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A Titratable Two-Step Transcriptional Amplification Strategy for Targeted Gene Therapy Based on Ligand-Induced Intramolecular Folding of a Mutant Human Estrogen Receptor

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Abstract

Purpose—The efficacy and safety of cardiac gene therapy depend critically on the level and the distribution of therapeutic gene expression following vector administration. We aimed to develop a titratable two-step transcriptional amplification (tTSTA) vector strategy, which allows modulation of transcriptionally targeted gene expression in the myocardium.

Procedures—We constructed a tTSTA plasmid vector (pcTnT-tTSTA-fluc), which uses the cardiac troponin T (cTnT) promoter to drive the expression of the recombinant transcriptional activator GAL4-mER(LBD)-VP2, whose ability to transactivate the downstream firefly luciferase reporter gene (fluc) depends on the binding of its mutant estrogen receptor (ER_{G521T}) ligand binding domain (LBD) to an ER ligand such as raloxifene. Mice underwent either intramyocardial or hydrodynamic tail vein (HTV) injection of pcTnT-tTSTA-fluc, followed by differential modulation of fluc expression with varying doses of intraperitoneal raloxifene prior to bioluminescence imaging to assess the kinetics of myocardial or hepatic fluc expression.

Results—Intramyocardial injection of pcTnT-tTSTA-fluc followed by titration with intraperitoneal raloxifene led to up to tenfold induction of myocardial fluc expression. HTV injection of pcTnT-tTSTA-fluc led to negligible long-term hepatic fluc expression, regardless of the raloxifene dose given.

Conclusions—The tTSTA vector strategy can effectively modulate transgene expression in a tissue-specific manner. Further refinement of this strategy should help maximize the benefit-to-risk ratio of cardiac gene therapy.

Keywords

Gene therapy; Drug-regulated gene expression; Transcriptional amplification; Transcriptional targeting; Intramolecular folding; Bioluminescence imaging

Introduction

The success of gene therapy depends critically on the level and the distribution of therapeutic gene expression after vector delivery. Although the spatiotemporal kinetics of therapeutic gene expression can be coarsely manipulated by adjusting the vector dose and the route of gene delivery, it would be more ideal to be able to finely regulate transgene expression after vector administration, so as to maximize treatment efficacy and minimize untoward side effects. Angiogenic gene therapy for myocardial ischemia is an example of such a scenario in which unregulated therapeutic gene expression (e.g., overexpression of vascular endothelial growth factor (VEGF)) can lead to undesirable side effects, such as aberrant neovasculature and hemangioma formation in small animal models and severe hypotension and tissue edema in humans [1]. Furthermore, unlike conventional drug therapy, the delivery of a therapeutic gene to the myocardium requires either percutaneous coronary intervention or open-heart surgery with finite risks of morbidity and mortality, which makes repeated gene delivery less clinically feasible. Therefore, a vector strategy that allows exogenous modulation of therapeutic gene expression after a single vector delivery should help maximize the clinical feasibility, safety, and efficacy of cardiac gene therapy.

Transcriptional targeting using a cardiac-specific promoter to drive therapeutic gene expression represents a promising strategy for spatial regulation of transgene expression by minimizing off-target gene expression. However, the practicality of this approach is limited by the weak transcriptional activity of most cardiac-specific promoters in adult cardiomyocytes [2, 3]. We have previously developed a generalizable two-step transcriptional amplification (TSTA) vector strategy, which can enhance the transcriptional activity of the cardiac troponin T (cTnT) promoter by as much as 42-fold in the mouse myocardium without significantly compromising its long-term tissue specificity (Fig. 1a) [3]. In the present study, we tested the hypothesis that this constitutive system can be further modified to allow modulation of the level of transcriptional amplification.

The titratable version of the TSTA vector system (tTSTA) constructed in this study takes advantage of the intramolecular folding pattern of the human estrogen receptor (ER) ligand binding domain (LBD) in response to ligand binding. We have previously found that the N-terminus and the C-terminus of the LBD approximate when the LBD core binds to an ER

ligand, more specifically, an ER antagonist [4]. Thus, we hypothesized that when the ER-LBD is inserted between the GAL4 DNA-binding domain and a peptide containing two copies of the herpes simplex virus type 1 transactivation domain (VP16), the functionality of the resultant recombinant transcriptional activator (GAL4-ER(LBD)-VP2) can be regulated using a selective estrogen receptor modulator (SERM) such as raloxifene. When used in a TSTA vector system to drive transgene expression (e.g., firefly luciferase (fluc) reporter gene expression), the overall level of gene expression can be finely modulated using varying levels of raloxifene. Here, we report the construction and characterization of an *in vivo*-ready variant of the tTSTA vector system (pcTnT-tTSTA-fluc) in which the ER-LBD has been mutated (ER_{G521T}) and previously shown to have no affinity for the endogenous ligand 17 β -estradiol (Fig. 1b) [4]. With further optimization, this vector strategy should help open the door for numerous gene therapy applications in which both the kinetics and the distribution of transgene expression need to be tightly regulated.

