Title: Robust, reversible gene knockdown using a single lentiviral shRNA vector

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Abstract

Manipulation of gene expression is an invaluable tool to study gene function *in vitro* and *in vivo*. The application of small inhibitory RNAs to knock down gene expression provides a relatively simple, elegant, but transient approach to study gene function in many cell types as well as in whole animals. Short hairpin structures (sh-RNAs) are a logical advance as they can be expressed continuously and are hence suitable for stable gene knockdown. Drug-inducible systems have now been developed; however, application of the technology has been hampered by persistent problems with low or transient expression, leakiness or poor inducibility of the short hairpin and lack of reversibility. We have developed a robust, versatile, single lentiviral vector tool that delivers tightly regulated, fully reversible, Doxycyline- responsive knockdown of target genes (FOXP3 and *MYB*) using single short hairpin RNAs. To demonstrate the capabilities of the vector we targeted FOXP3 because it plays a critical role in the development and function of regulatory T cells. We also targeted MYB because of its essential role in haematopoiesis and implication in breast cancer progression. The versatility of this vector is hence demonstrated by knockdown of distinct genes in two biologically separate systems.

Introduction

Conditional gene knockdown is a powerful tool both to study gene function and as a potential therapeutic. Systems for achieving this are constantly evolving. Short hairpin RNAs (shRNAs) provide an elegant and relatively straightforward approach for post-transcriptional down regulation of gene expression [1], because unlike siRNA these can be stably expressed within the cell, and are not lost with cell division. Drug-inducible activation of short hairpin RNAs provide a more finely tuned approach to gene knockdown, avoiding the artefacts that may result from constitutive gene knockdown [2]. There are still problems however in achieving effective and regulated knock-down by sh-RNAs, associated particularly with poor inducibility of the short hairpin[3], insufficient transcriptional activity of cell specific promoters [4], self-targeting of the shRNA sequence [5, 6], transient knockdown of the target gene, leakiness of short hairpin activity in the un-induced state [7, 8] and lack of reversibility [9].

Lentiviral vectors are an appealing vehicle with which to deliver genes or shRNAs, particularly because they are capable of transducing most non-dividing or quiescent cells [10]. This is important for transfer into haematopoietic cells or other primary human cell types which are typically refractory to transfection and transduce inefficiently with other virus types [11-13]. In addition, lentiviral vectors offer several advantages over other viral vectors: they are more efficient at delivering complex gene expression cassettes compared with other retroviral vectors [14], they can mediate long term gene expression [15] and are relatively safe.

The biological targets used to validate the vectors for this study were chosen as they are of clinical significance in autoimmunity and cancer. In the first example we focussed on the transcription factor FOXP3, the master transcription factor of regulatory T cells. Regulatory T cells (Tregs) are essential for normal immune homeostasis, balancing prevention of

autoimmunity and immune pathology on the one hand, whilst simultaneously allowing effective immunity to infection and tumours on the other. Many now regard these cells as "master regulators" of immune responsiveness[16]. The forkhead transcription factor, FOXP3, is expressed constitutively in 'natural' CD4+ Treg cells and has been shown to be necessary and sufficient for mouse Treg cell function [17-19] [20, 21]. Less is known about the role human FOXP3 plays in the development and function of the Treg cell (reviewed by [22]).

In order to demonstrate the robustness of the vector, we also chose a second target, the *MYB* oncogene, which encodes a transcription factor (MYB). MYB is predominantly associated with normal and leukemic haematopoiesis based on its pattern of expression [23, 24] the consequences of loss of expression [25] [26] and its ability to induce leukemogenesis in several species (reviewed in [27]). MYB is also expressed at relatively high levels in at least two epithelial tumors: colon [28] and breast cancer [29]. Evidence of a causal role in carcinogenesis includes the correlation of expression level with poor prognosis [30] and the presence of mutations that are likely to dysregulate MYB expression [31, 32].

We describe here a lentiviral system able to knockdown target genes reversibly using Doxycycline (Dox) induction. This system differs from previously described vectors as it combines exquisite sensitivity to Dox with very fast kinetics, efficient induction of shRNA expression and reversibility in a single lenti-vector. Vectors described in prior work [2, 7, 8, 33, 34] do not combine all of these characteristics in a single vector. We have used two biologically distinct systems to model the robust gene silencing capability of this vector.

