Rapid and highly efficient inducible cardiac gene knockout in adult mice using AAV-mediated expression of Cre recombinase

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Aims
Inducible gene targeting in mice using the Cre/LoxP system has become a valuable tool to analyse the roles of specific genes in the adult heart. However, the commonly used Myh6-MerCreMer system requires time-consuming breeding schedules and is potentially associated with cardiac side effects, which may result in transient cardiac dysfunction. The aim of our study was to establish a rapid and simple system for cardiac gene inactivation in conditional knockout mice by gene transfer of a Cre recombinase gene using adeno-associated viral vectors of serotype 9 (AAV9).

Methods and results
AAV9 vectors expressing Cre under the control of a human cardiac troponin T promoter (AAV-TnT-Cre) enabled a highly efficient Cre/LoxP switching in cardiomyocytes 2 weeks after injection into 5- to 6-week-old ROSA26-LacZ reporter mice. Recombination efficiency was at least as high as observed with the Myh6-MerCreMer system. No adverse side effects were detected upon application of AAV-TnT-Cre. As proof of principle, we studied AAV-TnT-Cre in a conditional knockout model (Srf-flex1 mice) to deplete the myocardium of the transcription factor serum response factor (SRF). Four weeks after AAV-TnT-Cre injection, a strong decrease in the cardiac expression of SRF mRNA and protein was observed. Furthermore, mice developed a severe cardiac dysfunction with increased interstitial fibrosis in accordance with the central role of SRF for the expression of contractile and calcium trafficking proteins in the heart.

Conclusions
AAV9-mediated expression of Cre is a promising approach for rapid and efficient conditional cardiac gene knockout in adult mice.

Keywords
Conditional transgenic mouse • Gene regulation • Adeno-associated virus • Cardiomyopathy • Serum response factor

1. Introduction
Overexpression and deletion of genes in the myocardium of transgenic mice has become an important instrument for investigations of genetic circuits underlying proper long-term myocardial function and dysfunction. Temporally controlled gene ablation in adult mice allows bypassing embryonic lethality or malformations associated with constitutive gene knockout. Newer transgenic mouse models have allowed inducible Cre expression in cardiomyocytes. Notably, Myh6(α-MHC)-MerCreMer transgenic mice,1 where Cre activity is induced by the application of tamoxifen or raloxifene, have become a valuable tool for generating conditional knockout models.2–5

To circumvent time-consuming breeding strategies for the generation of double-transgenic mice, we aimed to develop an alternative system for cardiac-specific expression of Cre using adeno-associated viral (AAV) vectors of serotype 9, offering the advantage of direct applicability...
in mice carrying a LoxP-flanked (‘floxed’) gene. Previously, AAV9 vectors enabled an efficient cardiac gene transfer in adult mice after intravenous injection.6–10 However, as AAV9 also transduces cells outside of the heart, notably the liver, we sought to control AAV-mediated Cre expression by a tissue-specific promoter. Previously, the cardiac troponin T (TnT) promoter from chicken has been extensively studied in vitro. It showed good expression in cultured cardiac myocytes and has already been applied for cardiac gene transfer using AAV.10 The rat TnT promoter has also been successfully applied in vitro14 and additionally showed a reliable cardiomyocyte-specific expression in transgenic mice.15,16 We decided to further characterize the human cardiac TnT promoter, aiming at future clinical applications of AAV-based gene therapy in the human heart.

Here we report rapid, safe, and highly efficient LoxP switching in the heart through AAV9-mediated Cre expression. In addition to a reporter mouse line (ROSA26-LacZ17), we successfully applied this approach in a conditional serum response factor (SRF) knockout line (transgenic mouse strain with a floxed exon 1 of the murine Srf gene, Srf:flex116). Deletion of SRF in the myocardium of adult mice resulted in a severe cardiomyopathy, consistent with the central role of this transcription factor for the expression of myocardial proteins.2,19–22

2. Methods

Detailed methods are provided in Supplementary material online.

2.1 Generation of AAV vectors

All AAV transgenes were cloned into a self-complementary AAV vector backbone.23 Recombinant AAV9 vectors were produced and purified using iodixanol gradient ultracentrifugation as described previously.24 Titration was performed by quantitative polymerase chain reaction (qPCR) on vector genomes. Further details of the procedure are provided in the Expanded Methods section in Supplementary material online.

2.2 Animal procedures and in vivo vector delivery

All procedures involving the use and care of animals were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996), the German animal protection code, and approval of the local ethics review board (G87/08).

