Avoidance of Transient Cardiomyopathy in Cardiomyocyte-Targeted Tamoxifen-Induced MerCreMer Gene Deletion Models

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Cardiac myocyte targeted MerCreMer transgenic mice expressing tamoxifen-inducible Cre driven by the α-myosin heavy chain promoter are increasingly used to control gene expression in the adult heart. Here, we show tamoxifen-mediated MerCreMer (MCM) nuclear translocation can induce severe transient dilated cardiomyopathy in mice with or without loxP transgenes. The cardiomyopathy is accompanied by marked reduction of energy/metabolism and calcium-handling gene expression (eg, PGC1-α, peroxisome proliferator-activated α, SERCA2A), all fully normalized with recovery. MCM-negative/loxP-positive controls display no dysfunction with tamoxifen. Nuclear Cre translocation and equally effective gene knockdown without cardiomyopathy is achievable with raloxifene, suggesting toxicity is not simply from Cre. Careful attention to controls, reduced tamoxifen dosing and/or use of raloxifene is advised with this model.

The Cre-loxP system is widely used for selective cell-targeted manipulation of gene expression and has been further enhanced by generating tamoxifen-responsive fusion proteins for conditional Cre induction.2,3 Targeted cells constitutively express Cre flanked by mutated estrogen receptor ligand-binding domains (MerCreMer [MCM]) insensitive to endogenous estrogen but sensitive to tamoxifen (TAM). MCM is cytoplasmic via binding to heat shock protein 90 complex, but this complex dissociates on TAM-Mer binding, whereupon the MCM targeting sequence sends the construct to the nucleus for Cre-mediated excision of loxP flanked sequences.1 Sohal et al linked MCM with an α-myosin heavy chain (Myh6) promoter to create cardiomyocyte-specific gene targeting.1 However, Cre recombinase displays dose-dependent cytotoxicity impairing growth and causing DNA fragmentation,4,5 and a recent review raised a caution that TAM-stimulated MCM in adult hearts may also adversely influence heart function.6 Here, we report on these cardiac effects and provide methods to avoid them.

Materials and Methods
Myh6-MerCreMer fl/fl transgenic mice (no. 005650, Jackson Labs, Bar Harbor, Me) were used. Myh6-MCM−/−no-flox were generated by mating to C57Bl/6 mice. Two strains with floxed alleles coding for either R2 (Tgbr2fl/fl) or R1 (Alk5fl/fl) transforming growth factor (TGF)β receptors (both on C57Bl/6 backgrounds) were crossed with Myh6-MCM−/− mice to study gene knockdown. Cardiac function was assessed by serial echocardiography and invasive pressure-volume analysis. Gene expression was determined by real-time PCR, gene knockdown was determined by analysis of mRNA and TGFβ-stimulated Smad2 phosphorylation, and nuclear Cre was determined by immunohistochemistry and immunoblot. All animal protocols were approved by The Johns Hopkins University Animal Care and Use Committee and followed established NIH guidelines. Details are provided in the Online Data Supplement, available at http://circres.ahajournals.org.

Results
In both MCM−/−/Tgbr2fl/fl and MCM−/−/Alk5fl/fl mice, TAM administered at 20 mg/kg body weight (BW) IP for 5 days (proposed dose) was insufficient for gene and functional knockdown (latter assessed by suppression of TGFβ-stimulated Smad2 phosphorylation; Figure 1A and 1B; Online Figure I, A and B). Increasing the dose to 80 mg/kg BW per day for 5 days (IP) resulted in 60% mortality by 6 days after TAM treatment because of severe cardiomyopathy (Online Figure II). Oral delivery of the same dose for 7 days was tolerated (no mortality) and effective for gene and functional TGFβ-receptor knockdown (Figure 1A and 1B; Online Figure I, A and B). However, a marked though reversible dilated cardiomyopathy (Figure 1C and Online Figure III) was also observed in both floxed/MCM−/− models and MCM−/− mice without a floxed transgene. MCM-negative controls with or without floxed genes (eg, MCM−/−/Tgbr2fl/fl) developed no myopathy at any TAM dose. Cardiac-depression peaked ∼3 days after terminating TAM (day 10 of protocol) with fractional shortening declining from 61±1% to 26.5±5% (P<0.01) and end-diastolic dimension increasing (3.2±0.1 to 4.1±1.4 mm; P<0.01) in MCM−/− mice (with or without floxed alleles; n=19). In vivo pressure-volume analysis in MCM−/−/no flox mice confirmed transient systolic and diastolic depression (Figure 1D and Online Table I), with full recovery observed by day 28 (3 weeks after stopping TAM). MCM−/− controls had no TAM-induced dysfunction. Myocardium displayed patchy interstitial mononuclear infiltration at day 10 (mild myocarditis) that resolved by day 28 and no myocyte hypertrophy (Online Figure IV).