Materials and Methods

Vector construction

The third generation lentiviral vectors containing the human hepatitis virus PRE element and the HIV cPPT element (described in Barry *et al*) were used for all experiments. For gene over expression the backbone was re-engineered to contain the elongation factor 1alpha promoter (plvEIG), a gateway cloning site (att, CAT ccdB att), and an IRES to drive the marker gene (GFP). To generate the shRNAi delivery vector (plvTsh), this backbone was engineered to contain a gateway cassette (att, CAT ccdB att) upstream of the rev responsive element into which the shRNAi cassette is recombined. The regulatory protein (Trex™) was inserted under the control of the EF1alpha promoter, and the marker gene (GFP) is expressed from an IRES.

Short hairpin expression constructs

Five short hairpin (sh-RNA) sequences predicted to knockdown FOXP3 expression were designed using BLOCK-iT™ RNAi Designer (Invitrogen Corp. Carlsbad, CA, USA). The following FOXP3 sequences were chosen as potential shRNA targets and were numbered with respect to FOXP3 cDNA numbering:

1355: GAGTCTGCACAAGTGCTTTGT

485: GCCACATTTCATGCACCAGCT

1289: CACACGCATGTTTGCCTTCTTC

767: GCTGGCAAATGGTGTCTGCAA

981: GCACTGACCAAGGCTTCATCT

Using the above target sequences, sh-RNA constructs were generated in a sense-loop-antisense expression format, using a 9 mer: TTCATGAGA to form the loop. A 3-step PCR procedure was used to generate the shRNA targeting sequences. PCR primers were designed

to generate 2 PCR products: a 5′end with the *att*B1 site, tH1 promoter, sense strand of the stem structure and the loop, and a 3′ end with the loop, antisense strand of the stem structure, termination signal and *att*B2 site. In step 1, a 5′ PCR product was generated using pBS-tH1, containing the tet-H1 promoter as template DNA, an *att*B1 forward primer and a site-specific reverse primer (see below for primer sequences). In step 2, a 3′ PCR product was generated using a site-specific forward primer and an *att*B2 reverse primer (see below for primer sequences). In this reaction the forward and reverse primers overlap and no template DNA was required. Products from these two reactions were subsequently gel purified (Qiaquick Gel Extraction Kit, Qiagen GmbH, Germany), quantitated, combined and used as the template in a third PCR reaction using the *att*B1 forward primer and the *att*B2 reverse primer. The final PCR product was gel purified and inserted into the pDonr107 entry vector as described below.

Forward (5') and Reverse (3') Primers used to generate shRNAs:

*att*B1 Forward Primer: GGGGACAAGTTTGTACAAAAAAGCAGGCTT

*att*B2 Reverse Primer: GGGGACCACTTTGTACAAGAAAGCTGGGTA

1355 Forward Primer: TGTTTCATGAGAACAAAGCACTTGTGCAGA

1355 Reverse Primer: TGTTCTCATGAAACAAAGCACTTGTGCAGA

485 Forward Primer: GCTTTCATGAGAAGCTGGTGCATGAAATGT

485 Reverse Primer: GCTTCTCATGAAAGCTGGTGCATGAAATGT

1289 Forward Primer: GAATTCATGAGAAAGACTTCTTCCGTTTGT

1289 Reverse Primer: GAATCTCATGAAAAGACTTCTTCCGTTTGT

767 Forward Primer: CAATCTCATGAATTGCAGACACCATTTGCC

767 Reverse Primer: CAATTCATGAGATTGCAGACACCATTTGCC

981 Forward Primer: TCTTTCATGAGAAGATGAAGCCTTGGTCAG

981 Reverse Primer: TCTTGTCATGAAAGATGAAGCCTTGGTCAG

The Gateway® Technology (Invitrogen Corp.) universal cloning system was used for the transfer of short hairpin expression cassettes (FOXP3 and sh-β-Catenin) and FOXP3 cDNA into expression vectors. Short hairpin cassettes were inserted into a Gateway® (Invitrogen Corp.) compatible entry vector (pDonr) using Gateway® BP clonase recombination techniques and transferred from the entry clone to the Gateway® (Invitrogen Corp.) compatible lentiviral destination vector (plv), using Gateway® LR clonase recombination techniques. FOXP3 lentiviral expression vectors were generated in a similar fashion, inserting FOXP3 cDNA into lentiviral destination vector plvEIG. All constructs were sequenced (Big Dye® Terminator Cycle Sequencing Kit v3.1, Applied Biosystems) prior to use in experiments.