Materials and Methods

Starting Plasmids and Vector Construction

The construction of an unidirectional constitutive TSTA vector (pcTnT-TSTA(G5)-fluc) containing the cTnT promoter, the GAL4-VP2 transcriptional activator gene, 5 tandem repeats of GAL4 binding sites (Gal4bs), and the fluc gene has been previously described [3]. pcTnT-TSTA(G5)-fluc was used in this study as the starting vector. PCR amplification of a fragment containing the LBD of a mutant human ER (ER_{G521T}) [4], followed by insertion into EcoRI- and BamHI-digested pcTnT-TSTA(G5)-fluc, produced the titratable TSTA vector, pcTnT-tTSTA-fluc. pCMV-hRL and pCMV- β -gal (cytomegalovirus (CMV) promoter-driven vectors that express human synthetic *Renilla* luciferase and β -galactosidase reporter genes, respectively) were purchased from Promega (Madison, WI). pCMV-fluc, a CMV promoter-driven vector expressing fluc, was constructed as previously described [3]. Note that the firefly luciferase, *Renilla* luciferase, and β -galactosidase reporter genes are abbreviated herein as fluc, hRL, and β -gal, whereas their expressed enzymes are abbreviated as FLuc, RLuc, and β -GAL, respectively.

Cell Culture and Transfection

HL-1 mouse cardiomyocytes were maintained in flasks coated with 0.00125 % fibronectin (Sigma-Aldrich, St. Louis, MO) and 0.02 % gelatin (Sigma-Aldrich) and cultured in Claycomb medium (JRH Biosciences, Lenexa, KS) supplemented with 10 % charcoal-stripped fetal bovine serum (Thermo Scientific HyClone, Logan, UT), 1 % penicillin/streptomycin solution (Invitrogen, Carlsbad, CA), 0.1 mM norepinephrine (Sigma-Aldrich), and 2 mM L-glutamine (Invitrogen). NIH3T3 mouse embryonic fibroblasts purchased from American Type Culture Collection (ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10 % fetal bovine serum (Invitrogen) and 1 % penicillin/streptomycin solution.

Triplicates of HL-1 and NIH3T3 cells plated in 12-well plates for 24 h were co-transfected with 1 μ g/well of pcTnT-tTSTA-fluc and 10 ng/well of pCMV-hRL (normalization plasmid) using lipofectamine 2000 (Invitrogen) at 2 μ l/ μ g total DNA and exposed to varying

concentrations of raloxifene (0–250 nM) (Sigma-Aldrich). HL-1 and NIH3T3 cells co-transfected with pCMV-fluc (1 µg/well) and pCMV-hRL (10 ng/well) in the absence of exposure to raloxifene served as positive controls. After 24 h, the cells were assayed for FLuc and RLuc enzyme activities using *in vitro* reporter enzyme assays, as well as protein content using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Duplicates of both cell lines plated in six-well plates underwent the same transfection experiments, except with twice the amount of pcTnT-tTSTA-fluc and pCMV-hRL, followed by exposure to varying concentrations of raloxifene (0–50 nM). Twenty-four hours later, Western blot was performed to qualitatively assess the FLuc, cTnT, and β-actin protein levels.

In Vitro Reporter Enzyme Assays

Cells lysed in 1X Passive Lysis Buffer (Promega) were centrifuged to obtain protein, whose concentration was determined using the Bio-Rad protein assay. The FLuc and RLuc enzyme activities were determined using a TD 20/20n luminometer (Turner Designs, Sunnyvale, CA) as previously described [3]. The FLuc activity was normalized to both total protein and RLuc activity, and expressed in units of micrograms of cellular protein.

Protein Determination by Western Blot

HL-1 or NIH3T3 cells co-transfected with pcTnT-tTSTA-fluc and pCMV-hRL and exposed to raloxifene were harvested by trypsinization, lysed by sonication in RIPA buffer (Cell Signaling, Danvers, MA), and centrifuged to obtain the supernatant, whose protein content was determined using the Bio-Rad protein assay. Ten micrograms of protein from each sample was resolved by 4–12 % gradient SDS/PAGE (Invitrogen) and electroblotted to a 0.2-µm nitrocellulose membrane (Schleicher & Schuell, Keene, New Hampshire). The membrane was subsequently blocked with 5 % nonfat dry milk in TBS containing 0.01 % Tween 20 (TBST buffer) for 3 h and probed overnight at 4 °C on a rotating platform with mouse monoclonal anti-human ERα antibody (Santa Cruz, Santa Cruz, CA). Afterwards, the blot was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Cell Signaling) for 2 h at room temperature and further developed using the LumiGlo enhanced chemiluminescence substrate (Cell Signaling) following the manufacturer's protocol. Afterwards, the blot was stripped and reprobed first with goat polyclonal anti-human cardiac troponin T antibody (Santa Cruz) and later with mouse anti-human β-actin antibody (Sigma-Aldrich) using HRP-conjugated donkey anti-goat secondary antibody (Promega) and HRP-conjugated goat anti-mouse secondary antibody (Cell Signaling), respectively.