TAM/MCM-induced cardiomyopathy was accompanied by marked changes in stress response, energy/metabolism, and calcium-handling genes (Figure 2). Natriuretic peptide expression (Nppa and Nppb) rose markedly in MCM−/− versus MCM−/− by day 10 and then returned to normal, although β-myosin heavy chain (Myh7), which typically rises with
cardiac stress, was unchanged. Peroxisome proliferator-activated receptor (PPAR)α, PPARγ-coactivator (PGC)1α, and transcription factor A-mitochondrial (TFAM) genes, which are centrally involved with coordinating mitochondrial function, energetics, and metabolism and suspected to play a key role in dilated human cardiomyopathy, all declined substantially with cardiac depression and then fully recovered to normal levels. Lastly, both sarcoplasmic reticular ATPase and phospholamban expression declined transiently, correlating with cardiac function. Although these changes are observed with various cardiac failure conditions, the insult in this instance started in the nucleus and its striking reversibility unusual. Although Cre toxicity might be suspected, reversibility would be less anticipated from DNA fragmentation, particularly in differentiated tissue without a high rate of cell regeneration.

Raloxifene (RAL) is an alternative selective estrogen receptor modulator with similarities but also differences when comparing TAM-regulated transcription. This may be attributable to differential binding to estrogen-related receptors (eg, ERR) and/or recruitment of different coactivators. Because RAL interacts with Mer, albeit at lower...
binding affinity, we tested whether RAL could induce gene knockdown without cardiomyopathy. Because of poor solubility, DMSO was required for IP dosing, limiting the dose to 40 mg/kg BW per day, which was suboptimal for gene knockdown in MCM/Alk5fl/fl. However, higher oral doses were feasible, tolerated, and effective. Myh6-MCM mice fed 160 mg/kg BW per day RAL PO displayed effective gene knockdown but without cardiac dysfunction (Figure 3A and 3B; Online Figure V). Nuclear Cre targeting was similar with TAM or RAL treatments, as shown by histochemistry (Figure 3C) and nuclear fraction immunoblot (Online Figure VI). Stress, metabolic, and Ca2+-handling gene changes (eg, Figure 2) were not observed (data not shown). Importantly, Cre recombinase activity (Online Figure VII), gene knockdown efficacy (Figure 3B), and corresponding functional suppression of TGFβ-induced SMAD2 phosphorylation (Figure 3D) with RAL were similar as with 80 mg/kg BW per day TAM, although required longer exposure (21 days). Recombination (lox-P site excision) was observed earlier with RAL (7 days), although at lower levels. Both RAL and TAM resulted in a similar ≈10% decline in BW during the first week that subsequently recovered (n=6 to 7/group; P=0.95, 2-way ANOVA). Lower oral TAM dose (20 mg/kg BW per day, one-third previously reported) for 21 days also induced effective gene knockdown without dysfunction (Online Figure VIII).

