Temporally Regulated and Tissue-Specific Gene Manipulations in the Adult and Embryonic Heart Using a Tamoxifen-Inducible Cre Protein

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Abstract—The advent of conditional and tissue-specific recombination systems in gene-targeted or transgenic mice has permitted an assessment of single gene function in a temporally regulated and cell-specific manner. Here we generated transgenic mice expressing a tamoxifen-inducible Cre recombinase protein fused to two mutant estrogen-receptor ligand-binding domains (MerCreMer) under the control of the α-myosin heavy chain promoter. These transgenic mice were crossed with the ROSA26 lacZ-flox–targeted mice to examine Cre recombinase activity and the fidelity of the system. The data demonstrate essentially no Cre-mediated recombination in the embryonic, neonatal, or adult heart in the absence of inducing agent but >80% recombination after only four tamoxifen injections. Expression of the MerCreMer fusion protein within the adult heart did not affect cardiac performance, cellular architecture, or expression of hypertrophic marker genes, demonstrating that the transgene-encoded protein is relatively innocuous. In summary, MerCreMer transgenic mice represent a tool for temporally regulated inactivation of any loxP-targeted gene within the developing and adult heart or for specifically directing recombination and expression of a loxP-inactivated cardiac transgene in the heart. (Circ Res. 2001;89:20-25.)

Key Words: cardiac ▪ cre recombinase ▪ genetics ▪ inducible gene expression ▪ embryo

The use of site-specific recombinase, such as Cre or Flp, has permitted an evaluation of gene function within genetically modified animals in a tissue-specific manner. Typically, a gene of interest is flanked with recombinase recognition sequences (loxP sites for Cre), which permit subsequent recombination and gene disruption coincident with expression of the appropriate enzyme. Expression of a site-specific recombinase is achieved either through a tissue-restricted transgene or by inserting the recombinase cDNA into a genetic locus with a known tissue expression profile. Tissue-specific gene disruption is often used to circumvent developmental lethality, excluding an assessment of gene function in the neonatal or adult heart. To circumvent this limitation, we have generated transgenic mice expressing a tamoxifen-inducible Cre-fusion protein specifically within the heart.

Materials and Methods

All experiments were conducted in accordance with the Guide for the Use and Care of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

A cDNA encoding a double-fusion protein between two mutant estrogen receptor domains on either side of Cre recombinase was subcloned as a SalI fragment into the cardiac-specific α-myosin heavy chain (MHC) 5.5-kb promoter construct. The MerCreMer fusion cDNA (gift of Michael Reth, Max-Plank Institute, Freiburg, Germany) encodes the mutated murine estrogen receptor ligand-binding domain (amino acids 281 to 599, G525R), which is insensitive to estrogen but sensitive to tamoxifen. The resulting α-MHC-MerCreMer DNA fragment was injected into pronuclei of freshly fertilized oocytes from FVB mice to produce transgenic animals. FVB founder transgenic mice are presently being crossed into the C57BL/6SV129 genetic background (fourth generation at present) to generate a strain more suitable for traditional gene-targeting experiments. Transgene expression was verified by Western blotting of 100 μg of cardiac protein extract from 3- and 6-week-old FVB transgenic mice using Cre-specific antisera at a dilution of 1:5000 (Covance). Western blotting and the subsequent detection procedure

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were performed as previously described. Similar levels of protein expression were observed between FVB transgenic mice and outbred C57BL/6SJW/SV129 transgenic mice (data not shown).

To induce Cre recombination, adult MerCreMer transgenic mice (3 to 12 weeks of age) were treated with tamoxifen (Sigma) by intraperitoneal injection once a day for 4 to 6 days at a dosage of 20 mg/kg per day. To examine recombination in embryos, pregnant mice were treated with tamoxifen by intraperitoneal injection once a day for 4 days at 20 mg/kg day. Tamoxifen was dissolved in 60% ethanol at a concentration of 5 mg/mL. Hearts from tamoxifen- or saline-treated control mice were removed and perfused in a retrograde manner with phosphate-buffered saline through the aorta, left ventricle, and coronary vasculature for 2 minutes followed by overnight perfusion at room temperature with X-gal staining solution to detect lacZ activity throughout the heart. RNA dot blotting and transtoracic echocardiography were performed as described previously. DNA Southern blotting to detect Cre-mediated recombination used a probe specific to an N-terminal sequence in lacZ and an EcoRI digest of genomic DNA. The lacZ Southern probe (470 base pairs) was generated by polymerase chain reaction using the following primers: lacZ up 5’ GTCAACACTAGCTGTGAACGT-3’ and lacZ down 5’ CTGCACATTCCGTTACG-3’. To examine the activity of the MerCreMer protein, line 1 transgenic mice were crossed with the ROSA26-lacZ-loxP (ROSA) background, and double-heterozygous mice were left untreated (left) or subjected to tamoxifen treatment (right) for 5 days and harvested thereafter for assessment of lacZ staining (blue).