Cell Culture

HEK293T cells were routinely cultured in D-MEM (Gibco, Invitrogen), 10% FCS (JRH Biosciences Inc. Lenoxa, KA, USA), 1% pen/strep, 1% L-Glutamine, and used between passage 11-20. Jurkat cells were routinely cultured in RPMI (SAFC Biosciences, Lenoxa, KA,USA), 10% FCS (JRH Biosciences Inc. Lenoxa, KA, USA), 1% pen/strep, 1% L-Glutamine and used between passage 15-20. For transient transfection of cells for Dual Luciferase® Reporter Assays, HEK293T cells were seeded at a density of $1x10^5$ cells in 500µl of medium (DMEM/10%FCS/1% pen/strep, 1% L-Glutamine) in a 24 well plate and transiently transfected with 25ng psi-check2™ vector and 775ng shRNA vector 4hr later using Lipofectamine™ 2000 reagent (Invitrogen Corp.) and Opti-MEM® (Invitrogen Corp) reduced serum medium, following the standard protocol. For transient transfection of Jurkat cells for Dual Luciferase® Reporter Assays, cells were seeded similarly in 500µl of medium (RPMI 10%FCS/1% pen/strep 1% L-Glutamine) and transiently transfected using

Lipofectamine™ LTX reagent (Invitrogen Corp.) using Opti-MEM® (Invitrogen Corp) reduced serum medium. All transfections were carried out in triplicates.

MCF7 cells, a human transformed malignant breast cancer line, cultured in RPMI transduced with the shMyb or shSCR scrambled trigger sequences were generated using the same protocols as described in [35]. GFP+ve cells were enriched by cell sorting. Transduced cells were cultured in the presence or absence of Dox (1µg/ml) for 48 hr unless otherwise stated.

Isolation and in vitro expansion of cord blood T cell populations

Cord blood was obtained with informed maternal consent as approved by the Children's, Youth and Women's Health Service Research Ethics Committee. Mononuclear cells (MNC) were isolated from 60-100ml venous blood collected directly post partum into pre-weighed blood collection bags (Fenwell) containing anticoagulant as previously described [36]. Cord blood CD4+ CD25+ (Treg) and CD4+ CD25- (Thelper) cells were isolated from MNC using a Dynabeads Regulatory CD4+CD25+ T cell kit (Invitrogen). The purity for each cell type was routinely greater than 90% by two colour flow cytomety for CD4 and CD25 expression. Cells were routinely expanded for 8 days in the presence of Dynabeads CD3/CD28 T cell expander beads (Invitrogen; Cat# 111-41D) at a bead to cell ratio of 3:1, prior to magnetic removal of the beads. *Ex vivo* expansion of isolated T cell populations ($1x10^6$ cells/well in a 24 well plate) were performed in X-Vivo 15 media (BioWhitticker) supplemented with 20mM HEPES-, pH 7.4, 5% heat inactivated pooled human serum (Lonza), 2mM l-glutamine and 500U/ml recombinant human interleukin-2 (rhIL2; R&D research)

Proliferation assays

MCF7 cells previously transduced with plv-T-sh-Myb or plv-T-sh-SCR were cultured in triplicate in the presence or absence of Dox for 12 days. Cell proliferation was measure by

cell counting using a Coulter counter. Replicate samples were also treated to washout the Dox from day 5. In one group of replicates of the sh-Myb transduced cells, Dox was washed out at day 5 and added back at day 7. Western blotting was also carried out as described in [35].

Lentiviral Packaging

For generating lentiviral stocks, HEK293T cells were seeded at a density of $6x10^6$ cells in 18ml medium in a $75cm^2$ tissue culture flask. Cells were transfected with 12.5 µg transfer vector (pvlEIG), 7.5 µg Gag/Pol (Δ 8.2), 6.25 µg Rev (pRSV-Rev), 3.75 µg Env (pCMV-VSV-G) using Lipofectamine™ 2000 reagent (Invitrogen) and using Opti-MEM® (Invitrogen Corp) reduced serum medium, following the standard protocol. The following day, the medium was replaced with 10ml fresh D-MEM and 48hr later supernatant was collected, filtered (0.45µm) and used immediately or stored frozen (-70°C). Virus was concentrated by unltracentrifugation at 22,000RPM for 90min at 4°C. The supernatant was aspirated and the pellet resuspended in 1/25 vol/vol of X vivo medium to give a 25x concentrated stock. Virus was titred by limiting dilution, as described previously [12]. Routinely we achieved titers of $5x10^5$-$1x10^6$ transducing units per ml