Study Design and Animal Groups

All animal studies were approved by the Stanford Institutional Animal Care and Use Committee. Eight groups of 8 to 10-week-old female Balb/c mice (total n=60) were purchased from the Stanford In-house Breeding Colony. Group 1 (n=10) received intramyocardial co-injections of pcTnT-tTSTA-fluc (80 µg/mouse) and pCMV-β-gal (16 µg/mouse). Afterwards, five mice were sacrificed on day 2 with their hearts assayed for β-galactosidase enzyme (β-GAL) activity, and the other five mice were imaged every 3 to 4 days with serial bioluminescence imaging (BLI) for 40 days. Groups 2 and 3 (n=5 each)

received the same vectors and underwent the same imaging procedures as group 1 except that these mice also received intraperitoneal administration of raloxifene, either at low dose (5 mg/kg; group 2) or high dose (10 mg/kg; group 3), 18 h before each BLI session. Group 4 ($n=10$) underwent the same procedures as group 1 except that pCMV-fluc (80 μ g/mouse) and pCMV- β -gal (16 μ g/mouse) were co-injected instead of pcTnT-tTSTA-fluc and pCMV- β -gal and that BLI was performed for 28 days instead of 40 days. Group 5 ($n=10$) underwent hydrodynamic tail vein (HTV) co-injections of pcTnT-tTSTA-fluc (20 μ g/mouse) and pCMV- β -gal (4 μ g/mouse). Five mice were subsequently sacrificed on day 2 with their livers explanted and assayed for β -GAL activity, whereas the other five mice were imaged every 3 to 4 days with serial BLI for 20 days. Groups 6 and 7 ($n=5$ each) also underwent HTV co-injections of pcTnT-tTSTA-fluc and pCMV- β -gal, followed by serial BLI for 20 days. Eighteen hours before each BLI session, the mice were intraperitoneally injected with either low-dose (5 mg/kg; group 6) or high-dose (10 mg/kg; group 7) raloxifene. Group 8 ($n=10$) underwent the same procedures as group 5 except that the mice were co-injected with pCMV-fluc (20 μ g/mouse) and pCMV- β -gal (4 μ g/mouse) instead.

Animal Surgery and Plasmid Vector Injection

Mouse surgeries and plasmid injections were performed as previously described [3]. Briefly, mice were orotracheally intubated and ventilated with 2 % isoflurane using a Harvard rodent ventilator (Harvard Apparatus, Holliston, MA). Following a left thoracotomy, two injections of 15 μ l each were made at one site near the base of the left ventricle. Afterwards, the chest was closed in layers and pneumothorax evacuated by suction using a 16G Angiocath (BD Biosciences, San Jose, CA). Animals received subcutaneous buprenorphine (0.1 mg/kg) for post-operative analgesia and recovered in 100 % oxygen.

Bioluminescence Imaging of Living Mice and Image Analysis

For each BLI session, mice were anesthetized with 2 % isoflurane, injected intraperitoneally with 375 mg/kg D-Luciferin (Biosynth International, Naperville, IL), imaged using the *In Vivo* Imaging System 100 (Xenogen product from Caliper Life Sciences, Alameda, CA), and recovered in 100 % oxygen via nose cone. Consecutive 5-min scans were acquired after substrate injection until the imaging signal peaked. Images were analyzed using Living Image 2.50 (Caliper Life Sciences) by placing a fixed-size region of interest over the target region (heart for groups 1 to 4 and liver for groups 5 to 8) and measuring the maximum radiance in units of photons per second per centimeter squared per steradian (p/sec/cm²/sr). Only the peak target signal following substrate injection was used for analysis. To correct for the differential *in vivo* transfection efficiencies of experimental and control vectors, the average target signal was normalized by the *ex vivo* target tissue β -GAL activity on day 2 and expressed in units of p/sec/cm²/sr/ β -GAL activity, where β -GAL activity corresponds to the absorbance at 420 nm obtained from the chromogenic β -GAL Enzyme Assay described below.

Tissue Harvest and Ex Vivo Reporter Assay

Mice were sacrificed by carbon dioxide asphyxiation, with either their hearts or livers explanted, then rinsed with PBS, immersed in 1 ml of 1X Passive Lysis Buffer, and

homogenized using a Kontes Duall tissue grinder (Fisher Scientific, Pittsburg, PA). Supernatants were obtained from tissue homogenates by centrifugation and assayed for β -GAL activity using β -galactosidase Enzyme Assay (Promega) according to the manufacturer's protocol, with the exception that 75 μ g of supernatant was used per reaction and the substrate incubation time was extended to 60 min for heart supernatants and 15 min for liver supernatants.

Statistical Analysis

All data are presented as mean $\pm$ standard error of the mean (SEM). The unpaired, two-tailed Student's *t* test was used for comparison between two measurements. Linear regression analysis was performed to assess the linearity between two variables. The strength of correlation was quantified in terms of the square of Pearson product-moment coefficient (r^2). The significance of correlation was calculated by performing the Student's *t* test against the null hypothesis that the correlation coefficient (*r*) is zero. *P* values less than 0.05 were considered statistically significant.

Results

Construction of the Titratable TSTA Plasmid Vector (pcTnT-tTSTA-fluc)

A plasmid vector (pcTnT-tTSTA-fluc) containing the cTnT promoter driving the expression of fluc via the titratable TSTA system was constructed (Fig. 1b). Specifically, this vector uses the rat cTnT promoter to drive the expression of the recombinant transcriptional activator GAL4-mER(LBD)-VP2, which can bind to either of the five tandem repeats of specific Gal4 binding sites (Gal4bs) elsewhere on the same vector and, in the presence of an ER ligand, recruit the transcriptional machinery necessary to transcribe the downstream fluc gene.