NMRI mice were obtained from the German branch of Jackson Laboratory and used for studies with the luciferase and GFP reporters. All transgenic lines were maintained in the C57BL/6 background and male littermates were used for the experiments. ROSA26-LacZ and Srf:flex1 mice were homozygous for the respective alleles. Mice were randomly assigned to the treatment groups. AAV vectors were intravenously injected into the tail vein as a 200 μL bolus. Tamoxifen (Sigma, Cat# T5648) was dissolved in peanut oil (PFA) and embedded in paraffin.

2.3 Luciferase assays

Frozen tissue samples were disrupted using a rotor-stator homogenizer in Luciferase Assay Lysis Buffer (Promega, Mannheim, Germany). Protein concentration was determined with a BCA assay and luciferase activity was measured in a luminometer (Lumat LB9501; Berthold, Bad Wildbad, Germany) using the Renilla Luciferase Assay System (Promega) according to manufacturer’s instructions.

2.4 Histological analysis

Frozen sections (7 μm thick) from the mid-part of myocardium and other examined tissues from ROSA26-LacZ mice were cut, fixed, and stained with X-Gal. Haematoxylin and eosin (HE) and Sirius red stainings were performed on 6 (HE) or 8 (Sirius red) μm thick paraffin-embedded tissue slices. For evaluation of inflammation or fibrosis, we analysed at least two HE and two Sirius red sections under light microscopy. Immunohistology for SRF was performed on PFA-fixed slides after deparaffinization and antigen retrieval in sodium citrate buffer, using a monoclonal rat primary antibody (Clone 2C5, Active Motif, Cat# 61385) in a 1: 500 dilution.

2.5 Western blot analysis

Primary antibodies for SRF (clone 2C5, Active Motif, Cat# 61385, 1: 1000 dilution) and GAPDH (Calbiochem, Cat# CB1001, 1: 2500 dilution) were incubated overnight at +4°C. HRP-conjugated secondary antibodies were incubated for 1 h at room temperature. Pierce ECL (GAPDH) and ECL Plus (SRF) western blotting substrates were used for detection.

2.6 Transthoracic echocardiography

Echocardiography (echo) was performed in two separate facilities with a VisualSonics Vevo 770 (ROSA26-LacZ experiments) or a Vevo 2100 (Srf:flex1 experiments) device. Echos for ROSA26-LacZ experiments were performed under isoflurane anaesthesia (0.8–1% in oxygen with spontaneous ventilation), whereas for Srf:flex1 experiments mice were awake during the procedure to avoid a potential risk by the anaesthesia. In both cases, the treatment and respective control groups were analysed by a blinded examiner. Left ventricular parasternal short-axis views were obtained in M-mode imaging at the papillary muscle level. Three consecutive beats were used for measurements of left ventricular end-diastolic diameter (EDD) and left ventricular end-systolic diameter (ESD). Fractional shortening (FS) was calculated as $FS = \frac{\text{EDD} - \text{ESD}}{\text{EDD}} \times 100%$.

2.7 Quantitative real-time polymerase chain reaction analysis

RNA from heart samples was reverse-transcribed to cDNA and equal amounts for each sample were used in duplicates in a qPCR reaction with the Sybr Green dye. Gene-specific primers were synthesized for Srf (Exon 1, not recombined by Cre), Cre, Nppb atrial natriuretic factor (ANF), Nppb brain natriuretic peptide (BNP), Myh7 β-myosin heavy chain (β-MHC), and Gapdh (as a housekeeping gene).

2.8 Statistical analysis

All data were expressed as mean ± standard error. To test for statistical significance between two groups, an unpaired two-sided Student’s t-test was applied. One-way ANOVA with a Bonferroni post-test was applied for comparisons among three or more groups. A two-way ANOVA for repeated measures with a Bonferroni post-test was applied for the longitudinal echocardiography studies. P-values of <0.05 were considered significant.
3. Results

3.1 Assessment of Cre-mediated recombination after systemic injection of AAV-MLC-Cre in adult mice

We first generated AAV9 vectors containing the open reading frame of the codon-improved Cre recombinase (Cre) under the control of the cytomegalovirus (CMV)-enhanced 260 bp myosin light chain-(CMVEnh/MLC0.26) promoter, which previously enabled an efficient cardiac expression in adult mice (AAV-MLC-Cre, Figure 1A). Self-complementary AAV vector genomes were used as they enable a significantly faster transgene expression compared with conventional single-strand AAV vectors.