Results
The estrogen receptor is a hormone-activated transcription factor that is usually sequestered by heat-shock proteins in the absence of ligand. In the presence of 17β-estradiol, the estrogen receptor is released, allowing participation in transcriptional regulatory complexes. Since its initial description, the ligand-binding domain of the estrogen receptor (amino acids 281 to 599) has been extensively used as a strategy for transcriptional regulatory complexes. Since its initial description, the ligand-binding domain of the estrogen receptor (amino acids 281 to 599) has been extensively used as a strategy for

A cDNA consisting of Cre recombinase fused to a Mer-encoding sequence at both the N- and C-termini (MerCreMer) was subcloned into the cardiac-specific α-MHC 5.5-kb promoter construct to permit generation of transgenic mice (Figure 1A). This double-fusion strategy was shown to more effectively reduce promiscuous Cre activity in mammalian

Figure 1. A, cDNA encoding the mutant estrogen receptor ligand-binding domain (Mer) flanking Cre recombinase (Cre) was subcloned downstream of the α-MHC 5.5-kb cardiac-specific promoter, which was used to generate transgenic mice (FVB strain). B, Western blotting of heart protein extract from wild-type (Wt) or MerCreMer (MCM) transgenic mice at 3 or 6 weeks of age using Cre-specific antisera demonstrated a band of the predicted size only in the transgenic samples (107 kDa, arrowheads).

cells. Interestingly, the double-fusion protein also demonstrates substantially greater Cre recombinase activity compared with a single CreMer fusion, suggesting that the double-fusion strategy somehow enhances the intrinsic recombinase activity of Cre or its ability to access DNA sites.

Two stable transgenic mouse lines were generated expressing the MerCreMer fusion protein in the heart, both of which were viable with phenotypically normal hearts. One of the transgenic lines (line 2) demonstrated only weak MerCreMer protein expression in the neonatal heart, which was down-regulated in adulthood (data not shown). However, line 1 transgenic mice demonstrated robust MerCreMer protein expression (107 kDa) in the juvenile and adult heart, as revealed with Cre-specific antisera and Western blotting at both 3 and 6 weeks of age (Figure 1B).

To examine the activity of the MerCreMer protein, line 1 transgenic mice were crossed with the ROSA26 lacZ-loxP reporter strain as an indicator of Cre-mediated recombination in the heart. The ROSA26 lacZ-loxP reporter strain contains a Cre-dependent, loxP-inactivated lacZ cDNA cassette targeted within the ubiquitously expressed ROSA26 locus (Figure 2A). Cre-mediated recombination of this allele deletes neomycin and a series of polyadenylation sequences, resulting in the juxtaposition of a splice acceptor site and the lacZ
heterozygous mice (MCM
tROSA) for subsequent analysis.

DNA was also analyzed from the brain (B), kidney (K), lung (Lu),
and skeletal muscle (SkM) from tamoxifen-treated
mice. Genomic
Southern blot analysis of genomic DNA from the hearts of
double-heterozygous mice with or without tamoxifen. D,
indication of Cre-mediated recombination. Remarkably, un-
regulated Cre activity result in irreversible recombina-
tion, which has a cumulative effect on lacZ staining. Whereas
the overall lack of lacZ staining up through 6 weeks of age
indicated extremely tight regulation, we did occasionally
observe a single cell with lacZ staining in histological tissue
sections (data not shown). To more carefully examine the
extent of background recombination, significantly older (3-
month-old) double-heterozygous mice were examined. By 3
months of age, untreated double-heterozygous mice demon-
strated approximately 1% spurious recombination within the
heart as estimated from whole mount and histological sec-
tions (Figure 2B and data not shown). These results indicate
that the MerCreMer transgenic protein is, on the whole,
tightly regulated within the heart so that only very low levels
of unregulated recombination are observed in the absence of
tamoxifen.

Treatment of double-heterozygous mice with tamoxifen for
only 5 days (4 separate intraperitoneal injections) was suffi-
cient to induce robust recombination and lacZ activity in a
uniform profile throughout the embryonic, neonatal, and
adult heart (Figure 2B). To assess tamoxifen-induced recom-
bi}
lacZ staining between male and female mice at 6 weeks of age (data not shown).

