RESEARCH COMMUNICATION

Construction of a Multi-Functional Helper-Dependent Adenovirus Based System for Cancer Gene Therapy

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Abstract

Adenovirus holds great promise as a gene delivery system; it can hold large amounts of exogenous DNA and can be chemically and genetically modified to improve targeting to specific cells and tissues. A recombinant adenovirus construct expressing p53 is currently in clinical use as a cancer therapy in China. However, the use of adenovirus constructs in therapy is limited due to patients' strong immune response against these viruses and their gene products. To overcome this problem helper-dependent adenoviruses which do not express any viral gene products have been developed. Because the helper-dependent viruses do not express any viral gene products, a helper virus is required for their replication and encapsidation into infectious particles. This manuscript describes the construction of a prototype helper-dependent adenovirus system built such that it can be easily modified. The helper-dependent virus described here is built of a series of four cassettes, each with its own function. Furthermore, each individual cassette can be removed and replaced with a cassette with a different function. In this way, different helper-dependent viruses can be readily created. This type of system could be very useful in cancer therapy: For example, libraries of different cassettes could be maintained, allowing rapid assembly of constructs able to provide therapy for individual tumor types.

Key Words: Adenovirus - helper-dependent - cancer gene therapy - ADMM - EVE

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Introduction

Adenovirus based vectors are being developed in many laboratories for use in gene therapy clinical trials (Cross and Burmester, 2006; Kim et al., 2008; Shirakawa, 2008; also see http://www.wiley.co.uk/genmed/clinical/), and in China a recombinant adenoviral vector expressing p53, marketed under the name gendicine, is in clinical use as a cancer therapy (Peng, 2005). The use of adenovirus itself as the basis of a gene therapy construct is limited due to patients' strong immunogenic response against these viruses and their gene products. To decrease the patient's immunogenic response against adenovirus based constructs, constructs are being made which do not express any viral gene products. These constructs are known as helper-dependent viruses (Alba et al., 2005; Jozkowicz and Dulak, 2005; Xu et al., 2005). Because helper-dependent vectors do not encode any viral gene products, they require a helper virus, which does encode and express viral proteins, for propagation and encapsidation into infectious particles.

This manuscript describes the construction of a prototype helper-dependent adenovirus based system which can be easily modified for use as a cancer therapy. We refer to our helper-dependent constructs as adenovirus-dependent molecular medicine (ADMM), and the helper virus is referred to as EVE. Our ADMM construct consists of two adenovirus inverted terminal repeats (ITRs) which act as DNA replication origins, the adenovirus packaging site which is required for encapsidation of the ADMM DNA into infectious particles, and four cassettes. Each of these cassettes has its own function. By building a vector able to manifest multiple functions, a versatile vector with high specificity and cancer killing ability can be created. Moreover, each cassette can be removed and replaced with a cassette with a different function, allowing ready construction of ADMMs with specificity for different tumor types.

The prototype ADMM described here contains cassettes with the following functions: (1) expression of a reporter gene, LacZ, under the control of a tetracycline responsive promoter; (2) expression of a suicide gene, HSV-TK, enabling killing of the host cell, also under the control of a tetracycline responsive promoter; (3) spacer DNA; and (4) expression of a transcription factor, tTA, which binds to tetracycline responsive promoters in the absence of tetracycline (Freundlieb et al., 1999) and activates transcription.

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Materials and Methods

Materials

pGEM®-T Easy (Cat. No. A3600) was purchased from Promega. The BD Adeno-X™ Expression System 1 kit (Cat. No. K1650-1) was purchased from BD Bioscience (Adeno-X™ Expression System 1 is now sold by Clontech). pIRES (Cat. No. 631605) was purchased from BD Biosciences (pIRES is now sold by Clontech). pEGFP-N1 (Cat. No. 6085-1) was purchased from BD Biosciences (pEGFP-N1 was sold by Clontech but EGFP has been replaced by more highly optimized versions of green fluorescent protein, such as ZsGreen1 and AcGFP1; pEGFP-N1 Cat. No. 6085-1 is now discontinued). The BD™ Tet-Off Gene Expression System (Cat. No. 630921) was purchased from BD Biosciences (630921 has been replaced by an improved Tet-Off Advanced Inducible Gene Expression System, 630934, and is now sold by Clontech). pxCANCre (Cat. No. 1675) was purchased from Riken BioResource Center with the permission of the depositor Izumu Saito. The Q-mate™ Inducible Expression System was purchased from Q-BIOgene (Q-mate™ Inducible Expression System is now sold by Krackeler Scientific, Inc.). pTRE-Tight (Cat. No. 631059) was purchased from Clontech. pCM-TK (Cat. No. 5953) was purchased from Riken BioResource Center with the permission of the depositor Hirofumi Hamada. FuGENE 6 was purchased from Roche Applied Science. SuperScript™ III reverse transcriptase was purchased from Invitrogen.

Molecular Biology

Unless otherwise noted, standard molecular biology techniques were used. Amplification of DNA sequences used in the construction of EVE and ADMM was initially performed using TaKaRa Ex TaqTM or TaKaRa LA TaqTM (RR006 and RR042; Takara Bio Inc.), and the amplicons were ligated into pGEM®-T Easy vectors. However, during the construction of ADMM, Takara Bio. Inc. introduced a new PCR polymerase named PrimeSTAR (Cat. No. R044). This polymerase has an extremely low mistake rate and was used in the construction of ADMM after it became available.

Amplification with PrimeSTAR results in blunt ended amplicons, consequently, these amplicons must be phosphorylated and ligated into blunt-ended vectors. Whenever PCR amplicons or DNA oligomers were inserted into blunt-ended plasmid backbones, the inserts were first phosphorylated (and the blunt-ended backbones were dephosphorylated); the DNA oligomers and amplicons were phosphorylated using polynucleotide kinase (Cat. No. 2021; Takara Bio Inc.) according to the manufacturer's instructions. DNA oligomers were purchased from Invitrogen. When constructing plasmids of 20 kb or greater, ligations were performed using TaKaRa DNA Ligation Kit LONG (Cat. No. 6024; Takara Bio Inc.) according to the manufacturer's instructions. Sequencing analysis was performed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

DNA less than 10 kb was purified using DNA purification kits purchased from Qiagen and Promega. Larger plasmids were purified using DNA purification kits purchased from Qiagen. DNA fragments > 10 kb were purified as follows: DNA fragments were separated by electrophoresis through an agarose gel; the fragment of interest was cut out of the gel; the gel was dissolved in Membrane Binding Solution from Wizard®SV Gel and PCR Clean-Up System (Promega); the dissolved gel containing the DNA fragment of interest was applied to a QIAprep Spin Miniprep column (Qiagen) and centrifuged for 30 sec; the column was washed once with Buffer PB and washed twice with Buffer PE; and the DNA was eluted with 50 µl Buffer EB heated to 80˚C. Because the DNA tended to be dilute after this purification, > 10 kb restriction digest fragments were obtained from approximately 2 µg of plasmid DNA.

Plasmids were propagated in Stbl3 cells purchased from Invitrogen (Cat. No. C7373-03). These cells were grown at 37˚C. If the plasmid of interest could not be propagated at 37˚C, cells were grown at 32˚C. If the plasmid of interest could not be propagated at 32˚C, cells were grown at 28˚C: In general, larger, complicated plasmids such as pEVE, pC4-H_Tight-LacZ, and pADMM were propagated in cells grown at 28˚C.

Stuffer DNA

Genomes were scanned for sequences similar to 5'-CCANNAGNNGGC-3' using BLAST (NCBI). Intergenic regions containing positive hits were considered acceptable candidates for stuffer DNA.

Construction of the helper adenovirus (EVE)

Construction of pBS_PITR_Pack-wt:

Oligomer 5'-TCACGTGAA-3' was annealed to itself and then ligated into pGEM®-T Easy to generate pGEM_closed. Oligomers 5'-GGCCGCTGGCCATTTT CGCGGGAAAACTGAATA-3' and 5'-CTCTTATTCAG TTTTCCCGCGAAAATGGCCAGC-3' were annealed together and oligomers 5'-AGAGGAAGTGAAATCTGA ATAATTTTGTGTTACTCATAGCGCGCG-3' and 5'-TCGACGCGCGCTATGAGTAACACAAAATTATTCAG ATTTCACTTC-3' were annealed together. pGEM_closed was digested with NotI and SalI, and the annealed oligomers were ligated into the pGEM_closed backbone (triple ligation). Oligomers 5'-CTAGAACGCGTAATATT TGTCTAGGGCCGCGGGG-3' and 5'-AAGTCCCCGC GGCCCTAGACAAATATTACGCGTT-3' were annealed together and oligomers 5'-ACTTTGACCGTTTACGTGG AGACTCGGGATCCTTC-3' and 5'-CCGGGAAGG ATCCCGAGTCTCCACGTAAACGGTCA-3' were annealed together. pBS_KS+ was digested with XbaI and XmaI, and the annealed oligomers were ligated into the pBS_KS+ backbone (triple ligation). The pGEM_ oligomer construct was digested with ScaI and BssHII. The pBS_oligomer construct was digested with ScaI and MluI, and the insert from the pGEM_oligomer was ligated into the pBS_oligomer backbone to generate pBS_Pack-3'. The adenovirus inverted terminal repeat (ITR) and a 5' PacI site and the 5' portion of the adenovirus packaging site was amplified from Adeno-X (Adeno-X is part of the BD Adeno-X™ Expression System 1 kit) using primers

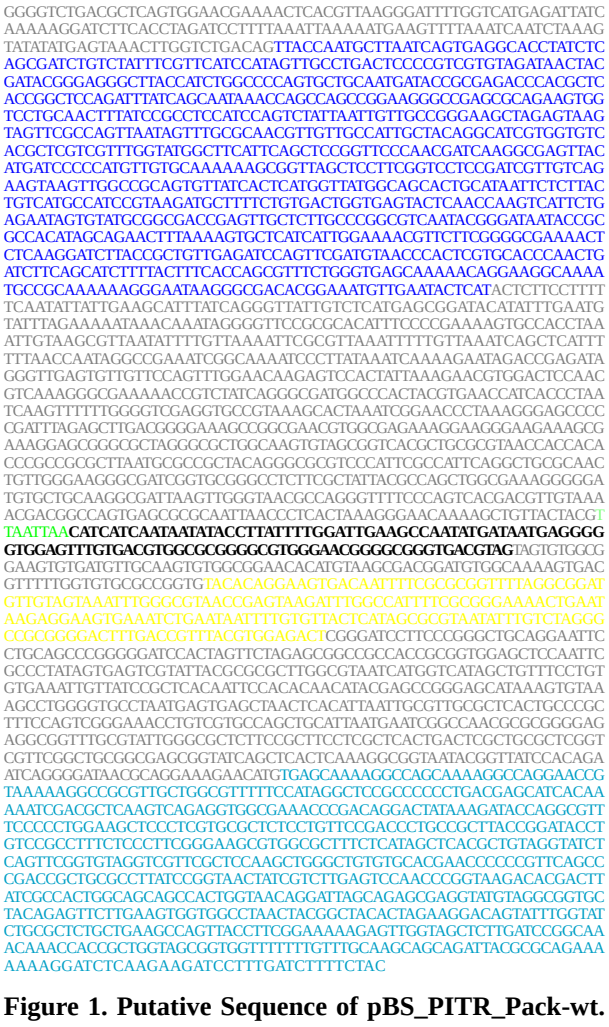

Figure 1. Putative Sequence of pBS_PITR_Pack-wt. The ampicilin resistance gene is in blue; the PacI site is in green; the inverted terminal repeat is in black; the packaging site is in yellow; and the origin of replication is in cyan. During insertion of the PacI site into the blunted KpnI site of pBS_rKS a "G" residue was lost during the blunting reaction. (Sequence data is best viewed by copying the sequence and pasting it into a program such as Gene Construction Kit or into a word processing program)

5'-CCCCAGGCTTTACACTTTATGC-3' and 5'-CTTTCGCTACCTTAGGACCGTTAT-3' and ligated into pGEM®-T Easy to generate pGEM_PITR_Pack-5' (pGEM_[PacI-Inverted Terminal Repeat]_[5' portion of the wild type packaging site]). pGEM_PITR_Pack-5' was digested with MscI and NotI. pBS-Pack-3' was digested with MscI and NotI, and the PITR-Pack-5' insert was ligated into the pBS-Pack-3' backbone to generate pBS_PITR_Pack-wt_Ex (pBS_[PITR]_[wild type packaging site]_[with extraneous sequences]). pBS_rKS was constructed by digesting pBS_KS+ with BssHII and ligating the BssHII fragment back into the parent pBS_KS+ backbone, and selecting clones with the MCS oriented in the reverse direction. pBS_rKS was digested with KpnI, blunted and dephosphorylated, and a PacI site (5'-TTACTACGTTAATTAACGATT-3') ligated into the pBS_rKS backbone to generate pBS_rKS-PacI. pBS-PITR_Pack-wt_Ex was digested with PacI and EcoRI. pBS_rKS-PacI was digested with PacI and EcoRI, and the PITR_Pack-wt insert was ligated into the pBS_rKS-PacI backbone to generate pBS_PITR_Pack-wt. The sequence of pBS_PITR_Pack-wt is shown in Figure 1.