Lentiviral Transduction

For transduction of cell lines, cells were routinely seeded at a density of $3x10^5$ cells per well in a 6 well plate. Medium was replaced with 3ml viral supernatant (unconcentrated) containing polybrene at a final concentration of 5 µg/ml. GFP positive cells were sorted by flow cytometry (Beckman Coulter FACSCalibur). For transduction of primary human cells 1-$3x10^5$ $CD4^+$ $CD25^+$ T reg cells were transduced with lentivirus at a multiplicity of infection (MOI) of approximately 20 24-48hr after isolation. Cells were expanded for 8 days in the

presence of Dynabeads prior to magnetic removal of the beads and isolation of GFP^+ cells and GFP^- cells by flow cytometry.

Intracellular staining

For detection of FOXP3 expression, cells ($1x10^5$) were washed in flow cytometry staining buffer (0.1% sodium azide, 1% FCS, in phosphate buffered saline) and stained intracellularly with phycoerythrin- (PE) conjugated anti-human FOXP3 antibody (PCH101, eBioscience, Inc) using fixation/permeabilisation and permeabilisation reagents (eBioscience, Inc) and standard protocols. Cells were resuspended in fixation buffer (1% formaldehyde, 2% glucose, 0.02% sodium azide in phosphate buffered saline) and analysed by flow cytometry (Beckman Coulter FACSCalibur).

Purification of DNA and RNA

DNA was prepared using Qiaprep® Spin Miniprep Kit (Qiagen GmbH, Germany) and HiSpeed® Plasmid Midi Purification Kit (Qiagen GmbH, Germany). RNA was prepared using miRNeasy mini kit (Qiagen GmbH, Germany).

Analysis of RNA expression by real-time PCR

RNA was reverse transcribed by Quantitect RT kit (Qiagen) Real Time PCR (RT-PCR) was performed on a Rotor-Gene 6000 (Corbett) using FastStart *Taq* DNA polymerase kit (Roche) and SYBR Green fluorescent dye. Transcription factor mRNA expression profiling was performed at various time points as described in the figure legends, and cells were analysed for relative abundance of FOXP3 compared to an internal control (Beta Actin).

FOXP3F: GAA ACA GCA CAT TCC CAG AGT TC

FOXP3R: ATG GCC CAG CGG ATG AG

Beta Actin F: AAG AGC TAC GAG CTG CCT GAC

Beta Actin R: GTA GTT TCG TGG ATG CCA CAG

Stem-loop Reverse Transcription-Polymerase Chain Reaction (SL-RT-PCR)

Reverse transcriptase (RT) reactions were performed on 150ng of total RNA. First strand complementary DNA (cDNA) was synthesized using 0.05μM of stem loop primer (SL-sh1355) (5'-GTTGGCTCTGGTAGGATGCCGCTCTCAGGGCATCCTACCAGAGCCAACACAAAG-3'), 2μM of antisense primer for the reference gene hypoxanthine phosphoribosyltransferase (*Hprt-1)* (5'-CAGGCAAGACGTTCAGTCCT-3'*)* using the Superscript™ III Reverse Transcriptase Kit (Invitrogen) according to manufacturer's protocol. SL-sh1355 contains a binding site for a universal reverse primer (URevP; 5'-GTAGGATGCCGCTCTCAGG-3'), a binding site for a Universal Probe Library (UPL) probe #21 (Roche Diagnostics) and a six base 3' overhang sequence, which is complementary to the last six bases at the 3' end of sh1355. Pulse RT was carried out using previously described conditions with modifications [37, 38]. Briefly, RNA was initially incubated at 65°C for 5 minutes to uncoil the RNA. cDNA synthesis was then performed at 16°C for 30 minutes followed by 60 cycles of 20°C for 30 seconds, 42°C for 30 seconds and 50°C for 1 second. Final incubation at 75°C for 15 minutes was performed to inactivate the reaction. Additional RT minus (without reverse transcriptase) reactions served as negative control. For quantitative PCR, a 20μl reaction containing 0.005x synthesized cDNA (from either RT positive or minus reaction), 1x LC480 Master Probe Mix (Roche Diagnostics), 250nM of forward (sh1355 forward primer 5'-CGGCCGAGTCTGCACAAGTG-3', *Hprt-1* forward primer 5'-TGACCTTGATTATTTTGCATACC-3') and reverse primers (URevP, *Hprt* antisense primers) (GeneWorks Pty Ltd, Aus.), and 100nM of UPL probe #21 (for sh1355), #73 (for *Hprt1*) (Roche Diagnostics), was prepared for each sample. PCR reactions were performed using the LightCycler® 480 System (Roche Diagnostics) with a pre-denaturing step of 95°C