At 6 weeks of age, lacZ-stained hearts were histologically sectioned to more carefully evaluate the extent of recombination after 5 days of tamoxifen treatment (Figures 3B and 3C). Nearly all of the myocytes demonstrated lacZ activity in double-heterozygous mice (MCM×ROSA) treated with tamoxifen, whereas untreated mice showed undetectable or extremely low levels of lacZ staining (Figures 3B and 3C). Qualitative assessment of histological sections suggested a recombination frequency of >80% after 5 days of tamoxifen administration in 5- to 6-week-old mice. To additionally evaluate the extent of Cre-mediated recombination, Southern blotting was performed with a probe specific to the ROSA 26 locus (neomycin-lacZ cassette). Untreated double-heterozygous mice showed no recombination at baseline at 6 weeks of age (lane 2), whereas 5 days of tamoxifen treatment revealed >70% recombination in the heart (lane 1) (Figure 3D). However, given that the heart contains a fair number of nonmyocytes (fibroblasts, infiltrates, and endothelial cells) that do not express the MerCreMer protein, the myocyte-specific recombination frequency is undoubtedly >70% as measured at 6 weeks of age. No recombination was observed in the brain, kidney, lung, liver, and skeletal muscle of double-heterozygous mice treated with tamoxifen (Figure 3D). Collectively, these data demonstrate efficient tamoxifen-induced Cre-mediated recombination specifically in the heart of MerCreMer transgenic mice. More importantly, the Southern blot data also confirm the histological data, demonstrating nearly undetectable levels of background recombination at 6 weeks of age.

MerCreMer transgenic mice represent a valuable genetic tool for inducing the inactivation of any loxP-targeted gene within the heart. However, for such a reagent to be useful, the MerCreMer protein itself should not adversely affect the heart. Accordingly, echocardiography was performed in 8-week-old MerCreMer transgenic mice (± tamoxifen for 5 days) and compared with nontransgenic littermate controls. The results show no alterations in cardiac chamber dimensions, wall thicknesses, or fractional shortening in MerCreMer transgene either with or without tamoxifen treatment (Table). It should be noted that MerCreMer transgenic mice were allowed to recover from tamoxifen treatment for 6 days before echocardiography to eliminate any potential nonspecific effects associated with momentary tamoxifen administration.

Heart weights of MerCreMer transgenic mice were similar to nontransgenic littermate control hearts at 8 weeks of age, and H&E- and trichrome-stained histological sections failed to identify any pathology (Figure 4A). More importantly, tamoxifen-treated MerCreMer transgenic mice (6 days after treatment) did not show upregulated expression of hypertrophy marker genes compared with wild-type control mice (Figure 4B). In contrast, control MEK1 transgenic mice with demonstrable hypertrophy showed significant upregulated expression of atrial natriuretic factor, brain natriuretic peptide, skeletal α-actin, and β-MHC mRNA (Figure 4B). Collectively, these data demonstrate that the MerCreMer transgene is relatively innocuous to the heart.

<table>
<thead>
<tr>
<th>Physical Characteristics of MerCreMer Transgenic Hearts at 8 Weeks of Age</th>
<th>Wild Type (n=4)</th>
<th>MerCreMer (n=4)</th>
<th>MerCreMer/Tamox (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Septum (sys)*</td>
<td>0.85±0.21</td>
<td>0.83±0.13</td>
<td>0.98±0.21</td>
</tr>
<tr>
<td>Septum (dia)*</td>
<td>0.59±0.08</td>
<td>0.64±0.08</td>
<td>0.77±0.19</td>
</tr>
<tr>
<td>LVpw (sys)*</td>
<td>1.25±0.16</td>
<td>1.14±0.19</td>
<td>0.8±0.11</td>
</tr>
<tr>
<td>LVpw (dia)*</td>
<td>0.56±0.09</td>
<td>0.56±0.11</td>
<td>0.44±0.1</td>
</tr>
<tr>
<td>LVES*</td>
<td>1.83±0.17</td>
<td>1.78±0.21</td>
<td>2.18±0.3</td>
</tr>
<tr>
<td>LVED*</td>
<td>3.78±0.27</td>
<td>3.38±0.25</td>
<td>3.93±0.4</td>
</tr>
<tr>
<td>FS% †</td>
<td>51±6</td>
<td>47±5</td>
<td>45±3</td>
</tr>
<tr>
<td>Heart/body‡</td>
<td>5.29±0.18</td>
<td>5.14±0.33</td>
<td>5.3±0.1</td>
</tr>
</tbody>
</table>

sys indicates systole; dia, diastole; LVpw, left ventricular posterior wall; LVES, left ventricular end-systolic dimension; LVED, left ventricular end-diastolic dimension; and FS, fractional shortening.

Transgenic (MerCreMer) and wild-type littermate mice were subjected to echocardiography and gravimetric analysis to characterize cardiac dimensions and weight. Each mouse was measured 3 times. None of the values were significantly different between the 3 groups (P=0.05).