Figure 2. Putative Sequence of pBS_PITR_LΔ5L_SC. The ampicilin resistance gene is in blue; the PacI site is in green; the inverted terminal repeat is in black; LoxP sites are in magneta; the packaging site is in yellow; the PI-SceI and I-CeuI sites are in green; and the origin of replication is in cyan. During insertion of the second LoxP site into the blunted SpeI site of pBS_PITR_LoxP-PackΔ5 a "G", a "C", and a "T" residue were lost during the blunting reaction

Construction of pBS_PITR_LΔ5L_SC (pBS_[PITR]_[LoxP-PackΔ5-LoxP]_[PI-SceI_I-CeuI]):

pBS_PITR_Pack-wt was used to generate a sequence containing a PITR and a shortened packaging site by PCR amplification using primers 5'-GTCGAGGTGCCGTAAAGCA-3' and 5'-GGATCCACGCGCTATGAGTAACACAAAA-3'. The amplicon was ligated into pGEM®-T Easy to generate pGEM_PITR_PackΔ5. pGEM_PITR_PackΔ5 was digested with PacI and BamHI. pBS_rKS-PacI was digested with PacI and BamHI, and the PITR_PackΔ5 insert was ligated into the pBS_rKS-PacI backbone to generate pBS_PITR_PackΔ5. pBS_PITR_PackΔ5 was digested with SgrAI, blunted and dephosphorylated, and a loxP site (5'-ATAACTTCGTATAATGTATGCTATACGAAGTTAT-

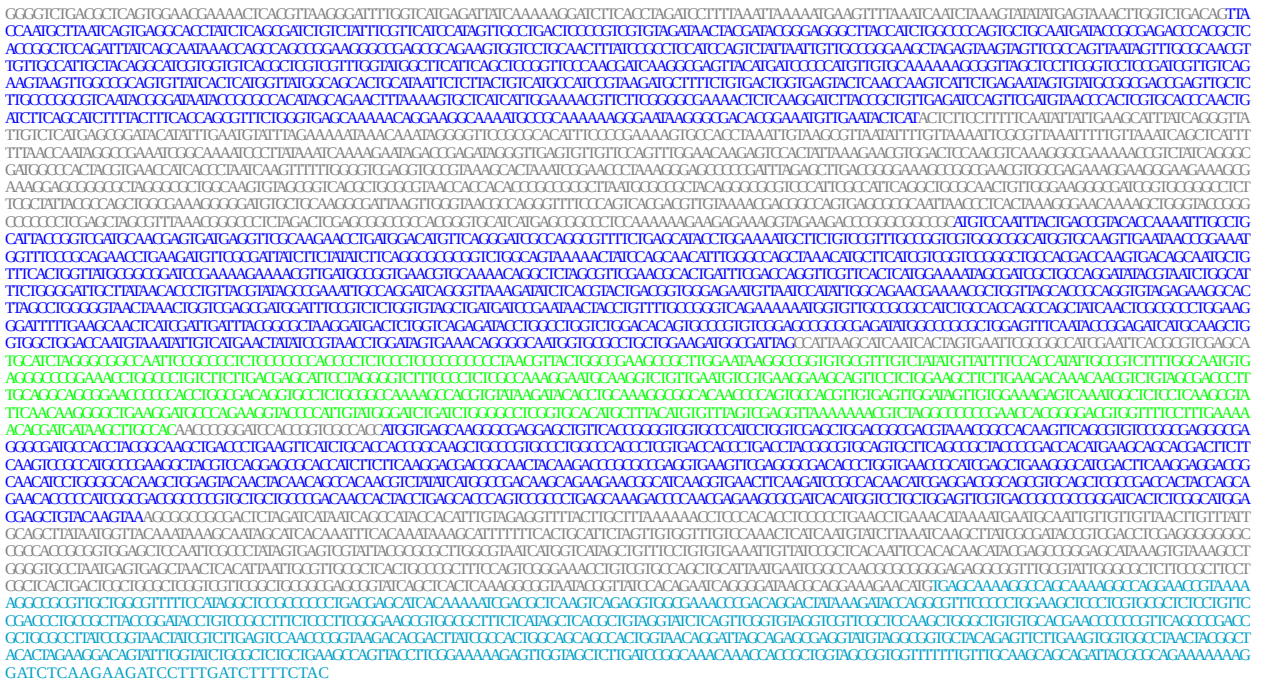

Figure 3. Putative Sequence of pBS_Cre-IRES-EGFP. The ampicilin resistance gene is in blue; the Cre and EGFP open reading frames are in blue; IRES is in green; and the origin of replication is in cyan

3') was ligated into the pBS_PITR_LoxP-PackΔ5 backbone to generate pBS_PITR_LoxP-PackΔ5-LoxP (pBS_PITR_LPΔ5L). A PI-SceI site was generated by annealing together the oligomers 5'-AATTCATCTATGTCGGGTGCGGAGAA AGAGGTAATGAAATGGCATCGACTCGAAGATCT-3' and 5'-AGATCTTCGAGTCGATGCCATTTCATTA CCTCTTTCTCCGCACCCGACATAGATG-3'. pBS_KS+ was digested with EcoRI and EcoRV, and the PI-SceI site was ligated into the pBS_KS+ backbone to

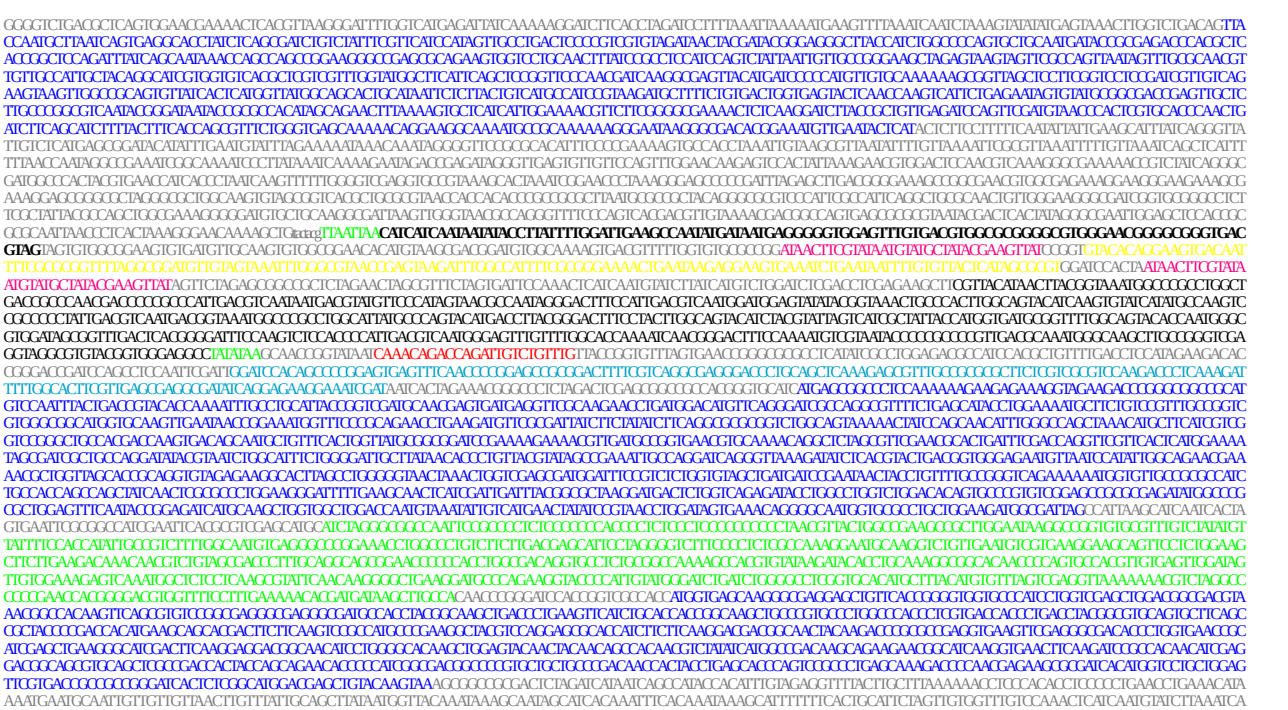

Figure 4. Putative Sequence of pBS_PITR-LΔ5L-CoE-CIE-SC. The ampicilin resistance gene is in blue; the PacI site is in green; the inverted terminal repeat is in black; LoxP sites are in magneta; the packaging site is in yellow; the CMV promoter is in black and the TATA box is in green; the CymR binding site is in red; the E2 Late Leader is in cyan; the Cre and EGFP open reading frames are in blue; IRES is in green; the PI-SceI and I-CeuI sites are in green; and the origin of replication is in cyan

Figure 5. Putative Sequence of pSH_SPC. The kanamycin resistance gene is in blue; the PacI site is in green; the PI-SceI and I-CeuI sites are in green; and the origin of replication is in cyan

generate pBS_PI-SceI. An I-CeuI site was generated by annealing together the oligomers 5'-GATCTGAATTTAACTATAACGGTCCTAAGGTAGCGAAAGCTCAGATC-3' and 5'-GATCTGAGCTTTCGCTACCTTAGGACCGTTATAGTTAAATTCA-3'. pBS_PI-SceI was digested with KpnI, blunted, then digested with BglII, and the I-CeuI site was ligated into the pBS_PI-SceI backbone to generate pBS_SC. pBS_PITR_LΔ5L was digested with BssHII, blunted, and then digested with NotI. pBS_SC was digested with AleI and NotI, and the PITR_LΔ5L insert was ligated into the pBS_SC backbone to generate pBS_PITR_LΔ5L_SC. The sequence of pBS_PITR_LΔ5L_SC is shown in Figure 2.