for 10 minutes, and 45 cycles of 95^{o}C (10 seconds), 60^{o}C (30 seconds) and 72^{o}C (10 seconds) followed by a cooling step at 40^{o}C for 1 minute. Semi-quantitative PCR was performed using the exact reaction setup and conditions for only 34-36 cycles without the presence of UPL probe. Cycle threshold value (Ct) was calculated from the amplification curve using Second Derivative Maximum method [39] provided in LightCycler® 480 Quantification Software version 1.5 (Roche). To compare the Ct value with gel electrophoresis results, we normalized the value to *Hprt*-1 using the comparative Ct method.

Dual Luciferase® Reporter Assays.

For luciferase assays, FOXP3 and β-Catenin cDNAs were inserted into the multi-cloning site of psiCHECK™-2 (Promega Corp. Madison, WI) vector. PsiCHECK™-2 is a dual luciferase reporter construct in which the cDNA of interest is cloned into the 3`UTR of the Renilla luciferase gene. Renilla luciferase activity is normalised with respect to Firefly luciferase activity, which is carried on the same vector to control for transfection efficiency. All constructs were sequenced (Big Dye® Terminator Cycle Sequencing Kit v3.1, Applied Biosystems) prior to use in experiments. Cells were transiently transfected as described above, before incubation in the absence or presence of Doxycyline (1µg/ml). Subsequently, culture medium was removed and cells were lysed using Dual Luciferase® Reporter Assay reagents (Promega Corp Madison, WI) following the standard protocol. Samples were assayed using Luciferase Assay Reagent II (LAR II) and Stop & Glo® reagents and analysed using a Veritas Micro Plate Luminometer (Turner Biosystems).

Results

A single lentiviral vector to conditionally knockdown gene expression.

A lentiviral vector was designed to achieve tightly regulated, drug inducible knockdown of target gene expression. Short hairpin RNA expression cassettes generated by PCR are inserted into the expression vector via donor vector intermediaries using Gateway® technology. The shRNA sequence is expressed from the tet operator-H1 promoter fusion (tH1) in either donor vectors for validation, (pDONR) or transfer vectors for lentiviral delivery (plv-T-sh). The TRex repressor cassette transcribed from an EF1α promoter is included in the LV backbone thereby delivering all the components required for tetracycline-induced de-repression of sh-RNA transcription in one vector. An internal ribosome entry site (IRES) downstream of the TRex cassette is inserted for expression of the marker gene GFP (**Fig. 1a**).

Cloning and screening of short hairpin RNAs

Construction of shRNA triggers as cassettes with the tet operator-H1 promoter fusion (tH1), facilitates the rapid screening for functional shRNA molecules in the pDONR vector prior to their transfer into the LV backbone. A panel of five 21-mer short hairpin sequences were designed to knockdown expression of human FOXP3 (Invitrogen BLOCK-iT RNAi designer). The gateway adaptor tailed tH1-shRNA cassettes generated by a two-step PCR reaction (**Fig 1b**) were inserted into a pDONR vector and sequenced. To screen for functional shRNA triggers, HEK293T cells were transiently co-transfected with vectors constitutively expressing the short hairpin RNAs (pDONR-shRNA) and the reporter vector Psi (ψ) check 2™, Promega Corp containing FOXP3 (Psi-check-FOXP3). Functional shRNA triggers were then identified by a reduction in Renilla activity from the Psi-check-FOXP3 reporter. To confirm on-target specificity of our short-hairpin sequences, a control β-catenin reporter

vector (Psi-check-β-cat) and a validated β-catenin short hairpin RNA (sh-β-cat) [40] were used in all experiments.