In Vitro Ligand-Regulated Transcriptional Amplification and Cardiac Specificity

To study how well a SERM such as raloxifene can modulate the degree to which the titratable TSTA system can amplify the transcriptional activity of the cTnT promoter, we co-transfected HL-1 cardiomyocytes and NIH3T3 fibroblasts with pcTnT-tTSTA-fluc and pCMV-hRL (normalization vector) and exposed them to increasing concentrations of raloxifene (0–250 nM). HL-1 and NIH3T3 cells co-transfected with pCMV-fluc and pCMV-hRL served as positive controls. After 24 h, the cells were assayed for FLuc and RLuc activities using *in vitro* reporter enzyme assays. The dose-response curve obtained by plotting the amount of FLuc induction in the HL-1 cells co-transfected with pcTnT-tTSTA-fluc and pCMV-hRL versus raloxifene concentration simulated that of a classic sigmoid-shaped receptor binding curve (Fig. 2a). In the absence of raloxifene binding, pcTnT-tTSTA-fluc led to a minimal level of FLuc activity in HL-1 cells compared to pCMV-fluc (1.7 %). The FLuc activity increased to 12 % of that of pCMV-fluc (sevenfold compared to baseline; $P<0.005$) when the raloxifene concentration was increased to 5 nM. At 10 nM raloxifene, the FLuc activity saturated at 57 % of that of pCMV-fluc (35-fold compared to baseline; $P<0.0005$). There was no statistically significant change in fluc induction at doses beyond 10 nM.

To assess the cardiac specificity of pcTnT-tTSTA-fluc relative to pCMV-fluc, we first defined a cardiac specificity index (CSI) using the following formula:

$$CSI_i = \left(fluc_{i,HL-1} / fluc_{i,NIH3T3} \right) / \left(fluc_{CMV,HL-1} / fluc_{CMV,NIH3T3} \right)$$

where $fluc_{i,HL-1}$ and $fluc_{i,NIH3T3}$ are the fluc expression mediated by a given vector i in HL-1 and NIH3T3 cells, respectively, whereas $fluc_{CMV,HL-1}$ and $fluc_{CMV,NIH3T3}$ are the fluc expression mediated by pCMV-fluc in HL-1 and NIH3T3 cells, respectively. The CSI of pcTnT-tTSTA-fluc, when plotted against the raloxifene concentration, exhibited a sigmoid profile similar to that of the dose–response curve (Fig. 2b). The CSI increased significantly from 1.4 to 6 ($P < 0.03$) as raloxifene was increased from 0 to 10 nM, beyond which no statistically significant change in CSI was observed.

Western Blot Analysis of Ligand-Regulated Tissue-Specific Gene Expression

To rule out the possibility that an ER ligand such as raloxifene can achieve transcriptional amplification by directly increasing the transcriptional activity of the cTnT promoter (rather than inducing intramolecular folding of GAL4-mER(LBD)-VP2), we performed Western blot analysis on HL-1 cardiomyocytes and NIH3T3 fibroblasts co-transfected with pcTnT-tTSTA-fluc and pCMV-hRL and exposed to varying concentrations of raloxifene to determine the relative expression of FLuc and cTnT in these cells. Western blotting of the transfected HL-1 cardiomyocytes revealed increasing induction of FLuc expression with increasing raloxifene exposure, compared to negligible FLuc levels in NIH3T3 fibroblasts regardless of the raloxifene concentration (Fig. 3). The cTnT expression was equally low for all raloxifene concentrations tested in HL-1 cardiomyocytes and nonexistent in NIH3T3 fibroblasts. The β -actin expression (loading control) in both cell lines did not vary with raloxifene concentration.

BLI of Ligand-Regulated Reporter Gene Expression in the Mouse Myocardium

To assess how well the titratable TSTA vector strategy can be used to modulate transgene expression in the mouse myocardium, we performed serial BLI on mice intramyocardially co-injected with pcTnT-tTSTA-fluc and pCMV- β -gal (normalization plasmid) and exposed to different levels of raloxifene (0, 5, 10 mg/kg) to assess the kinetics of myocardial FLuc expression. Mice co-injected with pCMV-fluc and pCMV- β -gal served as controls. Initially following vector delivery, the average normalized FLuc signal on day 2 was minimal for all groups injected with pcTnT-tTSTA-fluc regardless of the raloxifene dose (Fig. 4). The average normalized FLuc signal for the group without exposure to raloxifene remained negligible for the remainder of the study period. In the two groups injected with pcTnT-tTSTA-fluc and exposed to raloxifene, the average normalized FLuc signal increased steadily over time until it reached a maximum around day 12, when the average normalized FLuc signal for the high-dose (10 mg/kg) group and the low-dose (5 mg/kg) group was tenfold ($P < 0.03$) and fivefold ($P = \text{NS}$) greater than that of the group without exposure to raloxifene, respectively. The average normalized FLuc signal in these groups declined steadily over the next 2 weeks. In contrast, serial BLI of mice co-injected with pCMV-fluc

and pCMV- β -gal showed a fairly robust average normalized FLuc signal on day 2, which declined steadily over 3 weeks until the signal became undetectable by day 24.