Construction of pBS_Cre-IRES-EGFP:

Previously we had constructed pBS_tTA-IRES-EGFP as follows. The NheI site containing oligomer 5'-TCGAGCTAGC-3' was annealed to itself. pBS_rKS was digested with XhoI and dephosphorylated, and the NheI containing oligomer was ligated into the pBS_rKS backbone to generate pBS_rN: When ligated into the XhoI site, the annealed NheI oligomers generate the sequence 5'-CTCGAGCTAGCTCGAG-3'. pIRES was digested with EcoRI and SmaI. pBS_rN was digested with EcoRI and SmaI, and the IRES sequence was ligated into the pBS_rN backbone to generate pBS_IRES. pEGFP-N1 was digested with SacI and AflII and blunted. pBS_KS+ was digested with EcoRV and dephosphorylated, and the EGFP sequence was ligated into the pBS_KS+ backbone to generate pBS_EGFP. pBS_EGFP was digested with ClaI, blunted, and recircularized. This construct was then digested with SmaI and ApaI and blunted. pBS_IRES was digested with SmaI and EagI, blunted and dephosphorylated, and the EGFP insert was ligated into the pBS_IRES backbone to generate pBS_IRES-EGFP. The tTA sequence of pTet-Off (pTet-Off is part of the BD™ Tet-Off Gene Expression System) was amplified using primers 5'-ATCGATCCGTCAGATCGCCTGGAGAC-3' and 5'-GATATCTTGTCCAAACTCATCAATGTATCTTATCA-3' and ligated into pGEM®-T Easy to generate pGEM_tTA. pGEM_tTA was digested with NotI and blunted. pBS_IRES-EGFP was digested with EcoRV, and the tTA insert was ligated into the pBS_IRES-EGFP backbone to generate pBS_tTA-IRES-EGFP.

pShuttleX was previously constructed by annealing together the oligomers 5'-CTGGAATCTAGACTCGAGCGGCCGCCACGGGTAC-3' and 5'-ACACGACCTTAGATCTGAGCTCGCCGGCGGTGCC-5' and ligating them into BstXI-KpnI digested pShuttle2 (pShuttle2 is part of the BD Adeno-X™ Expression System 1 kit).

pShuttle_Cre was previously constructed as follows. Cre recombinase was amplified from pxCANCre using primers 5'-CCCGGGTGCATCATGAGCGGCCC-3' and 5'-TCTAGAGCGCTTAATGGCTAATCGCCATCTTCC-3', and the amplicon was digested with SmaI and XbaI. pShuttleX was digested with BstXI and blunted, then digested with XbaI and dephosphorylated, and the Cre recombinase insert was ligated into the pShuttleX backbone to generate pShuttle_Cre.

pShuttle_Cre was digested with NheI and AfeI. pBS_tTA-IRES-EGFP was digested with NheI and EcoRV, and the Cre insert was ligated into the pBS_(tTA)-IRES-EGFP backbone to generate pBS_Cre recombinase-IRES-EGFP (pBS_CIE). The sequence of pBS_CIE is shown in Figure 3.

Construction of pBS_PITR_LΔ5L_CoE_CIE_SC:

The E2 late leader was cloned as follows. HEK293 cells were transfected with an Adeno-X_EGFP: the Adeno-X_EGFP was made according to the manufacturer's instructions and transfected into cells using FuGENE 6 according to the manufacturer's instructions. Total RNA was extracted using Isogen according to the manufacturer's instructions. cDNA was made using SuperScript III according to the manufacturer's instructions. The E2 Late Leader was amplified using primers 5'-GGATCCACAGCCCCGGAGTGAGTT-3' and 5'-ATCGATTTCCTTCTCCTGATATCGCCTC-3' and ligated into pGEM®-T Easy to generate pGEM_E2. The CMV-ML-CuO sequence from pCMV5CuO (pCMV5CuO is part of the Q-mate™ Inducible Expression System) was amplified using primers 5'-CCAAACTCATCAATGTATCTTATCATG-3' and 5'-AAATTTCCTTTATTAGCCAGAGGTC-3' and ligated into pGEM®-T Easy to generate pGEM_CMV-ML-CuO. pGEM_E2 was digested with EcoRI and blunted. pGEM_CMV5-CuO was digested with SacII, blunted and dephosphorylated, and the E2 late leader was ligated into the pGEM_CMV-(ML)-CuO backbone to generate pGEM_CoE (pGEM_[CMV promoter]-[Cym operator][with the E2 Late Leader inserted]). pGEM_CoE was digested with SpeI and blunted. pBS_CIE was digested with PmeI and dephosphorylated, and the CoE sequence was ligated into the pBS_CIE backbone to generate pBS_CoE-CIE. pBS_CoE-CIE was digested with SacI and blunted, and then digested with NheI. pBS_PITR_LΔ5L_SC was

Figure 6. Putative Sequence of pEVE

Figure 6 (contined). Putative Sequence of pEVE

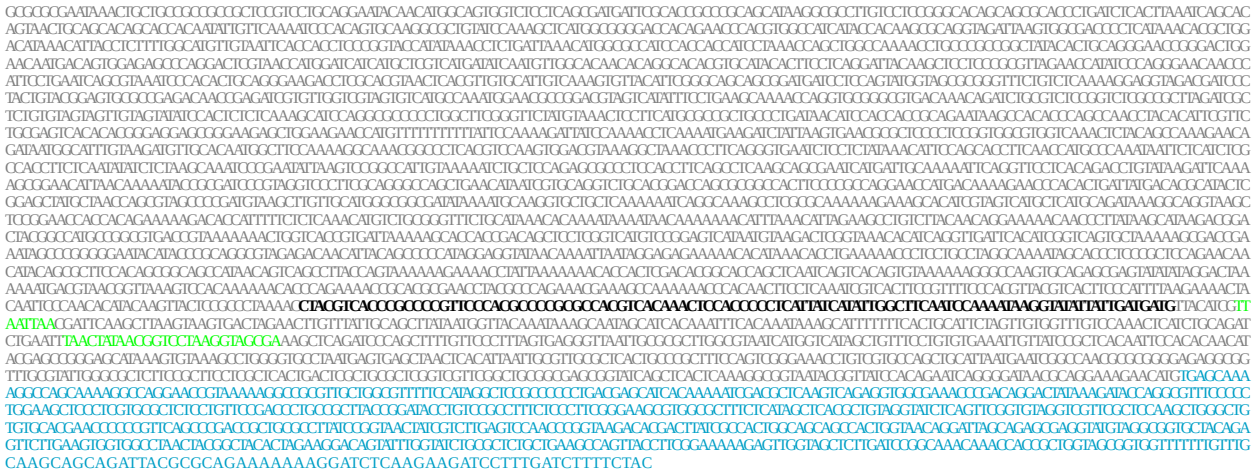

Figure 6 (cont). Putative Sequence of pEVE. The ampicilin resistance gene is in blue; the PacI sites are in green; the inverted terminal repeats are in black; LoxP sites are in magneta; the packaging site is in yellow; the CMV promoter is in black and the TATA box is in green; the CymR binding site is in red; the E2 Late Leader is in cyan; the Cre and EGFP open reading frames are in blue; IRES is in green; the PI-SceI and I-CeuI sites are in green; and the origin of replication is in cyan. The sequence between the PI-SceI site and the 3' inverted terminal repeat are viral sequences from Adeno-X: Adeno-X has not been sequenced in its entirety; Adeno-X sequences were obtained from the BD Sciences web site

digested with SpeI and SmaI, and the CoE-CIE insert was ligated into the pBS_PITR_LΔ5L_SC backbone to generate pBS_PITR_LΔ5L_CoE-CIE_SC. The sequence is shown in Figure 4.

Construction of pSH_SPC:

pShuttle2 was digested with EcoRI and SapI, blunted and dephosphorylated, and an I-CeuI site (5'-TAACTATAACGGTCCTAAGGTAGCGA-3') was ligated into the pShuttle backbone. This construct was then digested with BstXI and KpnI, blunted and dephosphorylated, and a PacI site (5'-GTTACTACGTTA ATTAACGATT-3') was ligated into the backbone. This construct was then digested with DraIII and XbaI, blunted

Figure 7. Putative Sequence of pSH_PSPSC. The kanamycin resistance gene is in blue; the I-PpoI, I-SceI, PI-PspI, PI-SceI and I-CeuI sites are in green (the PI-PspI site inserted in duplicate); and the origin of replication is in cyan. After digestion of pSH_CS with BsrDI, a "G" residue was lost during the blunting reaction. After digestion of pSH_PSPS with FauI, a "C" residue was lost during the blunting reaction

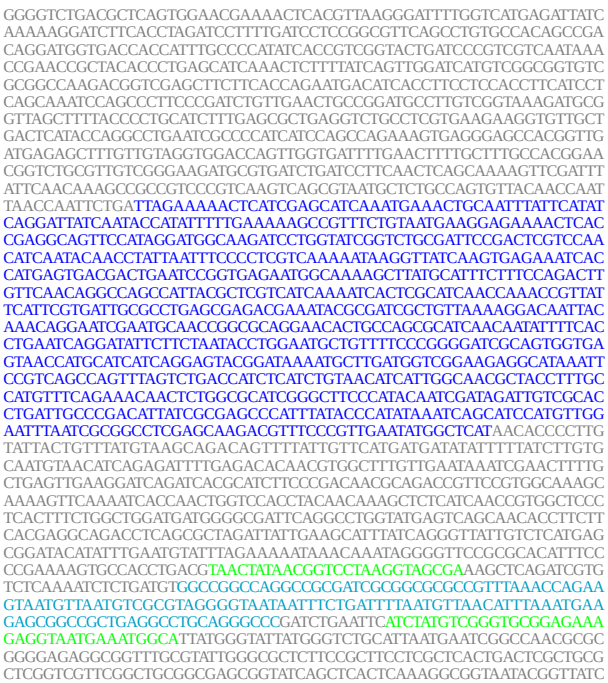

Figure 8. Putative Sequence of pSHCB. The kanamycin resistance gene is in blue; the PI-Scei and I-CeuI sites are in green; the sequences obtained from pSH_CST-BsrDI are in cyan; and the origin of replication is also in cyan

Figure 9. Putative Sequence of pSHPB. The kanamycin resistance gene is in blue; the PI-SceI and I-CeuI sites are in green; the sequences obtained from pSH_CST-BsrDI are in cyan; the PmeI site contained within this sequence is in black; the PmeI sites inserted into the HpaI site of pSHCB are also in black; and the origin of replication is in cyan

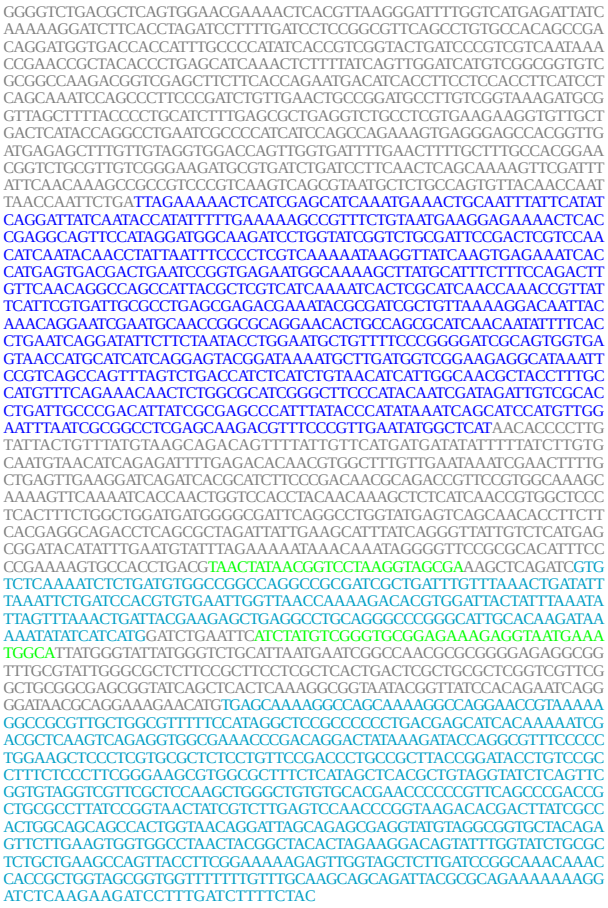

Figure 10. Putative Sequence of pSHcnB. The kanamycin resistance gene is in blue; the PI-SceI and I-CeuI sites are in green; the inserted oligomers are in cyan; and the origin of replication is also in cyan

and dephosphorylated, and a PI-SceI site (5'-ATCTATGT CGGGTGCGGAGAAAGAGGTAATGAAATGGCA-3') was ligated into the backbone to generate pSH_SPC. The sequence of pSH_SPC is shown in Figure 5.