Screening of the predicted FOXP3 shRNA targeting sequences identified one trigger, sh-1355, that substantially reduced (≈ 40% knockdown) renilla activity of the Psi-check-FOXP3 reporter compared with cells transfected with the reporter alone. No significant reduction in renilla activity was observed in cells co-transfected with Psi-check-FOXP3 and the sh-485, sh-767 or sh-981 expression vectors, while sh-1289, intriguingly, led to increased FOXP3 expression (60% higher) (means of triplicate samples and representative of >3 experiments) (**Fig 2a**). On target specificity was confirmed in cells co-transfected with the β-catenin reporter vector (Psi-check-β-catenin) and the short-hairpin β-catenin (sh-βcat), where expression was significantly reduced (80% knockdown) compared with cells transfected with Psi-check-β-catenin alone. In contrast, no significant knockdown of renilla activity was observed in cells co-transfected with sh-1355 and the Psi-check-β-catenin reporter vector or in cells co-transfected with sh-βCat and Psi-check-FOXP3 reporter. This screening strategy allowed rapid identification a single short hairpin RNA sequence (sh-1355) able to specifically knockdown expression of FOXP3.

Conditional regulation of target genes

Next, we wished to demonstrate Doxycyline-regulated knockdown of our target genes. HEK 293T cells transduced with plvTsh-1355 and plvTsh-βcat were then transiently transfected with the Psi-check reporter constructs (Psi-check-FOXP3 or Psi-check-βcatenin) and Doxycyline (Dox) was added (1μg/ml) to de-repress shRNA transcription. Knockdown of the reporter genes was monitored by luciferase assay after 48hr. Efficient and specific Dox-dependent knockdown of the reporter gene activity was observed for both FOXP3 and β-catenin (**Fig 2b**). In the absence of Dox, cells transduced with LV-T-sh-1355 virus and

transfected with FOXP3 reporter vector (Psi-check-FOXP3) showed levels of renilla activity comparable with that in cells transfected with the Psi-check-FOXP3 alone (data not shown). However, addition of Dox (1μg/ml) substantially reduced FOXP3 expression (>70% knockdown) (**Fig. 2b**). In contrast, no change in renilla activity was observed from the β-catenin expression construct in cells transduced with plv-T-sh-1355 virus either in the absence or presence of Dox, indicating that sh-1355 activity is specific. This result also indicates that addition of Dox itself had no net effect on expression of the target gene. Target specific and Dox inducible knockdown was also observed with the plv-T-shβ–cat transduced cells transfected with Psi-check-β–catenin. Similar results were observed in the Jurkat T cell line and NIH3T3 cells for the sh-βcat vector (not shown).

Lentivirally delivered FOXP3 is conditionally regulated by sh-1355 in Jurkat T cells.

The previous experiments have shown that short hairpin RNAs could down-regulate renilla activity from a hybrid mRNA containing FOXP3 or β catenin sequences within its 3′ UTR. In this mRNA the target gene sequences are not translated. We therefore wished to determine whether sh-1355 could conditionally down regulate the intact FOXP3 mRNA. To do this Jurkat cells, which do not express endogenous FOXP3, were transduced with a lentivirus expressing FOXP3 from the EF1a promoter (plvEIG-FP3) and GFP from an IRES. Cells were sorted by flow cytometry, into a population with the highest GFP^+ expression (top 5%), as compared with all GFP^+ cells (pooled population) (**Fig.3a**). We confirmed that cells with the highest GFP fluorescence also exhibited the highest levels of FOXP3 expression by intracellular staining (**Fig. 3b**), suggesting that the IRES driven reporter was a robust marker of expression of the target gene in plvEIG-FOXP3. The sorted population of plvEIG-FOXP3 cells (top5% GFP^+) were then transduced a second time (super-infected) with plv-T-sh-1355 lentivirus. As the levels of GFP in the super infected cells were significantly higher than the

single transduced cells (**Fig. 3c),** a simple gating strategy was used to analyse FOXP3 expression levels in cells with the highest MFI for GFP. Cells were treated with Dox (1µg/ml) for 48hr, stained intracellularly and then analysed by flow cytometry for FOXP3 expression. We observed that FOXP3 levels in cells transduced with both plvEIG-FOXP3 and plv-T-sh-1355 cultured in the absence of Dox was very similar to that observed in cells transduced with plvEIG-FOXP3 alone (**Fig. 4a**). However, when Dox was added to the culture medium, FOXP3 expression in the double transduced cells was significantly reduced, approaching the background levels of staining observed in cells not expressing FOXP3 (**Fig. 4a**). This result indicated that plv-T-sh-1355 down regulated plvEIG-FOXP3 in a Dox-dependent manner in Jurkat populations expressing high levels of FOXP3. Real time PCR confirmed that FOXP3 mRNA expression was similarly down regulated **(Fig. 4b**). The comparable levels of FOXP3 observed in cells transduced with plvEIG-FOXP3 and cells transduced with both plvEIG-FOXP3 and plv-T-sh-1355 cultured in the absence of Dox, is consistent with the tight regulation of sh-1355 transcription by the Trex protein in the absence of de-repression by Dox. This was confirmed by highly sensitive stem-loop RT PCR analysis of the expression of the plvTsh1355 shRNA itself, showing no product in empty Jurkat cells or Jurkat cells transduced with plvTsh1355 in the absence of Dox, but significant sh1355 product when the Jurkats transduced with plvTsh1355 were cultured in the presence of Dox (Fig 4c).