To determine whether complete withdrawal and reintroduction of raloxifene can suppress and recover the FLuc-mediated signal, respectively, raloxifene was purposely withheld on day 32, restarted on day 36, and withdrawn again on day 40 in the two mouse groups injected with pcTnT-tTSTA-fluc and exposed to either low-dose or high-dose raloxifene. The average normalized FLuc signal in both groups dropped to a near-background level on day 32 ($P=NS$ compared to the mouse group injected with pcTnT-tTSTA-fluc but not exposed to raloxifene). There was a non-statistically significant trend for the average normalized FLuc signal recovered on day 36 to be greater than that before raloxifene withdrawal (i.e., day 28). The degree of signal recovery on day 36 was greater for the high-dose group ($P<0.005$) than the low dose group ($P=NS$). In both groups, the average normalized FLuc signal dropped significantly on day 40, when raloxifene was again withdrawn, to a level comparable to that before the reinstitution of raloxifene ($P=NS$ compared to the average normalized FLuc signal on day 32).

BLI Evaluation of the Effect of Ligand Regulation on Vector Tissue Specificity

To assess the degree to which the cardiac specificity of pcTnT-tTSTA-fluc can be preserved in the presence of raloxifene, we subjected mice to hydrodynamic tail vein co-injections of either pcTnT-tTSTA-fluc and pCMV- β -gal (normalization plasmid) or pCMV-fluc and pCMV- β -gal, followed by serial BLI to assess hepatic FLuc expression. Mice that received pcTnT-tTSTA-fluc were further subdivided into three groups, which additionally received different levels of raloxifene (0, 5, and 10 mg/kg) 18 h prior to each imaging session. Over the first 12 days, the average normalized hepatic signal was initially the greatest for the pCMV-fluc group, followed by the high-dose (10 mg/kg) raloxifene group, the low-dose (5 mg/kg) raloxifene group, and the pcTnT-tTSTA-fluc group without exposure to raloxifene (Fig. 5). In all groups, the average normalized FLuc signal dropped precipitously over the first 12 days until it stabilized at a more constant level. From day 12 and on, the average normalized FLuc signal was seen in only the pCMV-fluc group but not in any of the groups that received the pcTnT-tTSTA-fluc vector, regardless of the amount of raloxifene received (e.g., day 20: $P<0.001$ comparing the pCMV-fluc group to any of the groups injected with pcTnT-tTSTA-fluc).

Discussion

Current gene therapy for cardiac diseases is limited by the lack of a robust vector system that would enable fine spatiotemporal control of therapeutic gene expression to achieve the utmost balance between therapeutic efficacy and safety. The need to tightly regulate therapeutic gene expression is supported by numerous pre-clinical studies demonstrating a narrow therapeutic window for a powerful angiogenic gene such as VEGF [5, 6]. In these studies, both subtherapeutic and supratherapeutic levels of local tissue VEGF led to formation of aberrant neovasculature, whereas only a uniform local therapeutic level of VEGF was able to stimulate functional angiogenesis without causing hemangioma formation. Furthermore, although not yet substantiated in clinical trials of VEGF at the

vector doses tested, theoretical concerns exist regarding the possibility of excessive off-target overexpression of VEGF leading to tumor growth, proliferative diabetic retinopathy, accelerated atherosclerosis, and plaque destabilization [7]. Besides angiogenic genes, unregulated expression of insulin growth factor-1 for the treatment of diabetic cardiomyopathy [8], *Drosophila* shaker B potassium channel for the treatment of heart failure-associated long QT prolongation [9], and short hairpin RNA directed against phospholamban for the treatment of heart failure [10] have been associated with nephromegaly, myocardial contractile dysfunction, and direct cardiomyocyte toxicity, respectively. All together, these findings underscore the need to develop a powerful therapeutic vector that would allow fine control of the level of transgene expression in a cardiac tissue-specific manner.

In this study, we have attempted to address the aforementioned limitations of gene therapy by developing and validating a novel titratable TSTA vector strategy to precisely control the biodistribution and kinetics of transgene expression following plasmid vector-mediated gene delivery. Specifically, we built an experimental plasmid vector (pcTnT-tTSTA-fluc) that uses (1) the rat cTnT promoter to minimize transgene expression in non-cardiac cells and (2) the titratable TSTA system to regulate the level of fluc reporter gene expression (Fig. 1b). In cultured HL-1 cardiomyocytes transfected with pcTnT-tTSTA-fluc, the maximal degree of reporter gene expression inducible by raloxifene was found to be at least 35-fold of the baseline expression in the absence of raloxifene, reaching 57 % of that achievable by pCMV-fluc. There was a corresponding increase in both reporter gene induction and cardiac specificity with increasing raloxifene such that the maximal cardiac specificity associated with the maximal gene induction was at least six times greater than that of pCMV-fluc (i.e., CSI=6 at 10 nM raloxifene). Note that the modulation of reporter gene expression was specifically achieved by increasing the number of raloxifene binding to the mutant ER-LBD of the GAL4-mER(LBD)-VP2 fusion proteins, leading to their favorable intramolecular folding and gaining of efficient function to transactivate reporter gene expression. The possibility of raloxifene directly activating cTnT promoter activity to enhance reporter gene expression was specifically ruled out by Western blot analysis (Fig. 3). As the strength of the pcTnT promoter was only 5 % of that of the constitutive CMV promoter under a similar experimental condition [3], our cell culture data herein suggested that the titratable TSTA system can effectively enhance the transcriptional activity of pcTnT promoter by as much as tenfold, more than adequate for most *in vivo* gene therapy applications.