Construction of pSH_AdX:

pAdeno-X_EGFP was digested with PI-SceI and PacI. pSH_SPC was digested with PI-SceI and PacI, and the AdX sequence was ligated into the pSH_SPC backbone to generate pSH_AdX.

Construction of pEVE:

pSH_AdX was digested with PI-SceI and I-CeuI. pBS_PITR_LΔ5L_CoE-CIE_SC was digested with PI-SceI and I-CeuI, and the AdX sequence was ligated into the pBS_PITR_LΔ5L_CoE-CIE_SC backbone to generate pEVE. The sequence of pEVE is shown in Figure 6.

Construction of the helper adenovirus-dependent vector (ADMM)

During construction of ADMM, newly cloned DNA was sequenced in its entirety. However, in the later stages of construction, only ligation junctions were sequenced and restriction digestion (primarily with SacI) was used assess the constructs.

Construction of pSH_PSPSC:

pShuttle2 was digested with XbaI and SpeI and recircularized to generate pSH_CS. The oligomers 5'-GG CCGGCCAGGCCGCGATCGCGGCGCGCCGTTTAA ACCAGAAGTAATGTTAATGTCGCGTAGGGGTAAT AA-3' and 5'-CTACGCGACATTAACATTACTTCTGG TTTAAACGGCGCGCCGCGATCGCGGCCTGGCCGG CC-3' were annealed together, and the oligomers 5'-TTT CTGATTTTAATGTTAACATTTAAATGAAGAGCGGC CGCTGAGGCCTGCAGGGCCCGGG-3' and 5'-CCCG GGCCCTGCAGGCCTCAGCGGCCGCTCTTCATTTAA ATGTTAACATTAAAATCAGAAATTATTACCC-3' were annealed together. pSH_CS was digested with BsrDI, blunted and dephosphorylated, and the oligomers were ligated into the pSH_CS backbone (triple ligation) to generate pSH_CST-BsrDI. pSH_CST-BsrDI was digested with BglII, blunted and dephosphorylated, and a PI-PspI site (5'-ACCCATAATACCCATAATAGCTGTTTGCCA-3') was ligated into the pSH_CST-BsrDI backbone to generate pSH_CPS. pSH_CPS was digested with FseI, blunted and dephosphorylated, and an I-PpoI site (5'-GCTACCTTAAGAGAGCCGG-3') was ligated into the pSH_CPS backbone to generate pSH_PCPS. pSH_PCPS was digested with DraIII and I-CeuI, blunted and dephosphorylated, and an I-SceI site (5'-ATTACCCTGT

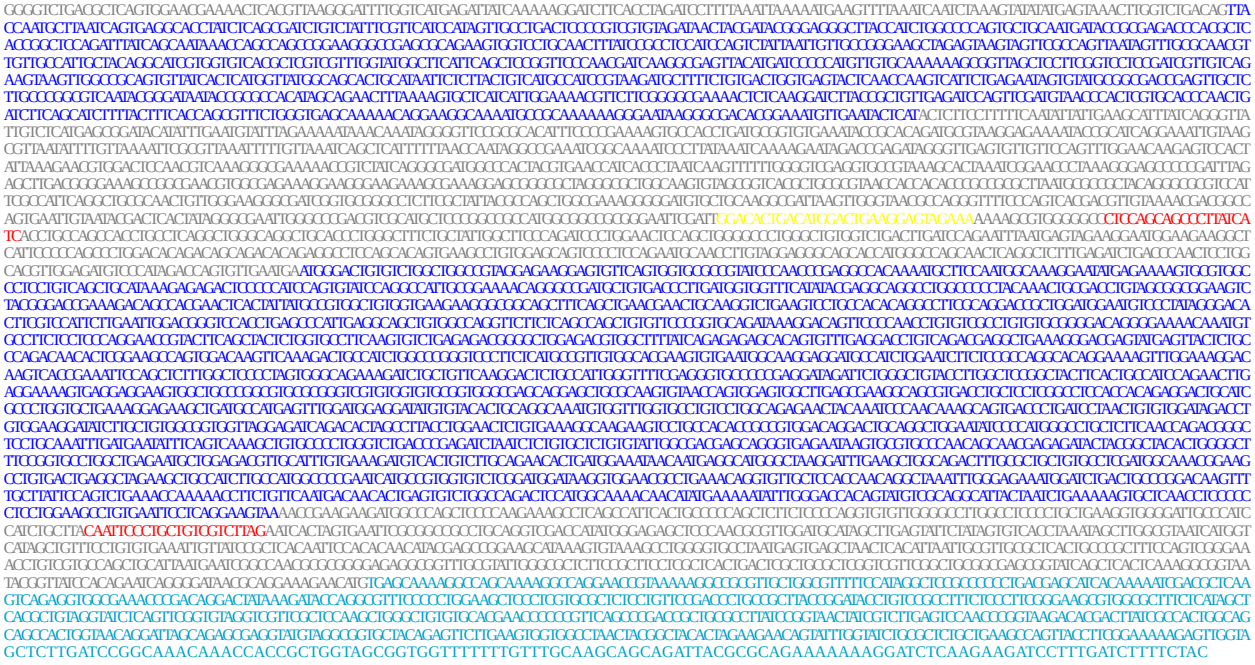

Figure 11. Putative Sequence of pGEM_gamma-lactoferrin (pGEM_gLF). The ampicillin resistance gene is in blue; the Gene Racer primer used to clone the 5' end of gLF is in yellow; the primers used to clone sub-gLF are in red; the ORF of gamma-lactoferrin is in blue; and the origin of replication is in cyan

TATCCCTA-3') was ligated into the pSH_PCPS backbone to generate pSH_PSPS. pSH_PSPS was digested with FauI, blunted and dephosphorylated, and an I-CeuI site (5'-TAACTATAACGGTCCTAAGGTAGCGA-3') was ligated into the pSH_PSPS backbone to generate pSH_PSPSC. The sequence of pSH_PSPSC is shown in Figure 7.

Construction of pSHCB:

pSH_CST-BsrDI was digested with DraIII and SmaI and blunted. pSH_CS was digested with BglII, blunted and dephosphorylated, and the insert from pSH_CST-BsrDI was ligated into the pSH_CS backbone to generate pSHCB. The sequence of pSHCB is shown in Figure 8.

Construction of pSHPB:

pSHCB was digested with HpaI and dephosphorylated, and a PmeI site (5'-AATTGGTTTAAACC-3') was inserted into the pSHCB backbone to generate pSHPB. The sequence of pSHPB is shown in Figure 9.

Construction of pSHcnB:

The oligomers 5'-GTGTCTCAAAATCTCTGATGTG GCCGGCCAGGCCGCGATCGCTGATTTGTTTAAAC TGATATTTAAATTCTGATCCACGTGTGAATTGGTT AACC-3' and 5'-CAATTCACACGTGGATCAGAATTT AAATATCAGTTTAAACAAATCAGCGATCGCGGCCT GGCCGGCCACATCAGAGATTTTGAGACAC-3' were annealed together, and the oligomers 5'-AAAAGACACG TGGATTACTATTTAAATATTAGTTTAAACTGATTAC GAAGAGCTGAGGCCTGCAGGGCCCGGGCATTGCA CAAGATAAAAATATATCATCATG-3' and 5'-CATGAT GATATATTTTTATCTTGTGCAATGCCCGGGCCCTGC AGGCCTCAGCTCTTCGTAATCAGTTTAAACTAAT ATTTAAATAGTAATCCACGTGTCTTTTGGTTAAC-3' were annealed together. pSH_CS was digested with BglII, blunted and dephosphorylated, and the annealed oligomers were ligated into the pSH_CS backbone (triple ligation) to generate pSHcnB. The sequence of pSHcnB is shown in Figure 10.

Construction of pSHCB_gSTOP, pSHPB_gSTOP, and pSHcnP_gSTOP:

We had previously cloned a putative intracellular form of lactoferrin which we named gamma-lactoferrin (gLF) (Alexander et al., 2007). Briefly, the cap-selective 5'-RACE procedure using Invitrogen's GeneRacer Kit was carried out according to the manufacturer's instructions: Total RNA was extracted from MRC5 cells using the Isogen RNA extraction procedure, dephosphorylated, treated with tobacco acid pyrophosphate, and an RNA oligonucleotide of known sequence (supplied in the Gene Racer Kit) was ligated to the 5' end of the RNA. The RNA was then reverse transcribed using a human lactoferrin specific primer (5'-ATCCTCCTTGCCATTCACAC-3'). Finally, the 5' region of the RNA was amplified using standard (Left primer, supplied by Invitrogen, 5'-CGACTGGAGCACGAGGACACTGA-3'; Right primer, anneals to lactoferrin, 5'-GCCAGATGGCAGTCTTTGA-3') and nested PCR (Nested left primer, supplied by Invitrogen, 5'-GGACACTGACATGGCTGAAGGAGT A-3'; Nested right primer, anneals to lactoferrin, 5'-TTGTCCACTGGCTTCCGAGTGTTGTC-3') and ligated into pGEM® -T Easy (pGEM_5'gLF). The 5' region was sequenced using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Nearly full length gLF was amplified using 5'-CTCCAGCAGCCCTTATCATC-3' (the left primer binds to the 5' UTR of gamma-lactoferrin) and 5'-CTAAG ACGACAGCAGGGAATTG-3' (the right primer binds to the 3' UTR of gamma-lactoferrin) and ligated into pGEM® -T Easy (pGEM_sub-gLF) and sequenced. Full

Figure 12. Putative Sequence of pSHCB_gSTOP. The kanamycin resistance gene is in blue; the PI-Scei and I-CeuI sites are in green; the Gene Racer primer used to clone the 5' end of gLF is in yellow; the gSTOP sequence is in red (the first 9 bases of the gLF ORF are in blue); and the origin of replication is in cyan

length gLF was generated by digesting pGEM_5'gLF with AatII and TthIII and ligating the 5'gLF sequence into AatII and TthIII digested pGEM_sub-gLF. The sequence of pGEM_gLF is shown in Figure 11.

pGEM_gLF was digested with SpeI and SalI, blunted and recircularized. The recircularized plasmid was digested with NotI, blunted and dephosphorylated, and a PmeI site (5'-AATTGGTTTAAACC-3') was ligated into the backbone to generate pGEM_PLF. pGEM_PLF was digested with PmeI and TthIII and blunted. pSHCB was digested with PmeI and dephosphorylated, and the STOP sequence from gLF was ligated into the pSHCB backbone to generate pSHCB_gSTOP. The sequence of pSHCB_gSTOP is shown in Figure 12.

pSHCB_gSTOP was digested with HpaI and dephosphorylated, and a PmeI site was ligated into the pSHCB_gSTOP backbone to generate pSHPB_gSTOP. The sequence of pSHPB_gSTOP is shown in Figure 13.

pSHPB_gSTOP was digested with AscI and SwaI and blunted. pSHcnB was digested with PmlI and dephosphorylated, and the gSTOP sequence ligated into the pSHcnB backbone to generate pSHcnP_gSTOP. The sequence of pSHcnP_gSTOP is shown in Figure 14.