We injected 10^{12} genomic particles (Vg, vector genomic particles) of AAV-MLC-Cre intravenously into 8- to 10-week-old ROSA26-LacZ reporter mice. This transgenic mouse strain expresses LacZ after Cre-mediated excision of a floxed transcription-silencing sequence (see Supplementary material online, Figure S1). Mice were sacrificed 5 and 10 weeks after vector injection and X-Gal staining was performed on cryosections of hearts showing LacZ expression in ≈50–70% of cardiomyocytes (Figure 1B). No stained cells were observed in control hearts. We also analysed liver, lung, spleen, kidney, and skeletal muscle cryosections of the 5-week group by X-Gal staining. A strong ubiquitous expression of LacZ was observed in the liver, suggesting that the hepatic leakiness of the CMVEnh/MLC0.26 promoter is sufficient to drive Cre expression in the majority of hepatocytes (Figure 1C). Also, a certain proportion of cells recombined in other tissues such as lung and kidney.

3.2 Analysis of human cardiac TnT promoter fragments

To improve the specificity of AAV-mediated recombination, we analysed several fragments of the 5′ region of the human cardiac troponin T (TnnT2) gene for their ability to drive cardiac-specific transgene expression. The human TnnT2 promoter shows high homology to the rat promoter, with known cis-acting elements being largely conserved (see Supplementary material online, Figure S2).

Four promoter fragments of the human TnnT2 promoter (abbreviated as TnT-1 to TnT-4) were cloned into AAV vector genomes to drive the expression of Renilla luciferase (Rluc) and compared with the CMVEnh/MLC0.26 promoter (Figure 2A and B). Six weeks after the injection of 10^{11} Vg of the respective AAV9 vectors in 6- to 8-week-old mice, the fragment TnT-4 showed the strongest cardiac expression, whereas the Rluc levels in the other analysed organs were reduced (Figure 2C). Notably, expression in the liver was ≈60× lower compared with CMVEnh/MLC0.26. Since intravenous injection of 10^{12} Vg of AAV9 harbouring enhanced fluorescent protein (EGFP) under control of the TnT-4 promoter (AAV-TnT-EGFP) resulted in a highly efficient cardiac transduction (see Supplementary material online, Figure S3), we have chosen the TnT-4 promoter for further expression of Cre (AAV-TnT-Cre, Figure 2B).

3.3 AAV-TnT-Cre affords highly efficient LoxP recombination in ROSA26-LacZ mice with strongly increased specificity for the heart

We next compared the efficiency of AAV-TnT-Cre-mediated LoxP switching with that of the widely used Myh6-MerCreMer mice, while at the same time assessing possible side effects.
Myh6-MerCreMer<sup>Tdf0</sup>, ROSA26-LacZ<sup>fox/fox</sup> mice were crossbred with ROSA26-LacZ<sup>fox/fox</sup> to obtain Myh6-MerCreMer<sup>Tdf0</sup>, ROSA26-LacZ<sup>fox/fox</sup> and Myh6-MerCreMer<sup>D00</sup>, ROSA-LacZ<sup>fox/fox</sup> littermates. At the age of 5–6 weeks, MerCreMer<sup>Tdf0</sup> mice were either administered five daily injections of 40 mg/kg of tamoxifen or vehicle (MCM-Tam and MCM-Ctrl groups), whereas MerCreMer<sup>D00</sup> littermates were either administered a single intravenous injection of 10<sup>12</sup> Vg AAV-TnT-Cre or AAV-EGFP, or served as controls (Figure 3A). Mice were sacrificed 4 weeks after the initial injection. Echocardiography at the indicated time points confirmed the appearance of a transient cardiac dysfunction in MCM-Tam mice following tamoxifen application (Figure 3B and see Supplementary material online, Table S1), which has been reported previously. We additionally observed an increase in heart rate in this group at Day 6, which is typical for compensatory sympathetic overstimulation due to insufficient cardiac output (see Supplementary material online, Table S1). No changes were observed in the AAV-TnT-Cre group. The heart function recovered in most of the MCM-Tam mice by 3 weeks after the last tamoxifen injection. Assessment of X-Gal staining on heart cryosections showed a surprisingly high and consistent amount of cardiac recombination after injection of AAV-TnT-Cre (Figure 3C). In the MCM-Tam group, the recombination efficiency was comparably high in the lateral wall of the left ventricle; however, we observed a weaker efficiency in the septum region in all of the mice in this group.