Indeed, further characterization of the titratable TSTA vector (pcTnT-tTSTA-fluc) in living mice revealed a great dynamic range of modulation (up to tenfold) of myocardial reporter gene expression after vector delivery with different doses of intraperitoneally administered raloxifene. The maximal transgene induction was achieved at the highest dose of raloxifene tested (10 mg/kg, close to the dose used clinically for osteoporosis and breast cancer prevention) and on day 12 when the cardiac-specific promoter- and TSTA-mediated transgene expression is expected to peak, as previously shown for plasmid-mediated gene delivery [3]. The ease of modulation with this titratable system was best demonstrated by the abrupt “turning off” and “turning on” of reporter gene expression with acute withholding and restarting of raloxifene at late time points (days 32 and 36, respectively; Fig. 4). The

fast-acting nature of this titratable system can be attributed to the binding of raloxifene to the already translated GAL4-mER(LBD)-VP2 proteins to provide instantaneous intramolecular conformation change and transactivation of reporter gene expression. The fast kinetics were also partly made possible by the relatively short plasma elimination half-life of raloxifene (28 h), compared to other SERMs (e.g., 5–7 days for tamoxifen) [11]. Interestingly, there was a non-statistically significant trend for the reporter gene expression upon reinstitution of raloxifene after a short period of withdrawal (4 days) to be greater than that predicted based on the kinetics of reporter gene expression prior to ligand withdrawal (Fig. 4). This finding suggests that the ligand-bound GAL4-mER(LBD)-VP2 transcriptional activator, like the ligand-bound human estrogen receptor alpha [12], may have a different degradation rate or pathway than that of the unbound counterpart, such that a period of ligand withdrawal allows more GAL4-mER(LBD)-VP2 to be available to bind to ligand and transactivate reporter gene expression. For therapeutic applications, this phenomenon, if proven to be true, can be exploited to develop a dosing regimen with the appropriate “holiday break” so as to maximize long-term efficacy.

Another favorable finding from the *in vivo* studies was that intramyocardial administration of pcTnT-tTSTA-fluc led to only minimal myocardial background transgene expression in the absence of raloxifene, as inferred from bioluminescence imaging. This trace level of basal gene expression was likely the result of residual GAL-mER(LBD)-VP2 transactivation activity in the absence of raloxifene binding and not a consequence of endogenous estrogen activating the titratable TSTA system, as the LBD of the mutant human ER within GAL4-mER(LBD)-VP2 has previously been shown to have no affinity for 17 β -estradiol, the endogenous ER ligand [4]. This performance characteristic is encouraging, as previous attempts at developing regulatable gene expression systems, such as the tetracycline inducible expression system (Tet-On), have been hampered by high basal transgene expression levels in the absence of an inducer—a limitation that ultimately required redesign of the transactivators or incorporation of transcriptional silencers for improved performance [13]. Nevertheless, with minimal basal transgene induction, our vector containing the cTnT cardiac-specific promoter was able to effectively minimize long-term hepatic reporter gene expression (after 12 days) following HTV vector administration, irrespective of the presence of raloxifene. This finding confirmed the preserved cardiac specificity of pcTnT-tTSTA-fluc (CSI=6 in cell culture) and illustrated the benefit of transcriptional targeting using cTnT *in vivo* over the use of a constitutively active promoter such as the CMV promoter (Fig. 5). It should be noted that the significant hepatic reporter gene expression immediately following pcTnT-tTSTA-fluc delivery was not a reflection of poor tissue specificity, as the HTV injection procedure itself has been shown to nonspecifically activate different cardiac-specific promoters (e.g., myosin light chain 2 and α -myosin heavy chain promoters) acutely, thus rendering the initial period (<12 days) unsuitable for analysis of promoter specificity [3]. Further incorporation of the plasmid vector into either a high-capacity adenovirus (HC-AD) [14] or a recombinant adeno-associated virus with cardiac tropism (AAV9) [15] could help simplify and expand the evaluation of vector tissue specificity to include other organs, thus circumventing the difficulty of assessing the tissue biodistribution of plasmid vectors due to their propensity to degrade rapidly in serum. Future replacement of the fluc reporter gene in the pcTnT-tTSTA-fluc vector with either a positron emission tomography reporter

gene (e.g., HSV1-sr39tk) [16] or an MRI reporter gene (e.g., ferritin) [17] will allow further validation and optimization of the titratable TSTA vector strategy in large animal models and potentially humans.