Figure 13. Putative Sequence of pSHPB_gSTOP. The kanamycin resistance gene is in blue; the PI-Scei and I-CeuI sites are in green; the Gene Racer primer used to clone the 5' end of gLF is in yellow; the gSTOP sequence is in red (the first 9 bases of the gLF ORF are in blue); the PmeI sites inserted into the HpaI site are in black; and the origin of replication is in cyan

Construction of pSHcnB_Ins50:

Stuffer DNA 50 was amplified from chicken genomic DNA using 5'-AGAGAAAGGGAAGGAACTTGTCAT-3' and 5'-CATTAGCGGCAGCACCG-3'. pSHcnB was digested with SwaI and dephosphorylated, and the stuffer DNA 50 amplicon was ligated into the pSHcnB backbone to generate pSHcnB_Ins50. The sequence of pSHcnB_Ins50 is shown in Figure 15.

Construction of pSHcnB_Ins50G:

Stuffer DNA G-3016 (InsG-3016) was amplified from rat genomic DNA using 5'-GGGCACTCCATACCAACCA-3' and 5'-GGACCCAGCAGCCTTTCAT-3'. pSHcnB_Ins50 was digested with SacII, blunted and dephosphorylated, and the InsG-3016 amplicon was ligated into the pSHcnB_Ins50 backbone to generate pSHcnB_Ins50G. The sequence of pSHcnB_Ins50G is shown in Figure 16.

Construction of pRcnSS:

pBS_KS+ was digested with BamHI, blunted and

Figure 14. Putative Sequence of pSHcnP_gSTOP. The kanamycin resistance gene is in blue; the PI-SceI and I-CeuI sites are in green; the PmeI sites inserted into the HpaI site are in black; the gSTOP sequence is in red (the first 9 bases of the gLF ORF are in blue); the Gene Racer primer used to clone the 5' end of gLF is in yellow; and the origin of replication is in cyan

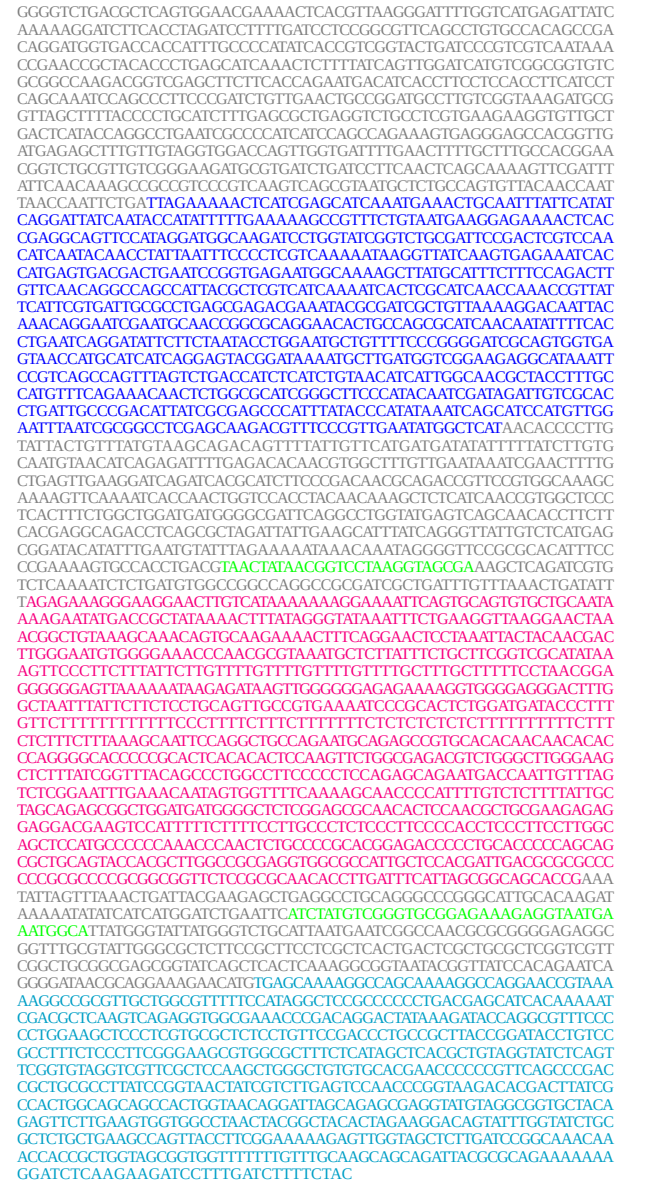

Figure 15. Putative Sequence of pSHCB_Ins50. The kanamycin resistance gene is in blue; the PI-Scei and I-CeuI sites are in green; Ins50 is in magneta; and the origin of replication is in cyan

dephosphorylated, and an I-CeuI site (5'-TAACTATAACGGTCCTAAGGTAGCGA-3') was ligated into the pBS_KS+ backbone to generate pBSC. mSTOP was amplified from mouse genomic DNA using 5'-TTTTTGAAATGGCTCGTTGC-3' and 5'-TGGTAACCGTATCGGTACAAGAT-3'. pBSC was digested with SmaI and dephosphorylated, and the mSTOP amplicon was ligated into the pBSC backbone to generate pBSCsma_mSTOP. pBSCsma_mSTOP was digested with PstI and NotI and blunted. pSHcnB was digested with I-CeuI, blunted and dephosphorylated, and the I-CeuI-mSTOP insert was ligated into the pSHcnB backbone to generate pRcnSx. pGEM_closed was digested with NcoI, blunted and dephosphorylated, and a PI-SceI site (5'-ATCTATGTCGGGTGCGGAGAAAGAGGTAATGAAATGGCA-3') was ligated into the pGEM_closed backbone to generate pGPS. dSTOP was amplified from human genomic DNA using 5'-CCTTAGGTAGATGCCGTAAGACAA-3' and 5'-AGCAAGACCGAGCCTTTA-3'. pGPS was digested with NotI, blunted and dephosphorylated, and the dSTOP amplicon was ligated into the pGPS backbone to generate pGPS_dSTOP. pGPS_dSTOP was digested with ApaI and BstXI and blunted. pRcnSx was digested with PI-SceI, blunted and dephosphorylated, and the PI-SceI-dSTOP insert was ligated into the pRcnSx backbone to generate pRcnSS. The sequence of pRcnSS is shown in Figure 17.

Construction of pRcnSS_Ins57-111:

Stuffer DNA 57-111 was amplified from chicken genomic DNA using primers 5'-TGCCAATCCATCAAGTTCCAT-3' and 5'-TGTCCAGCCTCATATCCAGTCA-3'. RcnSS was digested with PmlI and dephos-phorylated, and the stuffer DNA 57-111 amplicon was ligated into the pRcnSS backbone. The sequence of pRcnSS_Ins57-111 is shown in Figure 18.

Construction of pSH_PPS50G-111s_PSPSC_PITR:

The 3' PacI-ITR sequence (PITR) of Adeno-X was amplified from EVE using primers 5'-TTACTCCGCCCT

Figure 16. Putative Sequence of pSHcnB_Ins50G. The kanamycin resistance gene is in blue; the PI-Scei and I-CeuI sites are in green; Ins50 is in magneta; InsG-3016 is in yellow; and the origin of replication is in cyan

AAAACCTAC-3' and 5'-GCTATTGCTTTATTTGTAAC CATT-3'. pSHcnB was digested with SwaI and dephosphorylated, and the PITR amplicon was ligated into the pSHcnB backbone to generate pSHcnB_PITR-3'. pSHcnB_PITR-3' was digested with PmeI. pSH_PSPSC was digested with PciI, blunted and dephosphorylated, and the PITR insert was ligated into the pSH_PSPSC backbone to generate pSH_PSPSC_PITR. pBS_PITR_ Pack-wt was digested with BssHII and SmaI and blunted. pSH_PSPSC_PITR was digested with SwaI and dephosphorylated, and the PITR insert was ligated into the pSH_PSPSC_PITR backbone to generate pSH_PP_ PSPSC_PITR. pSHcnP_gSTOP was digested with PmeI. pSH_PP_PSPSC_PITR was digested with BamHI, blunted and dephosphorylated, and the gSTOP insert was ligated into the pSH_PP_PSPSC_PITR backbone to generate pSH_PPS_PSPSC_PITR. pSHcnB_Ins50G was digested with PmeI. pSH_PPS_PSPSC_PITR was digested with HpaI and dephosphorylated, and the Ins50G insert was ligated into the pSH_PPS_PSPSC_PITR backbone to generate pSH_PPS50G_PSPSC_PITR. pRcnSS_Ins57-111 was digested with PI-SceI, blunted and dephosphorylated, and an I-PpoI site (5'-GCTACCTTA AGAGAGCCGG-3') was ligated into the pRcnSS_Ins57-

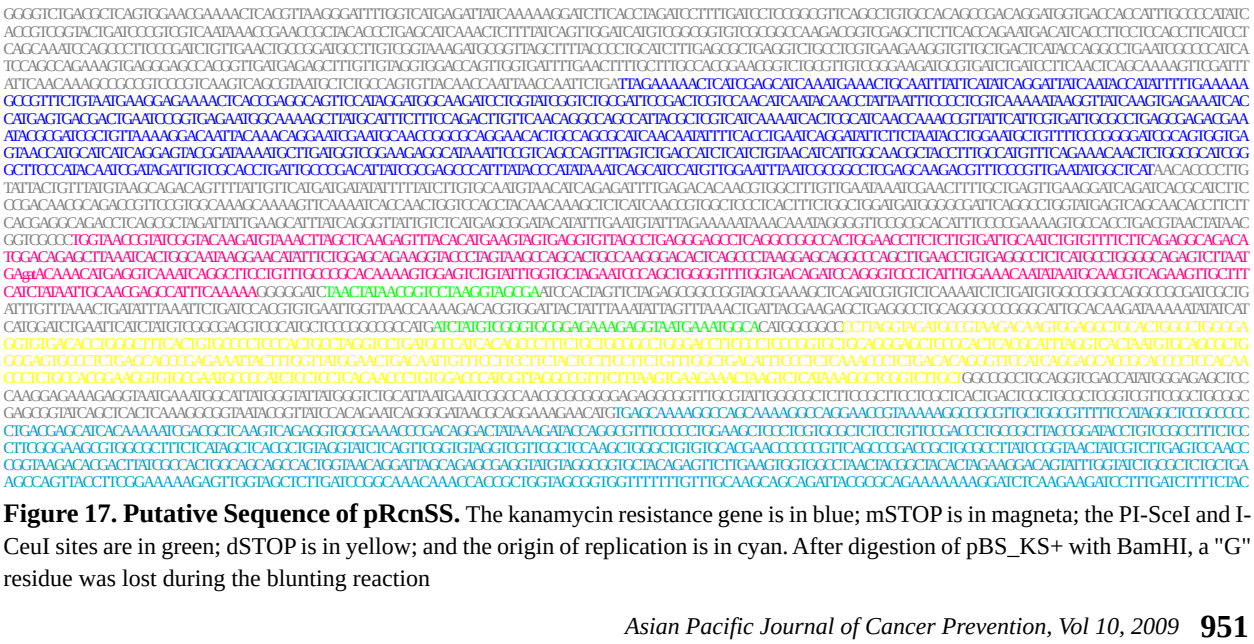

Figure 17. Putative Sequence of pRcnSS. The kanamycin resistance gene is in blue; mSTOP is in magneta; the PI-SceI and I-CeuI sites are in green; dSTOP is in yellow; and the origin of replication is in cyan. After digestion of pBS_KS+ with BamHI, a "G" residue was lost during the blunting reaction

Figure 18. Putative Sequence of pRcnSS_Ins57-111. The kanamycin resistance gene is in blue; the PI-SceI and I-CeuI sites are in green; mSTOP is in magenta; Ins57-111 is in cyan; dSTOP is in yellow; and the origin of replication is in cyan

111 backbone to generate pRcnP_Ins57-111. pRcnP_Ins57-111 was digested with I-CeuI, blunted, and then digested with I-PpoI. pSH_PPS50G_PSPSC_PITR was digested with PmeI and I-PpoI, and the Ins57-111 insert was ligated into the pSH_PPS50G_PSPSC_PITR backbone to generate pSH_PPS50G-111_PSPSC_PITR. pSH_PPS50G-111_PSPSC_PITR was digested with SpeI and recircularized to generate pSH_PPS50G-111s_PSPSC_PITR. The sequence of pSH_PPS50G-111s_PSPSC_PITR is shown in Figure 19.