Discussion

Our study did not precisely define the mechanism for TAM-MCM cardiac effects, and such analysis falls outside the scope and intent of this report. However, the data raise a novel hypothesis that the cardiotoxicity is not simply attributable to Cre. First, the striking reversibility is difficult to reconcile with mechanisms of cell damage attributed to Cre, namely targeting pseudo loxP sites to cause DNA fragmentation, cell growth arrest, and/or death. Second, the finding that both TAM and RAL induced similar nuclear Cre localization and gene suppression, yet with striking differences in cardiac phenotype, further questions a Cre-toxicity mechanism. An alternative relates to the specific nature of the ligand–MCM complex. In addition to recruiting different nuclear coactivators that can differentially target transcription, TAM but not RAL can inhibit ERRα, which, along with ERRγ, plays a central role in bioenergetic regulation. These differences could alter nuclear interactions that depend or are independent of Cre recombinase. Although TAM exposure at the same dose was not toxic, the MCM construct increases nuclear levels >4-fold, which could amplify interactions. Although similar energy/metabolic changes often accompany pathological cardiac stress, here, the triggering mechanism involved altered nuclear signaling; therefore, these hibernation-like reversible changes may indeed be primary. Further studies are needed to clarify this hypothesis.
Lastly, it remains possible that differences in the time course and/or nuclear Cre exposure between TAM and RAL play some role, and the finding that lower prolonged dosing of TAM was also effective without myopathy might suggest this. However, this could also reflect less nuclear exposure to TAM-MCM.

Our results have implications for existing and ongoing research with the MerCreMer model. Studies lacking Myh6-MCM<sup>+/−</sup>-flox<sup>−</sup> controls should be viewed cautiously, particularly if a significant cardiac phenotype is found within the 1 to 2 weeks after starting TAM. In such models, gene deletion without cardiodepression (eg, using RAL or longer-term low-dose TAM) is required. Both TAM and RAL dosing may need to be individualized depending on the floxed gene (perhaps related to gene accessibility and/or expression rate). For TAM, care to include MCM<sup>+</sup> controls and provide sufficient recovery time is strongly advised. Although RAL avoids the myopathy, the dose required was fairly high in the present floxed models (lower doses might work for other models). However, mice treated with \( \approx 10 \times \) this dose for up to 3 months had no systemic limiting effects.\(^{14}\) Because RAL may have antihypertrophic effects when given chronically,\(^{15}\) MCM<sup>+</sup> controls are also advised.

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**Disclosures**

None.

**References**


**Key Words:** inducible transgenic Cre recombinase selective estrogen receptor modulator ventricular function mouse models
Supplemental Materials and Methods:

**Mice:** *Myh6-MerCreMer*\(^{+/+}\) transgenic mice (B6/129-TG *Myh5*-cre/Esr1)1Jmk/J; #005650, Jackson Labs, Bar Harbor, ME) were used. *Myh6-MCM*\(^{+/−}\)/no-flox were generated by mating these to C57Bl/6 mice. TGFBR2\(^{fl/fl}\) (gift of Hal Moses)\(^1\) and Alk5\(^{fl/fl}\) (both on C57Bl/6 background) were mated to the *Myh6-MCM*\(^{+/−}\) to derive *Myh6-MCM*\(^{+/−}/tgfbr2^{fl/fl}\) or *Myh6-MCM*\(^{+/−}/alk5^{fl/fl}\), respectively. Genotype was confirmed with primers to *Cre* and *tgfbr2* or *alk5* floxed alleles.

**Tamoxifen/Raloxifene administration:** For IP injection, 10mg tamoxifen free base (T5648, Sigma) was dissolved in 1mL peanut oil (Sigma) and then sonicated. 50uL (0.5mg tamoxifen) was injected daily (20mg/kg/day, i.p.). Raloxifene was dissolved in warmed dimethyl sulfoxide (50mg/mL), then diluted ten-fold (40mg/kg/day, i.p.). For oral dosing, drugs were mixed in soft diet (Bioserve, MA), with daily intake ~4g/day (e.g. 500mg/kg tamoxifen-food = 80mg/kg/day, 1000 mg/kg raloxifene-food = 160mg/kg/day; 25 gm mouse).