Regulation of FOXP3 in primary human T regs

In order to test the broad scope of this lentiviral vector, we isolated primary human cord blood T regs ($CD4^+$ $CD25^+$) and transduced them with plv-T-sh-1355 viral supernatant (25x concentrated). Transduced cells (33.5% mean transduction efficiency, n=2) were then expanded *ex-vivo* for 8 days to increase cell numbers. Cells were sorted by flow cytometry

gating on GFP^+ events. GFP^+ and GFP^- cells were then cultured in the absence or presence of Dox (1µg/ml) for 48hr and subsequently stained intracellularly and analysed by flow cytometry for FOXP3 expression. We observed that FOXP3 expression in T reg cells transduced with plv-T-sh-1355 cultured in the absence of Dox, was similar to FOXP3 expression in non-transduced cells in the absence or presence of Dox (not shown). This result indicates that Dox has no non-specific effects on FOXP3 expression in non-transduced cells and also, importantly, that plv-T-sh-1355 is not leaky in the absence of Dox. When Dox was added to the culture medium of T reg plv-T-sh-1355 cells, FOXP3 expression was reduced by 36.6% (mean fluorescence intensity was reduced from 416 to 340 -/+ Dox respectively) compared with T reg plv-T-sh-1355 cells cultured in the absence of Dox (**Fig. 4d**). These results show that plv-T-sh-1355 conditionally down regulates the expression of endogenous FOXP3 in human Treg cells in the presence of Dox.

Activation of short hairpin is both sensitive and rapid.

A concentration curve to determine the kinetics of Dox induction of sh-1355 activity to knockdown expression of FOXP3 was then generated. The results of this experiment showed that the concentration of Dox routinely used, 1µg/ml, far exceeds that which led to maximal FOXP3 knockdown (between 20-40% FOXP3 remaining). At a Dox concentration of 10ng/ml FOXP3 expression was reduced to ≈ 40% of that expressed in non-Dox treated cells (**Fig. 5a**) indicating the sensitivity of the system. Likewise, using SL-RT PCR, we observed no plvTsh1355 shRNA in the absence of Dox but robust expression in the presence of just 10ng/ml of Dox, (Fig. 5b) confirming the exquisite sensitivity of the system. Activation of the short hairpin was also very rapid. In the experiments described here, cells were routinely treated with Dox for 48hrs prior to evaluating knockdown of FOXP3 expression, but we observed partial knockdown of FOXP3 as early as 12 hours after addition of Dox (68%

FOXP3 remaining) and maximal knockdown of FOXP3 protein by 24 hours 22 % FOXP3 remained, compared with non-Dox treated cells (**Fig 5c**).

Gene regulation is robust and reversible.

Having shown that target gene (FOXP3) expression can be significantly down-regulated in a Dox-dependent manner by lentiviral sh-1355, we next wanted to test the reversibility of the drug-induced knockdown of FOXP3 by performing a washout experiment. Jurkat cells expressing high levels of FOXP3 from plvEIG-FP3 and super-infected with plv-T-sh-1355 were cultured in the presence or absence of Dox (1µg/ml) for 48hr. After collecting an aliquot of cells for analysis, the remaining cells were washed and returned to culture. Over the next 120 hours, cells were collected every 24hrs or 48hrs followed by the re-addition of Dox at 120 hours. As seen previously, FOXP3 expression was reduced to ≈20% of original levels after incubation with Dox (1µg/ml) for 48 hours (**Fig.5d**). After washout of Dox the level of FOXP3 expression remained at ≈ 20% for the next 48hr, but increased at each time point thereafter until FOXP3 had returned to almost pre-knockdown levels (≈ 90%) by 120 hours post-Dox removal. This result shows that drug-induced knockdown of FOXP3 is efficient and reversible. To further test the robustness of the system, after the 120hr time point Dox (1µg/ml) was added back and the cells were incubated for a further 48hr. FOXP3 expression dropped back down to 20% of original levels (**Fig. 5d**). FOXP3 expression in cells continuously cultured in Dox for 120hr remained very low (3.8% of non-treated cells, (* on **Fig. 5d**) indicating that Dox-induced activation of the short hairpin is robust and prolonged. In all cases FOXP3 mRNA levels followed the same expression pattern (**Fig. 5e**). Over the course of the washout experiment, plvTsh1355 shRNA expression exactly mirrors expression of FOXP3. Expression of this RNA was high after 48hrs of Dox, but had diminished to near background levels after 120hrs washout. plvTsh1355 shRNA levels increased once more