Construction of pØØ_PSPSC:

pSH_PSPSC was digested with SmaI and PciI. pBS_KS+ was digested with ApoI and blunted, then digested with PciI, and the PSPSC insert was ligated into the pBS backbone to generate pØØ_PSPSC. The sequence of pØØ_PSPSC is shown in Figure 20.

Construction of the C-I cassette:

The LacZ ORF was amplified from pShuttle_LacZ (pShuttle_LacZ is part of the BD Adeno-X™ Expression System 1 kit) using primers 5'-CGAGGGGGGATCGAAAGAG-3' and 5'-ATGTAGCCAAATCGGGAAAAACG-3'. pSHPB_gSTOP was digested with SwaI and dephosphorylated, and the LacZ amplicon was ligated into the pSHPB_gSTOP backbone to generate pSHPB_gSTOP_LacZ. The poly(A) addition signal of rat glutathione S-transferase pi subclass (GST-P) was amplified from rat genomic DNA using primers 5'-CAGACTAATAAAGTTTGTAAGGCA-3' and 5'-GATCACAGCATTTGGGAGA-3'. pBS was digested with EcoRV and dephosphorylated, and the GSTP poly(A) addition signal was ligated into the pBS backbone to generate pBS_(rGSTP)pA. pBS_(rGSTP)pA was digested with SalI and SacII and blunted. pSHCB_gSTOP_LacZ was digested with NotI, blunted and dephosphorylated, and the (rGSTP)pA insert was ligated into the pSHPB_gSTOP_LacZ backbone to generate pSHPB_gSTOP_LacZ-pA. pTRE-Tight was digested with XhoI and EcoRI and blunted. pSHPB_gSTOP_LacZ-pA was digested with AscI, blunted and dephosphorylated, and the TRE-Tight promoter was ligated into the pSHPB_gSTOP_LacZ-pA backbone to generate pSHPB_Tight_gSTOP_LacZ-pA. pSHPB_Tight_gSTOP_LacZ-pA was digested with I-CeuI, blunted and dephosphorylated, and an I-PpoI site (5'-GCTACCTTAAGAGAGCCGG-3') was ligated into the pSHPB_Tight_gSTOP_LacZ-pA backbone to generate pSHP_Tight_gSTOP_LacZ-pA. pSHP_Tight_gSTOP_LacZ-pA was digested with PI-SceI, blunted and dephosphorylated, and an I-SceI site (5'-TAGGGATTACCCTGTTATCCCTACAGGGTAAT-3') was ligated into the pSHP_Tight_gSTOP_LacZ-pA backbone to generate

Figure 19. Putative Sequence of pSH_PPS50G-111s_PSPSC_PITR. The kanamycin resistance gene is in blue; the PacI sites are in green; the inverted terminal repeats are in black, the packaging site is in yellow; the Gene Racer primer used to clone the 5' end of gLF is also in yellow; the gSTOP sequence is in red (the first 9 bases of the gLF ORF are in blue); Ins50G is in magenta; Ins57-111s is in cyan; the I-PpoI, I-SceI, PI-PspI, PI-Scei and I-CeuI sites are in green; and the origin of replication is in cyan

pSHPS_Tight_gSTOP_LacZ-pA. Stuffer DNA 52 was amplified from chicken genomic DNA using primers 5'-TCATAGGGTTGTTGGGTTGTTT-3' and 5'-ATCCTCT TCTCACTGCTGCTACT-3'. pBS was digested with EcoRV and dephosphorylated, and the stuffer DNA 52 amplicon was ligated into the pBS backbone to generate pBS_Ins52. pBS_Ins52 was digested with EcoRI and HindIII and blunted. pSHPS_Tight_gSTOP_LacZ-pA was digested with PstI, blunted and dephosphorylated, and the Ins52 insert was ligated into the pSHPS_Tight_gSTOP_ LacZ-pA backbone to generate pSHPS_Tight_gSTOP_ LacZ-pA_Ins52. pSHPS_Tight_gSTOP_LacZ-pA_Ins52 was digested with PmeI (to remove the gSTOP sequence) and recircularized to generate pC-I_Tight-LacZ. The sequence of pC-I_Tight-LacZ is shown in Figure 21.

Construction of the C-II cassette:

The HSVTK ORF was amplified from pCM-TK using primers 5'-TCTAGATTGGTGGCGTGAAACTCCC-3' and 5'-GTCGACGGTTCCTTCCGGTATTGTCTCC-3' and ligated into pGEM®-T Easy to generate pGEM_HSVTK. pGEM_HSVTK was digested with

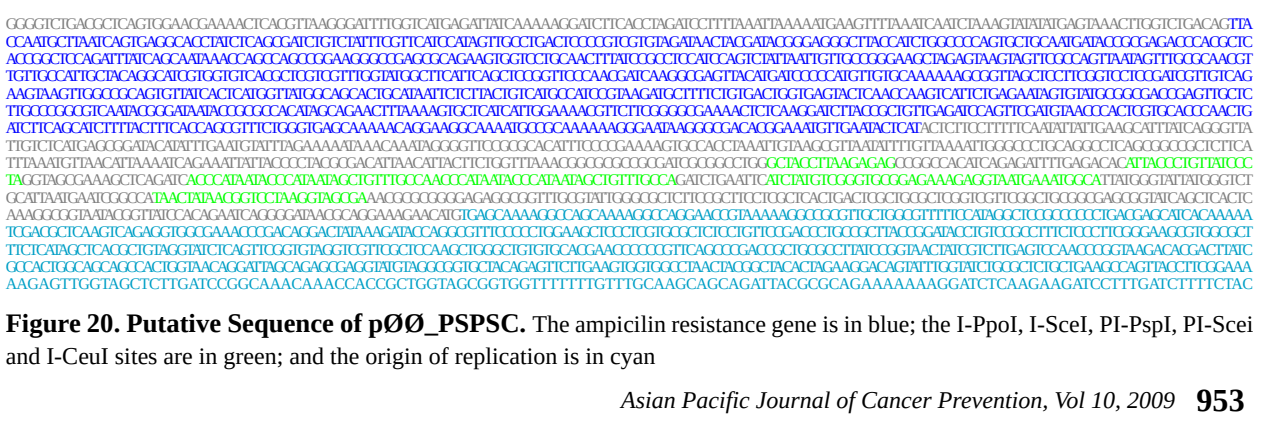

Figure 20. Putative Sequence of pØØ_PSPSC. The ampicilin resistance gene is in blue; the I-PpoI, I-SceI, PI-PspI, PI-Scei and I-CeuI sites are in green; and the origin of replication is in cyan

Figure 21. Putative Sequence of pC-I_Tight-LacZ. The kanamycin resistance gene is in blue; the I-PpoI site is in green; the TRE-Tight promoter is in black, the TATA box is in green; the LacZ ORF is in blue; the sequence containing the (rGSTP)pA is in red; Ins52 is in yellow; the I-SceI site is in gren; and the origin of replication is in cyan

EcoRI and blunted. pSHCB was digested with SwaI and dephosphorylated, and the HSVTK was ligated into the pSHCB backbone to generate pSHCB_HSVTK. The poly(A) addition signal of rat guanylate kinase 1 was amplified from rat genomic DNA using primers 5'-ACATCTATTCTCCCTGGGCTATTT-3' and 5'-TCC TTCCTGGCAAGTGGTGT-3'. pBS_KS+ was digested with EcoRV and dephosphorylated, and the guanylate kinase 1 poly(A) addition signal was ligated into the pBS backbone to generate pBS_(rGUK)pA. pBS_(rGUK)pA was digested with EcoRI and HindIII and blunted. pSHCB_HSVTK was digested with NotI, blunted and dephosphorylated, and the (rGUK)pA insert was ligated into the pSHCB_HSVTK backbone to generate pSHCB_HSPTK-pA. pTRE-Tight was digested with XhoI and EcoRI and blunted. pSHCB_stopHSVTK-pA was digested with AscI, blunted and dephosphorylated, and the TRE-Tight promoter was ligated into the pSHCB_HSVTK-pA backbone to generate pSHCB_Tight-HSVTK-pA. Stuffer DNA 53 was amplified from chicken genomic DNA using primers 5'-AACCGTCCA CCTACCACCAAC-3' and 5'-CTGTCAGCCTCATTTC ACAAGATT-3'. pBS_KS+ was digested with EcoRV and dephosphorylated, and the stuffer DNA 53 amplicon was ligated into the pBS backbone to generate pBS_Ins53. pBS_Ins53 was digested with SmaI and SalI and blunted. pSHCB_Tight-HSVTK-pA was digested with FseI, blunted and dephosphorylated, and the Ins53 insert was ligated into the pSHCB_Tight-HSVTK-pA backbone to generate pSHCB_Ins53_Tight-HSVTK-pA. pSHCB_ Ins53_Tight-HSVTK-pA was digested with I-CeuI, blunted and dephosphorylated, and a PI-PspI site (5'-ACCCATAATACCCATAATAGCTGTTTGCCA-3') was ligated into the pSHCB_Ins53_Tight-HSVTK-pA backbone to generate pSHP_Ins53_Tight-HSVTK-pA. pSHP_Ins53_Tight-HSVTK-pA was digested with PI-SceI, blunted and dephosphorylated, and an I-SceI site (5'-ATTACCCTGTTATCCCTA-3') was ligated into the pSHP_Ins53_Tight-HSVTK-pA backbone to generate pSHPS_Ins53_Tight-HSVTK-pA. pSHPS_Ins53_Tight-HSVTK-pA was digested with HpaI and PmeI and recircularized to generate pC-II_Tight-HSVTK. The sequence of pC-II_Tight-HSVTK is shown in Figure 22.

Construction of the C-III cassette:

Stuffer DNA 56 was amplified from Chicken DNA using primers 5'-AGGCTGAATAAGACAGTAGTGG TGA-3' and 5'-GTTTCGGAAGTGCCTGCTAAG-3'. pSHcnB was digested with PmlI and dephosphorylated, and the stuffer DNA 56 amplicon was ligated into the

Figure 22. Putative Sequence of pC-II_Tight-HSVTK. The kanamycin resistance gene is in blue; the PI-PspI site is in green; Ins53 is in yellow; the TRE-Tight promoter is in black, the TATA box is in green; the HSVTK ORF is blue; the sequence containing the (rGUK)pA is in red; the I-SceI site is in green; and the origin of replication is in cyan

pShcnB backbone to generate pSHcnB_Ins56. pSHcnB_Ins56 was digested with I-CeuI, blunted and dephosphorylated, and a PI-PspI site (5'-ACCCATAATAC CCATAATAGCTGTTTGCCA-3') was ligated into the pSHcnB_Ins56 backbone to generate pSHP_Ins56. pSHP_56 was digested with PacI, blunted, and recircularized to generate pC-III_Ins56. The sequence of pC-III_Ins56 is shown in Figure 23.