Analysis of cardiac sections at more early time points revealed a significant LacZ expression already 1 week after injection of AAV-TnT-Cre (Figure 3D). Two weeks after vector injection, mice showed a recombination efficiency similar to those after 4 weeks. We analysed extracardiac lacZ expression in tissues from mice 4 weeks after injection of AAV-TnT-Cre. Approximately 5–10% of cells had recombined in the liver, while LacZ-positive cells were almost absent in the parenchyma of lung, spleen, and kidney and undetectable in skeletal muscle (Figure 4A). We did occasionally observe LacZ-expressing cells in the smooth muscle of the media of some of the larger vessels in the lung and heart (Figure 4B), while large vessels (aorta) showed no LacZ expression (Figure 4B). In Myh6-MerCreMer mice, we did not observe any recombined cells outside of the heart, showing that the transgene allows for a cardiomyocyte-specific Cre expression (Figure 4A).

To further analyse the specificity of AAV-TnT-Cre-mediated recombination in cardiac tissue, hearts from ROSA26-LacZ mice were subjected to collagenase digestion with subsequent separate isolation of myocytes and non-myocytes 13 weeks after the injection of 10<sup>12</sup> Vg AAV-TnT-Cre. The late time point was chosen due to the fact that recombination events might continue to occur at later stages (compare Figure 1B, 5 vs. 10 weeks). After staining with X-Gal, we could confirm recombination in the vast majority of cardiomyocytes, while only ~5% of non-myocytes were weakly stained (Figure 4C).

**Figure 2** In vivo characterization of the efficiency and specificity of the human cardiac TnT promoter. (A) Schematic representation of the analysed promoter fragments from the human cardiac TnT gene (hTnnT2, promoter fragments were abbreviated with TnT). (B) Schematic representation of the AAV transgenes used in C and for further AAV-TnT-Cre experiments. (C) Comparison of Rluc expression in protein lysates of tissues from mice 6 weeks after intravenous injection of AAV9 vectors with respective promoters (n ≥ 4 for each group).
The applied dose of $10^{12}$ Vg AAV is typically used in our lab and has delivered robust overexpression for most experiments. In order to study the effect of different dosages, we performed a dose titration study with the doses of $1.4 \times 10^{12}$, $0.5 \times 10^{12}$, and $0.25 \times 10^{12}$ Vg AAV-TnT-Cre under the same conditions as above. Interestingly, we obtained a similar efficiency in the heart with $0.5 \times 10^{12}$ and $1 \times 10^{12}$ Vg as before with $1 \times 10^{12}$ Vg (see Supplementary material online, Figure S4A). The recombination events in the liver, however, appeared lower with $0.5 \times 10^{12}$ and higher with $1.4 \times 10^{12}$ Vg. The other analysed tissues in the $1.4 \times 10^{12}$ Vg group (see Supplementary material online, Figure S4B) were similar to the previous results with $1 \times 10^{12}$ Vg (Figure 4A). We conclude that a dosing range between $0.5 \times 10^{12}$ and $1.4 \times 10^{12}$ Vg of AAV-TnT-Cre can be applied for efficient recombination in the heart.

### 3.4 Analysis of possible side effects of AAV-TnT-Cre

AAV-TnT-Cre-injected mice ($1 \times 10^{12}$ Vg) did not show any alterations in the analysed morphometric parameters at the time of dissection (weights of heart, lung, liver, and spleen, bodyweight, tibia length; see Supplementary material online, Figure S5). In addition, the analysed blood parameters for possible liver (alanine amino transferase and aspartate amino transferase) and kidney (Crea) damage were also in the normal range for all mice (see Supplementary material online, Figure S5).

HE and Sirius red (fibrosis) stainings of hearts and livers showed no signs of inflammation or fibrosis in these organs. Also, no changes were observed in the HE histology of the kidney and lung tissues (see Supplementary material online, Figure S6).

We additionally determined local mRNA levels of the cytokines and cytokine receptors, which are typical for inflammation or myocarditis [Ccr1, Ccr2, Ccr5, Ccl2 (MCP-1), Il6, and Tnf (TNF-alpha)] from heart samples. No significant increase was observed in the AAV-injected group (see Supplementary material online, Figure S7A). To exclude a systemic inflammatory response, the levels of the inflammatory cytokines IL-1b, IL-6, and TNF-alpha were determined by ELISA in serum and showed no significant increase in the injected mice (see Supplementary material online, Figure S7B).