As the first prototype of its kind designed for cardiac gene therapy, the titratable TSTA plasmid vector presented herein has certain limitations that deserve extra attention, as well as further optimization. First, the use of a plasmid vector in this study led to non-sustained transgene expression overtime, regardless of whether HTV or intramyocardial plasmid injection was performed. The more acute decline in transgene expression within the first week could be attributed to plasmid loss from the transfected cells and, more importantly, promoter silencing; the more subacute decline after the first week could be attributed to the host immune response against the FLuc reporter enzyme [18]. This continual decline in transgene expression made it difficult for the fine titration of myocardial FLuc expression following pcTnT-tTSTA-fluc delivery. Although the use of the cTnT promoter in this study was able to prolong FLuc expression more so than the CMV promoter (Fig. 4b), the use of a viral vector (e.g., HC-AD, AAV, or lentiviral vector), with its superior transduction efficiency, should provide a more robust and longer lasting FLuc expression that is more suitable for large amplitude modulation with the titratable TSTA system. Furthermore, a viral vector will undoubtedly lead to more efficient myocardial transduction, thus making up for any loss of transgene expression due to the limited production of the GAL4-mER(LBD)-VP2 transactivator fusion protein secondary to cTnT's known weak promoter activity. Second, the caveat of using a plasmid vector is that it does not lead to appreciable retention and expression in non-cardiac tissues (e.g., liver) following intramyocardial delivery despite fair amount of leakage from the myocardium [19]. The rapid clearance of plasmid in blood has been attributed to the working of plasma endonucleases, tissue surface enzymes, and non-parenchymal liver cells that act to scavenge and degrade plasmid DNA. These pharmacokinetic properties make plasmid vectors very safe, biologically and clinically, as vector retention and transgene expression can be limited to the myocardium. However, biodistribution studies that are typically done to assess the cardiac specificity of a given vector become difficult to perform due to the lack of non-cardiac tissue transgene expression following intramyocardial vector delivery. In this study, we circumvented this problem by using HTV injection to purposely deliver high level of our titratable TSTA plasmid vector to the liver parenchymal cells so that the vector expression under the control of a cardiac-specific promoter can be adequately assessed. Future incorporation of our titratable TSTA construct into a viral vector will allow a comprehensive biodistribution study to be performed to further substantiate its cardiac specificity. Third, currently there is not a single regulatable gene expression system without some levels of basal expression in the absence of an inducer. This "leakiness" was also present, albeit at a very low level, in our tTSTA system likely as a result of basal GAL4-mER(LBD)-VP2 transactivation in the absence of raloxifene. This problem could be potentially remedied by replacing the linker sequence (GGGGS) between the mutant ER-LBD and GAL4 or VP2 with a more "rigid" sequence (e.g., EAAAAEAAAA or ESESESESES) so that the distance between VP2 and the adenovirus E4 minimal promoter TATA box could be increased to minimize transactivation in the absence of raloxifene. Alternatively, this "leakiness" could be due to the unintended tTSTA activation by estrogen mimics in the mouse diet [20] and could be addressed by

changing to a diet with less estrogen mimics. Fourth, raloxifene was chosen as the study ER ligand for the titratable TSTA system based on its relatively short elimination plasma half-life (28 h) compared to other SERMS (e.g., 5–7 days for tamoxifen), a characteristic that allowed more rapid modulation of transgene expression in the setting of limited duration of plasmid-mediated transgene expression (Fig. 4). Administration of raloxifene 18 h before BLI was performed in this study to specifically capture different levels of transgene induction over a period of time (i.e., sampling) as the plasmid vector was degraded. This mode of drug administration was sufficient to demonstrate the titratable nature of our TSTA system. However, in real therapy applications in which transgene expression needs to be persistent between imaging time points, a daily dosing regimen of raloxifene will be necessary and may impact the rapidity at which the transgene expression can be modulated. Lastly, the pharmacokinetics of raloxifene is such that it can be distributed to most cell types following systemic delivery and can traverse the cell membrane easily by simple diffusion [21]. Although the steady-state intracellular level of raloxifene determines the level of FLuc expression that can be induced via raloxifene's binding to the mER moiety of GAL4-mER-VP2, the overall level of FLuc expression in each tissue type will also depend on the tissue-specific variation in the expression of endogenous estrogen receptors [22], which can competitively bind to raloxifene. Ultimately, more pharmacokinetic studies using different drugs with varying affinities for the endogenous or mutant estrogen receptors, dosing regimens, concentrations of plasmid vectors, or gene delivery vehicles will be needed to optimize the overall level of transgene expression and the ease of its modulation with our titratable TSTA vector strategy.

Conclusions

In conclusion, we have developed and characterized a novel, robust titratable TSTA vector strategy that enables both transcriptional targeting and fine modulation of transgene expression. This vector strategy has the potential to improve both the safety and efficacy of experimental cardiac gene therapy and can be further modified using a previously reported bi-directional TSTA strategy to deliver and simultaneously monitor the expression of any therapeutic gene of choice after vector delivery. The application of this strategy could also be extended to include stem cell-based therapy applications where induction and titration of cell-mediated gene transfer under a specific stem cell or physiological condition (e.g., differentiation, hypoxia) using the appropriate promoter would be ideal. Further refinement of the titratable TSTA system presented herein should open the door for a wide range of possible biological or clinical applications as other regulatable gene expression systems [23] and gene targeting strategies [24] have in the past.

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Fig. 1.

Schematics of the titratable TSTA vector (pcTnT-tTSTA-fluc) compared to its constitutive counterpart. **a** The constitutive TSTA system (*top row*) uses the cardiac troponin T (cTnT) promoter to drive the expression of the recombinant transcriptional activator GAL4-VP2 composed of the yeast GAL4 DNA-binding domain (GAL4) linked to a peptide containing two copies of the herpes simplex virus VP16 transactivation domain (VP2). Each GAL4-VP2 molecule can bind to one of five tandem repeats of a specific Gal4-binding sequence (5xGal4bs) elsewhere on the same vector and recruit RNA polymerase to the adjacent adenovirus E4 minimal promoter TATA box (TATA) to transcribe the downstream firefly luciferase (fluc) reporter gene. **b** The titratable TSTA system expresses a modified recombinant transcriptional activator (GAL4-mER(LBD)-VP2) in which the ligand binding domain (LBD) of a mutant human estrogen receptor (ER_{G521T}) is inserted between GAL4 and VP2 to modulate their relative positions in space. In the absence of ligand binding (*middle row*), GAL4-mER(LBD)-VP2 binds to 5xGal4bs in a conformation that keeps VP2 away from the TATA box, leading to no transcription. With ligand binding to the mutant ER-LBD (*bottom row*), intramolecular folding of GAL4-mER(LBD)-VP2 brings VP2 closer to the TATA box and leads to efficient transcription of fluc. Additional abbreviation: poly(A) tail (PA).