Construction of the C-IV cassette:

The tTA ORF was amplified from pTet-Off using primers 5'-ATCGATCCGTCAGATCGCCTGGAGAC-3' and 5'-GATATCTTGTCCAAACTCATCAATGTATCT TATCA-3' and the amplicon was ligated into pGEM®-T Easy to generate pGEM_tTA. pGEM_tTA was digested with SpeI and EcoRV and blunted. pSHCB was digested with SwaI and dephosphorylated, and the tTA insert was ligated into the pSHCB backbone to generate pSHCB_tTA. The poly(A) addition signal of rat glucokinase was amplified from rat genomic DNA with primers 5'-GCAGGAATCATCTCCAACACTC-3' and 5'-TGTTCACCCGAAGGCATATTA-3'. pBS_KS+ was digested with EcoRV and dephosphorylated, and the glucokinase poly(A) addition signal was ligated into the pBS backbone to generate pBS_(rGck)pA. pBS_(rGck)pA was digested with EcoRI and HindIII and blunted. pSHCB_tTA was digested with NotI, blunted and dephosphorylated, and the (rGck)pA insert was ligated into the pSHCB_tTA backbone to generate pSHCB_tTA-pA. pTRE2hyg (pTRE2hyg is part of the BD™ Tet-Off Gene Expression System) was digested with XhoI and EcoRI. pBS_rN was digested with XhoI and EcoRI, and the TRE insert was ligated into the pBS_rN backbone to generate pBS_rN_TRE. pBS_rN_TRE was digested with XhoI and EcoRi and blunted. pSHCB_tTA-pA was digested with AscI, blunted and dephosphorylated, and the TRE insert was ligated into the pSHCB_tTA-pA backbone to generate

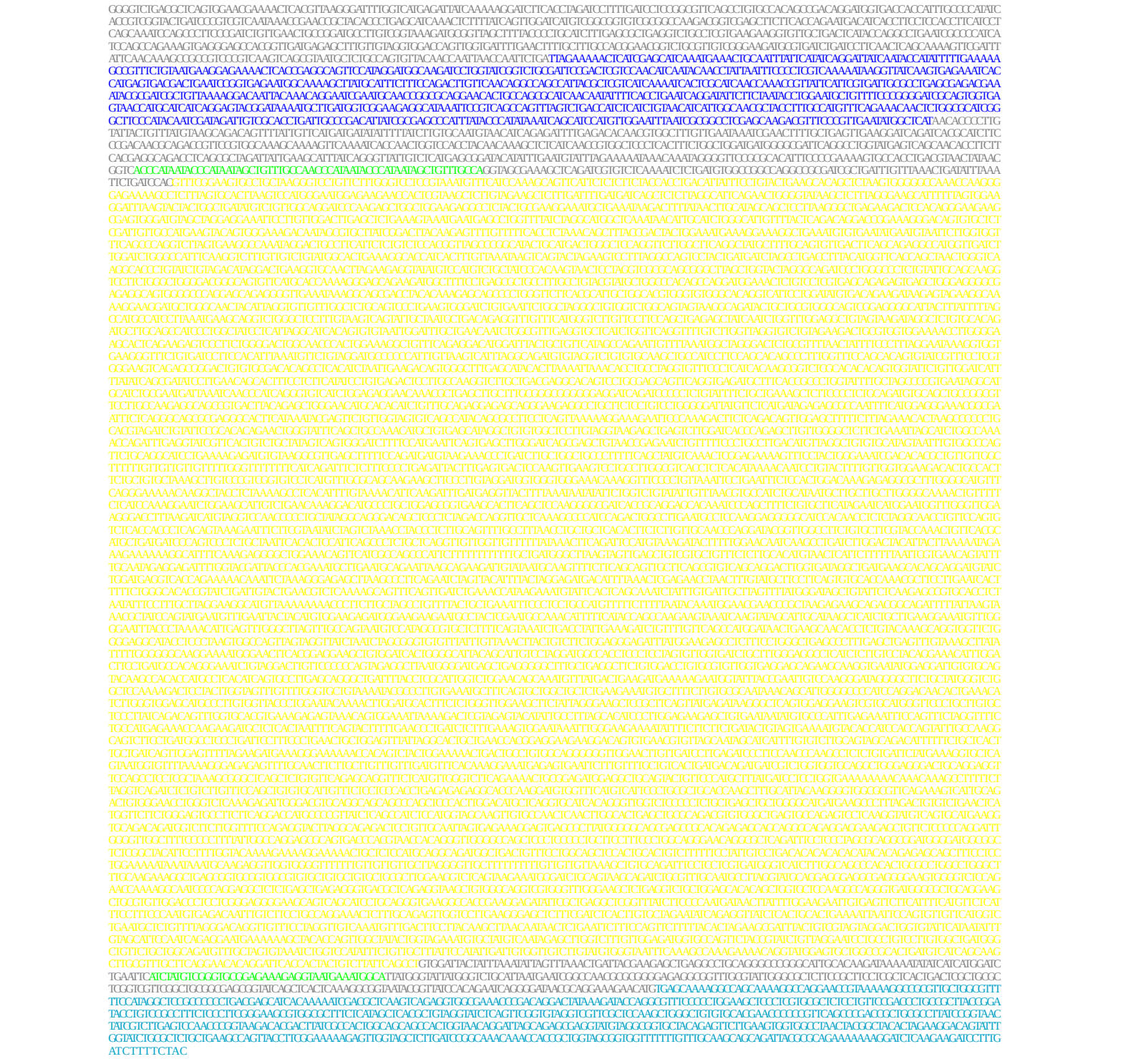

Figure 23. Putative Sequence of pC-III_Ins56. The kanamycin resistance gene is in blue; the PI-PspI site is in green; Ins56 is in yellow; the PI-SceI site is in green; and the origin of replication is in cyan

pSHCB_TRE-tTA-pA. pSHCB_ TRE-tTA-pA was digested with HpaI and PmeI and dephosphorylated, and the sequence 5'-GAAGTTCCTATACTTTCTAGA GAATAGGAACTTC-3' was ligated into the pSHCB_TRE-tTA-pA backbone to generate pSH_TRE-tTA-pA. pSH_TRE-tTA-pA was digested with I-CeuI, blunted and dephosphorylated, and an I-CeuI site was ligated into the pSH_TRE-tTA-pA backbone (I-CeuI sites in the reverse orientation were selected) to generate pSHC_TRE-tTA-pA. pSHC_TRE-tTA-pA was digested with PI-SceI, blunted and dephosphorylated, and a PI-SceI site was ligated into the pSHC_TRE-tTA-pA backbone (PI-SceI sites in the reverse orientation were selected) to generate pSHCS_TRE-tTA-pA. Part A of stuffer DNA 54 was amplified from chicken genomic DNA using primers 5'-GGCGGCTGCCTCTTCATA-3' and 5'-TCCACATTGAGAAGGGACCATAC-3'. pBS_KS+ was digested with EcoRV and dephosphorylated, and part A of stuffer DNA 54 was ligated into the pBS backbone to generate pBS_Ins54a. Part B of stuffer DNA 54 was amplified from chicken genomic DNA using primers 5'-AGGCTGTCTGTTGGCAGTCTTT-3' and 5'-CGTCTG TTTCCCAGGTGCTC-3'. pBS_KS+ was digested with EcoRV and dephosphorylated, and part B of stuffer DNA

Figure 24. Putative Sequence of pC-IV_TRE-tTA. The kanamycin resistance gene is in blue; the PI-SceI site is in green; Ins54 is in yellow; the TRE promoter is in black, the TATA box is in green; the tTA ORF is in blue; the sequence containing the (rGck)pA is in red; the I-CeuI site is in green; and the origin of replication is in cyan. After digestion of pSHCB_tTA with NotI, a "C" residue was lost during the blunting reaction

54 was ligated into the pBS backbone to generate pBS_Ins54b. pBS_Ins54b was digested with BamHI. pBS_Ins54a was digested with BamHI and dephosphorylated, and the Ins54b insert was ligated into the pBS_Ins54a backbone to generate pBS_Ins54. pBS_Ins54 was digested with HindIII and EcoRI and blunted. pSHCS_TRE-tTA-pA was digested with NaeI and dephosphorylated, and the Ins54 insert was ligated into the pSHCS_TRE-tTA-pA backbone to generate pSHCS_Ins54_TRE-tTA-pA. pSHCS_Ins54_TRE-tTA-pA was digested with PacI, blunted, and recircularized to generate pC-IV_TRE-tTA. The sequence of pC-IV_TRE-tTA is shown in Figure 24.

Construction of pC4-H_Tight-LacZ:

pSH_PSPSC was digested with SmaI and PciI. pBS_KS+ was digested with ApoI and blunted, and the PSPSC insert was ligated into the pBS_KS+ backbone to generate pØØ_PSPSC. pC-IV_TRE-tTA was digested with PI-SceI and I-CeuI. pØØ_PSPSC was digested with PI-SceI and I-CeuI, and the C-IV_TRE-tTA insert was ligated into the pØØ_PSPSC backbone to generate pØØ_PSPS_tTA. pC-II_Tight-HSVTK was digested with I-SceI and PI-PspI. pØØ_PSPS_tTA was digested with I-SceI and PI-PspI, and the C-II_Tight-HSVTK insert was ligated into the pØØ_PSPS_tTA backbone to generate pØØ_PS_HSV_PS_tTA. pC-III_Ins56 was digested with PI-PspI and PI-SceI. pØØ_PS_HSV_56_tTA was digested with PI-PspI and PI-SceI, and the C-III_Ins56 insert was ligated into the pØØ_PS_HSV_56_tTA backbone to generate pØØ_PS_HSV_56_tTA. pC-I_Tight-LacZ was digested with I-PpoI and I-SceI. pØØ_PS_HSV_56_tTA was digested with I-PpoI and I-SceI, and the C-I_Tight-LacZ insert was ligated into the pØØ_PS_HSV_56_tTA backbone to generate pC4-H_Tight-LacZ (pØØ_LacZ_HSV_56_tTA).

Construction of pADMM:

pC4-H_Tight-LacZ was digested with I-PpoI and I-CeuI. pSH_PPS50G-111s_PSPSC_PITR was digested with I-PpoI and I-CeuI, and the LacZ_HSV_56_tTA insert was ligated into the pSH_PPS50G-111s_PSPSC_PITR backbone to generate pADMM-H_Tight-LacZ.

Computer analysis

Trace files generated by the ABI Prism 3100 Genetic Analyzer were viewed using 4Peaks software (A. Griekspoor and Tom Groothuis, mekentosj.com). DNA sequences were aligned using ClustalW (Larkin et al., 2007) (http://www.ebi.ac.uk/clustalw). Primers were designed using Primer Premier (Premier Biosoft International: www.premierbiosoft.com/index.html).

