublished data). Consequently, injecting tamoxifen citrate could prove to be a suitable alternative (Bersell et al., 2013).

In summary, several conclusions can be drawn from the present report and a survey of the current literature. First, high levels of tamoxifen-induced MerCreMer can cause substantial cardiac phenotypes with short- and long-term effects. Second, genetic background is most likely a determining factor of the tamoxifen-induced MerCreMer phenotype. Third, delivery methods have varying effectiveness in terms of tamoxifen bioavailability, and consequently affect concentration and duration of MerCreMer in the nucleus. Therefore, the most appropriate delivery method for each model must be evaluated prior to extensive experimentation and the optimal dose of tamoxifen adjusted to the lowest concentration required for achieving sufficient recombination, as recently reported (Touvroun et al., 2012).

Given the potential of tamoxifen treatment to cause a substantial phenotype in MerCreMer mice at molecular, macrostructural and functional levels, we strongly support the inclusion of tamoxifen-treated MerCreMer single-genotype animals as controls in relevant studies, particularly in experiments involving myocardial-specific MerCreMer transgenes (Anastassiadis et al., 2010). Because we show that the observed phenotype is not related to cytoplasmic

MerCreMer or the presence of a floxed transgene, tamoxifen-treated α MHC-MerCreMer animals should be chosen over *flox/flox* mice as negative controls in studies in which time and resources prohibit the use of all applicable control groups.

MATERIALS AND METHODS

Animals

All animal work was performed in accordance with Imperial College London, UK institutional guidelines. *T β 4shRNAflox* animals were kindly provided by Paul Riley from University College London, UK. α MHC-MerCreMer animals were purchased from The Jackson Laboratory (JAX stock number 005657). Male *T β 4shRNAflox/MerCreMer* mice at 3–4 months of age and α MHC-MerCreMer mice between 8 weeks and 8 months were used in this study. The *tdTomato/Ai14* reporter line (Madisen et al., 2010) was a gift from Michael D. Schneider, Imperial College London, UK.

MerCreMer induction

Tamoxifen (Sigma-Aldrich, Gillingham, UK, # T5648) was dissolved in ethanol (100%) to a concentration of 100 mg/ml and further diluted in soybean oil (Sigma-Aldrich, Gillingham, UK, # S7381) to 10 mg/ml (Nakamura et al., 2006). Tamoxifen solution was injected IP (20 mg/kg body weight) once daily for 5 consecutive days or as a single injection of 40 mg/kg body weight (Sohal et al., 2001; Hougen et al., 2010). EtOH in soybean oil at identical concentrations was injected as control. Animals were maintained for 6–7 weeks (*T β 4shRNAflox/MerCreMer* study) or 1 month (α MHC-MerCreMer study) before sacrifice.

Echocardiography analysis

Mice were maintained under low anesthesia [1.5% (v/v) isoflurane, 1.5 ml/minute O₂]. Short-axis view transthoracic echocardiography

(ECHO) was performed on shaved mice at midpapillary level. Ejection fraction (EF) was determined in 2D and M-mode; fractional shortening (FS) and I dimensions were measured in M-mode using a 12.5 MHz transducer (Siemens Acuson Sequoia C256). Each animal was analyzed at least in duplicate on consecutive days and results averaged. All raw ECHO data was recorded within 10 minutes from induction of anesthesia. At baseline, all groups showed indistinguishable normal cardiac function. ECHO data are shown as \pm standard deviation (s.d.) of the mean.

Immunohistochemistry

Heart samples were incubated in formal saline (10%) overnight at 4°C and embedded in paraffin the following day using standard protocols. Staining for collagen was performed using a Trichrome (Masson's) kit (Sigma-Aldrich, Gillingham, UK, # HT15-1KT) following the manufacturer's instructions. Hematoxylin and eosin (H&E) staining to investigate cell infiltration and morphology was performed using standard protocols. For the *tdTomato/Ai14* reporter experiment, hearts were fixed for 4 hours in PFA (4%) at room temperature, incubated overnight at 4°C in sucrose (10%) and embedded in OCT using standard protocols. Frozen sections were stained with 4',6-diamidino-2-phenylindole (DAPI) for nuclei detection and examined for positive tdTomato staining induced by Cre recombination.

Real-time PCR

Total RNA from heart was isolated 3 days following 5 days of tamoxifen or control injections. Comparative ΔC_T method was used to identify relative changes in mRNA expression using Taqman® reagents and an ABI7500 fast thermocycler (Applied Biosystems, Carlsbad, CA). 18s ribosomal subunit expression served as an endogenous expression control. The following transcripts were analyzed: *CCL2* (Mm00441242_m1), *COL3A1* (Mm01254476_m1), *Nppa* (Mm01255747_g1), *Nppb* (Mm01255770_g1), *IL-1 β* (Mm00434189_m1), *IL-6* (Mm00446190_m1), *IL-10* (Mm00439614_m1), *IFN γ* (Mm01168134_m1), *TGF β 1* (Mm01178820_m1) and *TNF α* (Mm00443260_g).

Copy number analysis

Genomic DNA was isolated from tails samples using a DNeasy kit (Qiagen, Crawley, UK). *Cre* copy abundance was determined by real-time PCR using a Taqman® probe targeting *Cre* cDNA (Mr00635245_cn).

Statistical analysis

Experimental groups were compared by one-way analysis of variance (ANOVA) and Bonferroni post-testing. Results where $P < 0.05$ were considered statistically significant (*). Error bars indicate s.d.

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

J.L. and M.P.S. conceived and designed the experiments. J.L. and T.P. performed the experiments. P.S. performed sectioning and staining of histological specimen. J.L. and T.P. analyzed the data. J.L. and N.R. wrote the paper.

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