*Echocardiographic measurements are given in millimeters.
†Fractional shortening is unitless and was calculated as (LVED−LVES)/LVED×100.
‡Gravimetric analysis of heart weight in milligrams divided by body weight in grams.

**Discussion**

The ability to control tissue specificity of gene deletions in the mouse using Cre-lox technology has profoundly advanced mouse genetics and the ability to examine single gene function in vivo. However, one significant limitation of the traditional Cre-lox approach is an inability to temporally control genetic recombination. Typically, a loxP-targeted gene undergoes Cre-mediated recombination coincident with Cre expression directed by a promoter region of interest. In this study, we describe transgenic mice that express a modified Cre recombinase fusion protein in the heart under the transcriptional control of the α-MHC promoter. Whereas transgene expression from this promoter is largely constitutive throughout development and in the adult heart, the Cre recombinase fusion protein is inactivated by two mutant estrogen receptor ligand-binding domains. Such an approach only requires a single transgene to achieve cardiac-specific and temporally regulated recombination in vivo. Alternative cardiac-specific gene regulatory strategies, such as the tetracycline-responsive activator and transcriptional regulatory region, have also been recently described. However, the tetracycline-inducible system typically requires two independent transgenes (binary), one expressing the tet-activator/repressor in the tissue of interest and one containing a chimeric tetracycline-regulated promoter fused to a cDNA encoding Cre recombinase. Although such a tetracycline-inducible system for regulating Cre-mediated recombination in the heart has yet to be described, it remains entirely feasible, because the proof of concept has already been demonstrated. However, a binary tetracycline-inducible approach requires a more complicated breeding strategy compared with the monogenic MerCreMer approach described herein. In addition, the potential fidelity (leakiness) of a tetracycline-regulated Cre recombinase system is uncertain.
Figure 4. Histological and molecular marker analysis of MerCreMer transgenic mouse hearts. A, Eight-week-old wild-type mice and MerCreMer transgenic mice were subjected to histological assessment of gross morphology (top), and cellular architecture in H&E-stained sections or fibrosis was analyzed in trichrome stained (Trich) sections. B, Cardiac mRNA dot blot analysis from wild-type mice, tamoxifen-treated MerCreMer transgenic mice, or a MEK1 transgenic mouse (positive control). Sk.α-actin indicates skeletal α-actin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

While this manuscript was in review, Minamino et al. demonstrated drug-regulated Cre recombination in a temporally controlled manner in the mouse heart. These investigators used a construct in which Cre was fused to the ligand-binding domain of the human progesterone receptor under the control of the α-MHC promoter. Similar to our study, Minamino et al. demonstrated that even a single tamoxifen dose (7.5 mg/kg per day) administered daily between E12 and E18 in pregnant CD-1 mice did not compromise development or induce abortion, although endometrial hyperplasia and polyploid adenomas were identified in 30% to 50% of female offspring at 1 year of age. Despite these concerns, the results of the present study indicate that MerCreMer transgenic mice can be used to temporally regulate gene manipulations within the developing mouse heart without causing embryonic lethality.

Here we measured Cre-mediated recombination within the ROSA26 gene locus, which is ubiquitously expressed and constitutively active in the mouse. Given its active confirmation with respect to chromatin, it is formally possible that the ROSA26 locus is more permissive to site-specific recombination compared with other genetic loci. A final technical consideration relates to the use of tamoxifen as an inducing agent. Although echocardiographic assessment of tamoxifen-treated MerCreMer transgenic mice did not reveal obvious defects in cardiac chamber dimensions 6 days after drug treatment, nor were hypertrophic marker genes significantly activated, it remains possible that tamoxifen might alter some aspect of cardiac physiology or biochemistry. However, decreasing the dose of tamoxifen (10 mg/kg per day) or the time course of treatment might resolve any potential alterations. Indeed, we have observed that even a single tamoxifen injection in 3-week-old double-heterozygous mice induced significant lacZ expression (data not shown).

The most obvious application of the MerCreMer transgenic line is to promote temporally regulated deletion of loxP-targeted genes in vivo. However, the MerCreMer transgene can also be used to temporally regulate expression of another unrelated transgene within the heart. Such a transgene would be constructed such that a loxP flanked stuffer sequence is placed immediately upstream of a given cDNA, rendering it inactive. Cre-mediated excision of such a stuffer region would result in the juxtaposition of the downstream cDNA and the given promoter, as shown in Figure 2A. In this manner, a transgene could be specifically activated in the adult heart, bypassing developmental affects associated with unregulated transgenesis. In summary, MerCreMer transgenic mice will permit temporally regulated activation or inactivation of a properly designed transgene or a loxP-targeted genetic locus within the heart. Such applications will undoubtedly expand our ability to genetically dissect the function of putative disease-causing genes within either the developing or adult heart.
Acknowledgments
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References
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