**Western blot/Smad2 phosphorylation analysis** Isolated myocytes suspended in Tyrode’s solution were aliquotted and a sub-fraction stimulated by recombinant human TGFβ1(Sigma). After 30minutes incubation with gentle rotation at room temperature, cells were centrifuged, the pellet lysed (Cell Signaling Technology), and lysates subjected to Western blot using a NuPage system (Invitrogen) and probed with Phospho Smad2/3 antibody (Cell Signaling). Membranes were then reprobed with total Smad2 antibody or GAPDH (Cell Signaling). Band intensity was quantified by NIH Image J software. For Cre or MerCreMer fusion protein detection, we used rabbit polyclonal
anti-Cre antibody (Novagen).

**RNA analysis**  Total RNA was prepared using TRIzol reagent (Invitrogen) from left ventricular tissue or isolated myocytes. Messenger RNA was analyzed by quantitative real-time polymerase chain reaction (PCR) using either SYBR green or Taqman probe method. Complementary DNAs (cDNAs) were synthesized by standard techniques by using the TaqMan Reverse Transcription Reagent (Applied Biosystems) with random hexamer primers. Real-time PCR reactions were performed, recorded, and analyzed using the ABI PRISM 7900 (Applied Biosystems, Foster City, California). *Tgfbr2* and *Alk5* were assessed using each targeted exon specific primer-probe from Applied Biosystems: *Tgfbr2*, Mm00436973_m1; *Alk5*, Mm01353995_m1, and normalized with *Gapdh*, Mm99999915_g1. For tissue, the *Nppa* (atrial natriuretic peptide: ANF), *Nppb* (B-type natriuretic peptide: BNP), *Myh7* (beta myosin heavy chain: βMHC), *Atp2a2* (Sarcoplasmic reticulum calcium ATPase 2a: SERCA2a), *Pln* (phospholamban) and *gapdh* mRNA levels were quantified by using SYBR green PCR primers or TaqMan primer-probe method as previously described. Following primers were also used for SYBR green real-time RT-PCR; *Ppargc1a* (peroxisome proliferative activated receptor gamma coactivator 1 alpha: PGC1α), Forward: 5’-ATAGAGTGTGCTGCTCTGGTTGGT-3’, Reverse: 5’-TGGTCGCTACACCACTTCAATCCA-3’; *Ppara* (peroxisome proliferator activated receptor alpha: PPARα), Forward: 5’-GAAACGGAAACGGCCTCATGTGTT -3’, Reverse: 5’-AGAGTACAATCGCTTGCTGTCCCA-3’; *Tfam* (mitochondrial transcription factor A), Forward: 5’-TGAAAGCTTCCAGGAGGCAAAGGA-3’, Reverse: 5’-ACTTCAGCCATCTGCTCTTCCAA-3’. The specificity of the SYBR green assays was confirmed by dissociation curve analysis.

**Genomic DNA analysis in cardiomyocytes**  Genomic DNA was extracted from isolated myocytes using DNeasy tissue kit (Qiagen) according to the manufacturer’s protocol. Genotyping PCR for
alk5 excised allele was performed with the triple PCR method using primers as follows; ALK5-flox:Prim1, fw ACC CTC TCA CTC TTC CTG AGT; ALK5-flox:Prim2, Rev ATG AGT TAT TAG AAG TTG TTT; ALK5-flox:Prim3, Rev GGA ACT GGG AAA GGA GAT AAC. This PCR gives a band(s) for each genotype as follows; wild type, 150bp; alk5+/fl, 150+250bp; alk5fl/fl, 250bp; Cre excised allele, 350bp².

**Immunostaining and confocal microscopy** Cardiac myocytes were isolated and stained with anti-Cre antibody (1:1000, Novagen) and anti-sarcomeric α actinin antibody (1:500, Sigma). Confocal microscopic analysis was performed as previously described³ (RGS paper).

**Nuclear protein extraction** Nuclear protein was fractionated by NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce) according to the manufacturer’s protocol. Anti-Histon H3 antibody (Cell Signaling) was used as a nuclear extract marker.