To assess for possible long-term effects of AAV-TnT-Cre, we performed an additional study over 12 weeks where we included an additional high-dose ($1.4 \times 10^{12}$ Vg) group. Cre expression in the heart remained constant at 4 and 12 weeks after injection, showing that AAV transgenes continued to persist in the myocardium and that the TnT promoter was not being silenced (see Supplementary material online, Figure S8A). We observed no changes in echocardiographic parameters at 4, 8, and 12 weeks after injection (see Supplementary material online, Figure S8B and Table S2). The evaluated organ weights were unchanged and the blood values remained in the normal range after 12 weeks (see Supplementary material online, Figure S8C).

**Figure 3** Comparison of AAV-TnT-Cre with Myh6-MerCreMer in ROSA26-LacZ reporter mice. (A) Outline of the experimental procedure, Day 1 being the day of AAV or the first tamoxifen injection. (B) FS over the time course of the experiment. *P < 0.05 for MCM-Tam vs. MCM-Ctrl. (C) Representative X-Gal staining of heart cryosections. Overviews show individual mice. Scale bar in the overview = 1 mm and in the magnification = 150 μm. (D) Additional analysis of cardiac sections 1 and 2 weeks after AAV-TnT-Cre injection (scale bar sizes as in C). *P < 0.05. For A-C: MCM-Ctrl: n = 3; MCM-Tam, AAV-EGFP, Ctrl: n = 4; AAV-TnT-Cre: n = 5. For D: n ≥ 4.
Overall, we could not detect any negative side effects of AAV9-mediated continuous expression of Cre recombinase using the human cardiac TnT promoter at the applied doses. This is in agreement with previous findings, which indicate a very mild immune response to systemic AAV application in mice, which is limited to several hours post-injection.

### 3.5 Efficient depletion of SRF in hearts of Srf-flex1 mice after AAV9-mediated Cre expression

We then investigated whether AAV-TnT-Cre is suitable to mediate a gene knockdown in hearts of adult conditional SRF knockout mice and thereby induce cardiomyopathy, as previously shown with the Myh6-MerCreMer model. In Srf-flex1 mice, Cre recombinase mediates an excision of the floxed coding sequence of exon 1 of Srf, thereby ablating the expression of a functional, full-length SRF.

When 5- to 6-week-old Srf-flex1 mice were injected with 10^{12} Vg AAV-TnT-Cre, a strong reduction of both Srf mRNA and protein was observed in the heart after 4 weeks (Figure 5A and B). This was confirmed by immunohistochemistry (Figure 5C).

Using echocardiography we observed a pronounced phenotype with significant systolic dysfunction developing by 4 weeks after injection and increasing further by 7 weeks, at which point a diastolic dilation was also observed (Figure 6A and B, and see Supplementary material online, Table S3). Three mice in the AAV-TnT-Cre group (total n = 8), which previously showed the lowest ejection fractions in echo, died during the 8th week post-injection, at which point we sacrificed all groups. A quantitative PCR on heart biopsies confirmed a strong increase in foetal genes that are typically up-regulated in heart failure [Myh7 (β-MHC), Nppa (ANF), and Nppb (BNP); Figure 6C]. Histologically, we observed a modest but significant increase in left ventricular fibrosis (Sirius red staining), whereas HE staining did not reveal any obvious alterations (Figure 6D and E).

These findings mirror the phenotype obtained with the Myh6-MerCreMer-mediated SRF knockdown.

### 4. Discussion

Conditional gene deletion approaches in the myocardium currently rely on crossbreeding of several genetically engineered mouse lines. In the
brain, local application of AAV2-Cre has become one of the commonly used tools for Cre–LoxP switching in vivo. In the liver, adenoviral Cre expression has been established long ago, while more recent approaches have used AAV8 for this purpose. Although successful adenovirus-mediated Cre expression has been demonstrated in the heart using reporter genes, to our knowledge it has not been applied for gene knockout. This may be due to the relatively low recombination efficiency and the necessity for an invasive intracardiac or transcoronary vector delivery. Although AAV vectors, especially AAV9, have demonstrated a high efficiency for myocardial transduction, they have so far not been used for knockout experiments. One reason could be the high amount of extracardiac transduction by AAV9, as we observed with the less specific CMVenh/MLC0.26 promoter.