Fig. 2.

In vitro ligand-regulated transcriptional amplification and cardiac specificity. HL-1 cardiomyocytes and NIH3T3 fibroblasts co-transfected with pcTnT-tTSTA-fluc and pCMV-hRL (normalization vector) were exposed to increasing concentrations of raloxifene, a selective estrogen receptor modulator, and assayed for FLuc and RLuc expression 24 h later. **a** The amount of FLuc induction by raloxifene in HL-1 cells is plotted for various raloxifene concentrations shown. The level of FLuc expression in HL-1 cells as a percentage of that mediated by pCMV-fluc for each raloxifene dose is noted *within parentheses*. **b** The calculated cardiac specificity index of pcTnT-tTSTA-fluc is shown for the same range of raloxifene concentrations. * $P < 0.05$ compared to the FLuc expression at zero raloxifene concentration. ^ $P < 0.05$ compared to the cardiac specific index at zero raloxifene. Error bars represent SEM for triplicate transfections.

Fig. 3.

Western blot validation of ligand-regulated tissue-specific reporter gene expression. NIH3T3 fibroblasts and HL-1 cardiomyocytes co-transfected with fixed doses of pcTnT-tTSTA-fluc and pCMV-hRL (normalization vector) were exposed to increasing concentrations (nM) of raloxifene and assayed for the expression of FLuc, cTnT, and β -actin using Western blot. The Western blots corresponding to NIH3T3 fibroblasts (*left blot*) and HL-1 cardiomyocytes (*right blot*) are shown. The blot bands corresponding to FLuc (*top rows*), cTnT (*middle rows*), and the β -actin loading control (*bottom rows*) are displayed for various raloxifene concentrations shown.

Fig. 4. Bioluminescence imaging of ligand-regulated reporter gene expression in the mouse myocardium. Experimental mice intramyocardially co-injected with pcTnT-tTSTA-fluc and pCMV- β -gal (normalization vector) underwent serial BLI of myocardial FLuc expression for 40 days, with varying doses (0, 5, or 10 mg/kg) of raloxifene (Ral) injected intraperitoneally 18 h prior to each imaging session except for days 32 and 40. Control mice co-injected with pCMV-fluc and pCMV- β -gal underwent serial BLI without addition of raloxifene for 28 days. **a** Representative images of three experimental mice corresponding to different raloxifene doses (0 mg/kg, *top row*; 5 mg/kg, *second row*; 10 mg/kg, *third row*) and one control mouse (*bottom row*) are shown for the days listed. The heart signal intensity (*pseudocolor*) is displayed as radiance in units of photons per second per centimeter squared per steradian (p/sec/cm²/sr). **b** The average normalized heart signal for each experimental group that received 0 mg/kg (*light gray solid line*), 5 mg/kg (*dark gray solid line*), or 10 mg/kg (*black solid line*) of raloxifene is plotted over a 40-day study period, whereas the average normalized heart signal for the control group (*black dashed line*) is plotted over 28 days. The normalized heart signal corrects for the difference in transfection efficiency (i.e., *ex vivo* tissue β -GAL activity) between pcTnT-tTSTA-fluc and pCMV-fluc. Error bars represent SEM for five mice.

Fig. 5.

Bioluminescence imaging of ligand-regulated reporter gene expression in the mouse liver. Experimental mice that underwent hydrodynamic tail vein co-injections of pcTnT-tTSTA-fluc and pCMV-β-gal were serially imaged for hepatic FLuc expression for 20 days using BLI, with varying doses (0, 5, or 10 mg/kg) of raloxifene (Ral) given intraperitoneally 18 h before each imaging session. Control mice co-injected with pCMV-fluc and pCMV-β-gal were similarly imaged except that no raloxifene was given prior to imaging. **a**

Representative images of three experimental mice corresponding to different doses of raloxifene (0 mg/kg, *top row*; 5 mg/kg, *second row*; 10 mg/kg, *third row*) and one control mouse (*bottom row*) are shown for the days indicated. Hepatic signal (*pseudocolor*) is displayed in radiance in units of photons per second per centimeter squared per steradian (p/sec/cm²/sr). **b** The average normalized hepatic signal for each of the experimental groups that received 0 mg/kg (*light gray solid line*), 5 mg/kg (*dark gray solid line*), or 10 mg/kg (*black solid line*) of raloxifene, as well as the control group (*black dashed line*), is plotted for the entire 20-day study period. The normalized signal corrects for the difference in transfection efficiency (i.e., *ex vivo* tissue β-GAL activity) between pcTnT-tTSTA-fluc and pCMV-fluc, and is displayed here on a log scale. *Error bars* represent SEM for five mice.