Results and Discussion

Vector Construction

During construction of pBS_PITR_Pack-wt: After pBS_rKS was digested with KpnI, a "G" residue was lost during the blunting reaction; this loss had no effect on the function of the construct. During construction of

pBS_PITR_LΔ5L_SC: After digestion of pBS_PITR_LoxP-PackΔ5 with SpeI, a "G" residue, a "C" residue, and a "T" residue were lost during the blunting reaction; these losses had no effect on the function of the construct. During construction of pSH_PSPSC: After pSH_CS was digested with BsrDI, a "G" residue was lost during the blunting reaction; this loss had no effect on the function of the construct. During construction of pSH_PSPSC: After digestion of pSH_PSPS with FauI, a "C" residue was lost during the blunting reaction; this loss had no effect on the function of the construct. During construction of pRcnSS: After digestion of pBS_KS+ with BamHI, a "G" residue was lost during blunting reaction; this loss had no effect on the function of the construct. During construction of the C-IV cassette (pC-IV_TRE-tTA): After digestion of pSHCB_tTA with NotI, a "C" residue was lost during the blunting reaction; this loss had no effect on the function of the construct.

During construction of ADMM, the most stable constructs often had the PI-PspI site inserted in duplicate; the reason for this is unknown. During digestion of 5'-CTCGAGCTAGCTCGAG-3' containing sequences (pBS_rN and pBS_rN_TRE) with XhoI, only one of the XhoI sites was digested; the reason for this is unknown. During construction of the C-IV cassette (pC-IV_TRE-tTA), insertion of GAAGTTCCTATACTTTCTAGAGAA TAGGAACTTC is not necessary and can be omitted.

EVE

EVE is based on Adeno-X which is a replication incompetent, ΔE1/ΔE3, human adenoviral type 5 genome (Mizuguchi and Kay, 1998). Successful generation of helper adenovirus-dependent vectors (ADMM) requires removal of the helper virus (EVE) from the ADMM preparation. To achieve this goal, we modified the packaging site of EVE, inserted loxP sites on the 5' and 3' sides of the packaging site, and inserted a regulatable expression cassette based on the Q-mateTM Inducible Expression System which expresses Cre recombinase and enhanced green fluorescent protein (EGFP).

Due to patients' strong immune response against adenovirus and its gene products and because helper adenoviruses do express adenovirus gene products, contamination of helper-dependent virus preparations by the helper virus is one of the limiting factors in the use of these preparations in gene therapy. The most common method of removing the helper virus is by flanking the packaging site of the helper virus with LoxP sites and propagating the helper-dependent virus in Cre recombinase expressing packaging cells (such as HEK293 cells transfected with a Cre recombinase expression cassette) infected with helper virus: The Cre recombinase excises the floxed packaging site from the helper virus and removal of the packaging site severely limits encapsidation of the helper virus into infectious particles, but the helper virus retains the ability to express all the viral proteins required for propagation of the helper-dependent virus and its encapsidation into infectious particles (since the packaging site of the helper-dependent virus is not flanked by LoxP sites, its packing site is not removed by Cre recombinase). The expression of high levels of Cre recombinase, however, is detrimental to most cells, resulting in chromosomal aberrations (Loonstra et al., 2001). Therefore, we constructed EVE to express Cre recombinase. This allows high levels of Cre recombinase to be expressed during propagation of ADMM, resulting in efficient removal of EVE from ADMM preparations, but the packaging cell is not damaged prior to propagation of ADMM.

During the later stages of infection adenoviruses express proteins which inhibit Cap-dependent translation (Cuesta et al., 2000; Cuesta et al., 2004), but translation of adenoviral gene products continues via ribosomal binding to sequences in adenoviral mRNA known as leader sequences (Xi et al., 2004). Therefore, we constructed EVE such that the mRNA for Cre recombinase contained the adenoviral E2 late leader sequence.

Since EVE expresses Cre recombinase and also has its packaging site flanked by LoxP sites, Cre recombinase expression by EVE must be off when EVE is being propagated. We chose the Q-mateTM Inducible Expression System (Krackeler Scientific, Inc.) to regulate Cre recombinase expression by EVE. Therefore, we constructed EVE with a CymR binding site between the CMV promoter and the Cre recombinase sequence. When EVE is propagated in CymR expressing cells, CymR binds to the CymR binding site contained in EVE and blocks CMV driven transcription of Cre recombinase, keeping the packaging site of EVE intact and allowing EVE to become encapsidated into infectious particles.

EVE was constructed such that EVE contamination of ADMM preparations could be monitored using EGFP. This was done by inserting an internal ribosomal entry site (IRES) followed by an ORF coding for EGPF downstream of the Cre recombinase ORF. Infection of cells which do not express CymR by EVE results in expression of EGFP.

Recombination between EVE and ADMM can remove LoxP sites from EVE resulting in encapsidation of EVE. To reduce recombination between EVE and ADMM, we attempted to create a packaging site in EVE that contained less DNA than the packaging site in ADMM. Initially we generated a wild type packaging site and 3 different variant packaging sites with portions of the packaging sequence deleted. The variants were generated by amplification of the wild type packaging site sequence with the following primer pairs: Pack-Δ6, 5'-GTCCCATTCGCCATTCAG G-3' and 5'-GGATCCGCGGCCCTAGACAAATA-3'; Pack-Δ5, 5'-GTCGAGGTGCCGTAAAGCA-3' and 5'-GGATCCACGCGCTATGAGTAACACAAAA-3'; and Pack-Δ3, 5'-GTCCCATTCGCCATTCAGG-3' and 5'-GGATCCGGCCAAATCTTACTCGGTTAC-3' (constructs not shown). The EVEs containing Pack-wt, Pack-Δ6, and PackΔ5 were able to propagate in the HEK293_CymR packaging cell line, but the EVE containing Pack-Δ3 did not propagate in the HEK293_CymR packaging cell line (data not shown). Therefore, the EVE described in this manuscript contains PackΔ5.

When propagating plasmids containing both a floxed packaging site and the Cre Recombinase coding region in *E. coli*, it is imperative that the *E. coli* be grown at

28˚C or less. Also, the cultures should be harvested while they are still in the log phase of growth. Finally, after harvesting, the plasmids must be screened to confirme that the packaging site has not been lost.

ADMM

We have found that plasmids containing certain sequences are extremely difficult to propagate in E. coli. One possible explanation is that these sequences code for toxic peptides. We, therefore, inserted sequences which we call STOP sequences into four plasmids (generating pSHCB_STOP, pSHPB_STOP, pSHcnP_STOP, and pRcnSS: all based on pShuttle2) and used these plasmids as recipients of hard-to-propagate DNA sequences. The STOP sequences were obtained from human and mouse DNA; they contain a number of ATG codons followed by STOP codons, making translation of downstream ORFs a rare event.

A STOP sequence is incorporated into the ADMM described here (ADMM-H_Tight-LacZ) just downstream of the packaging site. This is because the adenovirus E1 promoter overlaps the packaging site sequence (Kovesdi et al., 1987), indicating that the packaging site itself can have gene promoter activity.

Enhancer-blocking insulators block interaction between a promoter and distal enhancer elements, and CCCTC-binding factor (CTCF) is the major protein implicated in establishment of insulators in vertebrates (Gaszner and Felsenfeld, 2006; Wallace and Felsenfeld, 2007). We scanned the chicken genome and the rat GSTP locus for putative CTCF binding sites (Kim et al., 2007; Xie et al., 2007). Intergenetic sequences with putative CTCF binding sites were used as stuffer DNA in ADMM-H_Tight-LacZ. Only Ins57-111s has no putative CTCF binding sites: The putative CTCF binding sites in Ins57-111 were lost after shortening Ins57-111 to generate Ins57-111s.

Stuffer DNA is also used to constrain the size of ADMM constructs. Type 5 human adenoviruses are not efficiently encapsidated if they are less than approximately 75% or more than approximately 105% of the wild type genome length (Bett et al., 1993; Parks and Graham, 1997): the genome length of type 5 human adenoviruses is approximately 36 kb. The length of ADMM-H_Tight-LacZ is approximately 33,945 bp; note that this ADMM construct itself has not been sequenced in its entirety: during construction of ADMM-H_Tight-LacZ, all newly cloned DNA was completely sequenced, however, in the later stages of construction, only ligation junctions were sequenced and restriction digestion was used assess the constructs.

ADMM-H_Tight-LacZ consists of 2 inverted terminal repeats (ITRs), a packaging site, and four cassettes: a LacZ expressing cassette, an HSV-TK expressing cassette, a large stuffer cassette, and a tTA expressing cassette. Digestion with PacI removes the entire ADMM-H_Tight-LacZ sequence from the carrier plasmid, resulting in a sequence which can propagate and be encapsidated into infectious adenovirus type 5 particles when transfected into packaging HEK293 cells which have been infected with EVE.

The tTA expressing cassette is modeled on the tetracycline responsive system originally developed by Hermann Bujard (Freundlieb et al., 1999). In ADMM-H_Tight-LacZ, the transcriptional activator tTA is itself under the control of the tetracycline responsive element (TRE), therefore, once tTA begins to be expressed in the absence of tetracycline, a positive feedback loop is established resulting in high expression levels of tTA. These high levels in turn result in robust expression of LacZ and HSV-TK, which are under the control of another tTA/tetracycline responsive element TRE-Tight. Because of these high expression levels, ADMM-H_Tight-LacZ is propagated in the presence of tetracycline (data not shown).

Importantly, ADMMs are built of a series of cassettes, and these cassettes can be changed. Therefore, different pathways can be targeted. For example, by utilizing different promoters and different positive and negative feedback loops, ADMM activity can be targeted to cells which do not express p53 (most human tumors) or cells which overexpress glutathione S-transferases (in the case of tumors resistant to chemotherapy), thereby specifically targeting ADMM activity to different kinds of tumors. One possibility is that libraries of different cassettes could be maintained. In this case, ADMM constructs able to respond to individual tumor types could be assembled in as little as a few weeks, making ADMM a practical cancer therapy given today's technology.

Propagation of EVE and ADMM in vitro

EVE is propagated in HEK293 cells expressing the cumate repressor protein CymR: CymR represses expression of Cre recombinase by the helper virus, allowing the helper virus to propagate normally.

ADMMs are propagated in HEK293 cells (not expressing CymR) which are also infected with helper virus. In the absence of CymR expression, Cre recombinase is expressed by EVE and catalyses excision of the packaging site out of the EVE genome. Consequently, the helper virus is not efficiently encapsidated and, therefore, not purified along with ADMM. However, EVE without its packaging site is still able to express all of the viral proteins required for propagation of ADMM and its encapsidation into infectous particles (data not shown). EGFP expressed by EVE is used to help monitor helper virus contamination of ADMM preparations.

Naming Conventions

In addition to ADMM-H_Tight-LacZ, we have generated ADMMs which express human delta-lactoferrin (ΔLF) (Mariller et al., 2007; Siebert and Huang, 1997) under the control of the human actin promoter. In this construct, the TRE-Tight-HSVTK sequence was removed from cassette pC-II by digestion of pC-II_Tight-HSVTK with SalI followed by recircularization, and the TRE-tTA was removed from pC-IV by digestion of pSHCS_Ins54_TRE-tTA-pA with NheI and PacI followed by blunting and recircularization. (Also, in the ΔLF construct, the TRE-tight promoter was replaced with the human actin promoter.) This construct is named pADMM-

54_AP-ΔLF. We also have a series of constructs in which the TRE-Tight-HSVTK sequence was removed from cassette pC-II_HSVTK, but cassette pC-IV_TRE-tTA was not altered. These ADMMs are named pADMM-53_XXX: for example, pADMM-53_LacZ. Finally, we constructed an ADMM in which the TRE-Tight-[downstream ORF] sequence has been removed from cassette pC-I, but the C-II, C-III, and C-IV cassettes were not altered. This construct is named pADMM-52_Tight-HSVTK. In sum, we have four series of pADMMs which we named the pADMM-52, pADMM-53, pADMM-54, and pADMM-H series.

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