**Echocardiography** *In vivo* cardiac morphology was assessed by transthoracic echocardiography (Acuson Sequoia C256, 13MHz transducer; Siemens) in conscious mice. M-mode LV end-systolic and end-diastolic dimensions were averaged from 3-5 beats. LV percent FS and LV mass were calculated as described previously³⁵. Studies and analysis were performed by investigators blinded to genotype or treatments.

**In vivo hemodynamics** *In vivo* LV function was assessed by PV catheter as described previously³⁶. Briefly, mice were anesthetized with 1%–2% isoflurane, 750–100 mg/kg urethane i.p., 5–10 mg/kg etomidate i.p., and 1–2 mg/kg morphine i.p.; were subjected to tracheostomy; and were ventilated with 6–7 µl/g tidal volume and 130 breaths/min. Volume expansion (12.5% human albumin, 50–100 µl over 5 min) was provided through a 30-gauge cannula via the right external jugular vein. The LV apex was exposed through an incision between the seventh and eighth ribs,
and a 1.4-Fr PV catheter (SPR 839; Millar Instruments Inc.) was advanced through the apex to lie along the longitudinal axis. Absolute volume was calibrated, and PV data were measured at steady state and during transient reduction of venous return by occluding the inferior vena cava with a 6-0 silk snare suture. Data were digitized at 2 kHz, stored to disk, and analyzed with custom software. From the 10–15 successive cardiac cycles during the inferior vena cava occlusion, the end-systolic PV relation slope (i.e., end-systolic elastance) and stroke work–end-diastolic volume relation (i.e., preload recruitable stroke work) were derived.

**Statistics**  All values are expressed as mean ± SEM. Group comparisons were performed by 1-way ANOVA or non-paired 2-tailed Student’s t test. Sample sizes and individual statistical results for all analyses are provided in the figures, supplemental figures, and supplemental tables.
References


Online Figure I
A

Baseline Day3 Day5 Day8

FS 61.5% EDD 3.4mm FS 40.3% EDD 3.4mm FS 13.7% EDD 5.1mm

Death

B

Survival (%)

TAM 80mg/kg IP/5days

Online Figure II
Online Figure III

**A**

<table>
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<tr>
<th>MCM flox</th>
<th>+/-</th>
<th>-/-</th>
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<tr>
<td>+/- fl/fl</td>
<td>+/</td>
<td>fl/fl</td>
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<tr>
<td>Tgfrb2</td>
<td>-/-</td>
<td>fl/fl</td>
</tr>
<tr>
<td>Alk5</td>
<td>-/-</td>
<td>fl/fl</td>
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</table>

**B**

- **FS (%)**
  - Baseline: 60
  - Day 10: 20
  - Day 14: 40
  - Day 28: 60

- **EDD (mm)**
  - Baseline: 4
  - Day 10: 6
  - Day 14: 3
  - Day 28: 2

- Legend:
  - MCM^+/−/ALK5^+/− fl/fl
  - MCM^−/−/ALK5^+/− fl/fl
  - MCM^+/−/ALK5^−/− fl/fl
All mice treated with tamoxifen 80 mg/kg/day P.O. for 7 days.

Online Figure IV
Online Figure V
**Online Figure VI**

A

- **MCM**\(^{+/+}\)/flox\(^{-/-}\)
- **MCM**\(^{-/-}\)/flox\(^{-/-}\)
- **Control Lysate**

- 100kD
- IB:Histone H3
- IB:GAPDH
- IB:Cre

B

- **MCM**\(^{-/-}\)/flox\(^{-/-}\)