This study shows the feasibility and effectiveness of an AAV9-mediated cardiac transfer of Cre recombinase through a single intravenous injection. When using the human cardiac TnT promoter (AAV-TnT-Cre), we were able to achieve a high degree of recombination in cardiomyocytes, which is at least comparable with the efficiency obtained using the Myh6-MerCreMer transgene, even at the relatively high dose of 40 mg/kg/days × 5 days of tamoxifen. Although the strength of Cre expression for efficient recombination may vary between different transgenes, our data suggest that a dose in the range of 0.5–1 × 10^12 Vg injected at the age of 5–6 weeks allows for a highly efficient myocyte transduction and can be used as a starting point. Furthermore, since AAV-TnT-Cre enabled significant recombination already 1 week after injection, onset of recombination appears to be suitable also for approaches requiring a rapid conditional knockout.

Using AAV-TnT-Cre we obtained a low level and dose-dependent off-target recombination, mostly in the liver and occasionally in vessels, especially in the lung. This remaining off-target recombination could be a disadvantage for applications where absolute specificity is important. If analysis of extracardiac tissue results in significant alterations of the target protein, e.g. in the liver, it might become necessary to balance extracardiac effects of the AAV-Cre approach against transient cardiac dysfunction in double-transgenic lines. Alternatively, ectopic hepatic expression could be further reduced through post-transcriptional detargeting using miR-122-target sites.

We could not observe any adverse effects in animals treated with AAV-TnT-Cre (despite ongoing cardiac expression of Cre recombinase) as confirmed by echocardiography, histology, cytokine expression analysis and unchanged heart weight, lung weight, and body weight parameters. Also, no changes were observed for the other analysed organs (liver, lung, kidney, and spleen). Most importantly, we did not observe any signs of an ongoing or past inflammation or autoimmune reaction, which could have been triggered by viral vectors. This was additionally confirmed by unchanged plasma levels of cytokines, which are most commonly elevated during immunological processes.

Using AAV-TnT-Cre, we achieved a strong knockdown of Srf in the hearts of conditional Srf-deficient mice (Srf-flex1). Decreased Srf expression was associated with heart failure, as observed in the reduced FS.

**Figure 5** AAV-TnT-Cre-mediated knockout of Srf, 4 weeks after injection into 5- to 6-week-old male homozygous Srf-flex1 mice. (A) Quantification of wild-type (non-recombined) Srf mRNA in heart tissue (normalized to Gapdh and to the AAV-EGFP group). (B) Western blot analysis of SRF protein (quantification normalized to GAPDH and to the AAV-EGFP group). (C) SRF immunostaining (bar = 100 μm). Typical nuclear staining is observed in the AAV-EGFP group, which is largely absent in the AAV-TnT-Cre group. For all panels: AAV-EGFP: n = 3; AAV-TnT-Cre: n = 4. *P < 0.05.
which was mainly due to a systolic dysfunction. We also observed an increased fibrosis and a strong up-regulation of heart failure genes, such as \textit{Myh7} (\(\beta\)-MHC), \textit{Nppa} (ANF), and \textit{Nppb} (BNP). A corresponding phenotype has previously been reported for \textit{Srf} disruption in adult mice using the \textit{Myh6}-MerCreMer transgene.\(^2\)

An advantage of the AAV-TnT-Cre approach compared with conventional inducible transgenes is the reduction in time and breeding efforts required for conditional knockout experiments. Breeding of double-transgenic lines requires at least two mating rounds (>4 months) in order to cross in a Cre-driver line into a homozygous floxed background, while AAV-TnT-Cre can be applied directly in the homozygous line.

A disadvantage of the AAV-TnT-Cre approach might be the formation of neutralizing antibodies against AAV9, which could interfere with re-administration of AAV9 vectors expressing therapeutic gene products in such conditional knockout model. Simultaneous administration of an AAV9 vector with a therapeutic sequence together with AAV-TnT-Cre or alternatively the use of double-transgenic \textit{Myh6}-MerCreMer lines might be preferable in such a scenario.

Overall, AAV-TnT-Cre appears to be a fast and robust system to mediate efficient conditional Cre-dependent gene inactivation in the heart of adult transgenic mice.

### Supplementary material

Supplementary material is available at \textit{Cardiovascular Research} online.

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Conflict of interest: A patent application concerning the human TnT promoter was filed by S.W., J.K., H.A.K., and O.J.M. No further conflicts of interest.

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