- MerCreMer
- IB:Cre
- Cre
- IB:Histone H3
- IB:GAPDH

- Drug (-)
- TAM
- RAL
Drug(-) TAM 80mg/kg p.o. 7d

500 400 300 200

Cre excised allele
Recombinant allele

500 400 300 600

bp

MerCreMer
Control

Online Figure VII
Online Figure VIII
## Supplemental Table: Hemodynamic parameters and indices derived from PV loop analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MCM(-)+TAM Day10 N=3</th>
<th>MCM+TAM Day10 N=4</th>
<th>MCM+TAM Day28 N=3</th>
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<tr>
<td>HR (min)</td>
<td>458±35</td>
<td>424±37</td>
<td>574±18 *</td>
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<tr>
<td>ESP (mmHg)</td>
<td>85.1±3.2</td>
<td>75.7±3.4</td>
<td>92.1±6.0</td>
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<td>EDP (mmHg)</td>
<td>7.5±2.3</td>
<td>10.5±2.0</td>
<td>4.8±2.0</td>
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<tr>
<td>EDV (μL)</td>
<td>31.0±7.5</td>
<td>57.0±5.6*</td>
<td>27.4±3.6</td>
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<tr>
<td>ESV (μL)</td>
<td>13.5±5.0</td>
<td>44.5±4.7†</td>
<td>8.9±3.3</td>
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<tr>
<td>CO (mL/min)</td>
<td>7.4±1.7</td>
<td>5.4±0.8</td>
<td>11.0±0.7‡</td>
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<tr>
<td>Ea (mmHg/μL)</td>
<td>6.7±1.5</td>
<td>5.3±1.1</td>
<td>5.0±0.3</td>
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### Systolic indices

- EF (%)                 | 60.0±8.8          | 21.7±3.8*        | 70.0±8.1         |
- dP/dt\(_{max}\) (mmHg/sec) | 7711±1144        | 6467±543        | 11033±425 *     |
- PWR\(_{max}\)/EDV (mmHg/μL) | 28.7±9.9         | 6.6±1.1*        | 41.1±11.3       |

### Diastolic indices

- -dP/dt\(_{min}\) (mmHg/sec) | 6000±510         | 3477±366 §       | 9152±890*       |
- Tau (S) (msec)            | 6.4±0.4          | 9.1±0.7*        | 4.3±0.4*        |
- PFR/EDV (/sec)            | 19.9±4.0         | 9.8±0.8*        | 34.3±4.6*       |

HR, heart rate; ESP, end-systolic pressure; EDP, end-diastolic pressure; EDV, end-diastolic volume; ESV, end-systolic volume; CO, cardiac output; Ea, arterial elastance; EF, ejection fraction; dP/dt\(_{max}\), peak rate of pressure rise; PWR\(_{max}\)/EDV, peak power index; -dP/dt\(_{min}\), peak rate of pressure decline; Tau (S), relaxation time constant calculated by Suga method; PER/EDV, peak filling rate/end-diastolic volume. * p<0.05 vs other two groups, † p<0.005 vs other two groups, ‡ p<0.005 vs MCM+TAM; § p=0.06 vs MCM(-)+TAM; 1-way ANOVA; tukey test for multiple comparisons.
Online Figure Legends:

Online Figure I.  A) Gene expression changes in Alk5 in MCM^{+/}Alk5^{fl/fl} mice treated with tamoxifen TAM) at two dosing regimens. Data are as displayed in Fig 1A; normalized to Gapdh and to results in MCM^{+/} controls. Only the higher TAM dose yielded effective gene knockdown. This result was similar to that in the other flox’d model shown in Fig 1A,B.  B) Summary data for Smad phosphorylation assay results shown in Fig 1B. (n=4/group, *p<0.05 vs MCM^{+/}).

Online Figure II  A, An example of tamoxifen-mediated cardiomyopathy in MCM^{+/}/Tgfbr2^{fl/fl} mouse. This mouse was administered TAM 80mg/kg i.p. injection for only 2 days and then followed by echocardiography. Marked cardiac dysfunction developed and the animal succumbed at day 8.  B, Kaplan-Meier analysis of MCM^{+/}/flox^{+/} versus MCM^{+/}/flox^{+/} mice after receiving the same i.p. dose of tamoxifen for only 2 days.

Online Figure III . A, Representative M-mode echocardiographic images showing the temporal changes of cardiac function before and after oral tamoxifen treatment (80mg/kgBW/day for 7days). Results are shown at baseline, day 10 (3-days after terminating tamoxifen treatment; and peak of cardiac depression), and day 28 (21 days after terminating tamoxifen). Reversible dilated cardiomyopathy was observed in all models that included the heterozygote merCremer (MCM) transgene, with or without a flox’d gene target. MCM-negative controls did not develop dysfunction., (B) Summary data for fractional shortening (FS) and left ventricular end-diastolic dimension (EDD) before and after tamoxifen administration; additional time point at day 14 is shown. (N=5 of each group. *P<0.05 vs MCM^{+/}/Alk5^{0/fl}).
**Online Figure IV**

Hematoxylin/eosin stained myocardium from mice treated with oral tamoxifen (80 mg/kg/day for 7 days). All mice received this treatment. Mice lacking the MCM transgene show normal histology. Those with MCM+/- (no flox’d gene) show patchy inflammation with mononuclear infiltrates at day 10 (3 days after completing tamoxifen) – middle pane - , and this essentially resolved with little residual damage when examined at day 28 (same time when functional recovery was documented). These results were typical of samples from 3-4 separate hearts for each condition.

**Online Figure V**

Temporal changes in fractional shortening (FS) and left ventricular end-diastolic dimension (EDD) during raloxifene (RAL) or tamoxifen (TAM) treatment in MCM+/−/Alk5^fl/fl^ mice. RAL 320mg/kgBW-7d p.o for 7 days treatment; RAL160mg/kgBW-21d p.o.; TAM 20mg/kg-21d p.o for 21 days. N=5 of each group. None of these dosing regimens induced changes in ventricular function.

**Online Figure VI A) Western blot for Cre showing constitutive expression of MerCreMer expression in cardiac myocardium (whole tissue lysates) in MCM+/− but not MCM−/- left ventricles. The ~100kDa band represents the MerCreMer fusion protein. Cell lysate of COS7 cells transfected with Cre-adenovirus (Cell Biolabs, Inc) is used as a control. GAPDH is shown as a loading control.**

**B) Western blot of nuclear extract from isolated cardiomyocytes in MCM+/−/flox−/− or MCM+/+/flox−/− mice immediately after completing 7 day TAM (80mg/kg/d) or 21 day RAL (160mg/kg/d) treatments. Nuclear localization of Cre was detected similarly with both treatments (top bands). Histone H3 is used as a marker for nuclear fraction, and GAPDH as a negative control (used for cytosolic fraction).**

**Online Figure VII** Recombination of the targeted allele in MCM+/−/alk5^fl/fl^ mouse. A) A scheme showing Alk5 recombinant allele (upper) and Cre excised allele (lower). PCR primers are indicated
by small arrow heads \textbf{B}) Genotyping by PCR of genomic DNA extracted from isolated cardiomyocytes. Upper panel shows the triple primer PCR for detecting the Cre excised allele using the primers shown in \textbf{A}. Lower panel shows genotyping for MerCreMer. Seven-day raloxifene treatment showed some induction of the Cre-excised allele, but longer exposure was required to achieve levels similar to TAM. Part of this may relate to a fall in food intact with RAL-mixed food during the initial week (possibly related to taste) which rose after that.

\textbf{Online Figure VIII}

Smad2 phosphorylation by rhTGFβ: 5ng/mL, 30min stimulation in cardiomyocytes from MCM\(^{-/}\)/Alk5\(^{fl/fl}\) treated with 21d RAL. Representative immunoblot (left) and summary data for \%rise in pSmad2 (right) are shown (n=4 for each group, \(\dagger p<0.01\) vs No Tx group).

\textbf{Online Figure IX}

Summary data for fractional shortening (FS) and Alk5 gene expression (normalized to GAPDH, and then to MCM(-/-) data) showing efficacy of low dose sustained tamoxifen (20 mg/kg/d for 21 days) without significant cardiac changes. There was somewhat greater variance in the cardiac response to TAM versus 21d raloxifene (RAL) treatment, however. Data not presented in Fig 3B is denoted by the dashed box.