when Dox was added back to the cells. These results indicate that as the Dox is washed out, plvTsh1355 is concomitantly switched off and FOXP3 expression is de-repressed (**Fig. 5f, g**).

Validation of reversible tet regulated gene ablation in breast cancer cells

To rigorously establish the utility and reversibility of the vector, we chose to target an endogenous cellular gene, the oncogene *MYB* in the breast cancer cell line MCF7. When a pre-validated *MYB* specific shRNA or a scrambled shRNA [35] were delivered stably into the MCF7 cells, no impact on viability or growth characteristics was seen in the absence of Dox, as measured in a proliferation assay. However, when Dox was added to the culture at 1ug/ml, a significant reduction in growth and proliferation was observed only in the plv-T-sh-Myb transduced cells. When Dox was left on the cells for 5 days a stable gene silencing effect was observed both at the level of protein expression and proliferation. This growth suppression was reversible, as demonstrated by washout on day 5 and restoration of growth kinetics. Furthermore in the sh-Myb line only, the growth inhibition could be re-established by a second Dox treatment on day 7 resulting in growth arrest for a second time in the same culture (**Fig. 6a**). The impact of the sh-Myb construct on MYB protein levels was confirmed by western blot analysis of the parent cell line, the cell line transduced with sh-Myb, and two controls where the cells were transduced with a scrambled shRNA (sh-SCR) or transduced with an empty lentiviral vector. In the plv-T-sh-Myb transduced MCF7 cells in the absence of Dox, the levels of Myb were comparable with the parent cells or the two controls. MYB was significantly reduced when Dox was added, and returned to control levels after washout (**Fig. 6b**).

Discussion

The main goal of this study was to build a simple, efficient system with which to study gene function, and we chose two genes to model this. Conditional gene knockdown has the potential to be a powerful tool, with short hairpin RNAs emerging as a highly effective and relatively straightforward mechanism to achieve this goal. However, for this technology to reach its full potential safe, efficient and tightly regulated delivery systems need to be further developed. A system that allows regulated control of gene expression (including full reversibility) has huge advantages over systems that rely on absolute knockout or Cre-mediated excision [9, 41] potentially overcoming issues including lethality. It also allows knock-down in the appropriate temporal- or stimulus- specific manner. Critically, developing such tools allows for the controlled manipulation of gene-expression in a variety of primary cell types and diverse organisms, rather than being restricted to mice. Additionally, for a system to be robust, particularly for an *in vivo* model, manipulation of gene expression should also be enduring.

There have been many advances in the design of gene delivery systems, with lentiviruses emerging as a relatively safe system of delivery [42]. We have based the vectors described herein on the third generation self-inactivating lentiviral vectors first described and shared by Trono *et al* [10, 43, 44] and modified by us [12]. We, and others, have demonstrated that these lentiviral vectors have improved transduction efficiency, function and specificity, particularly into primary cells [45] and are also well tolerated *in vivo* [15, 46-48] making them ideal for *in vivo* studies. We chose to combine the shRNA and regulatory elements into a single vector as we felt that it is less practical to perform multiple transductions or to generate clones when targeting rare primary cells or using *in vivo* models. By utilising Gateway® recombination technology we are able to rapidly screen potential short hairpins before the production of lentiviral particles. This is of potential benefit as there

are still persistent problems with short hairpin design, leading to inefficient knockdown of the target gene, and thus requiring a number of targeting sequences to be tested for each gene. Screening a bank of potential short hairpin RNAs in this way revealed that one sequence out of five was able to knockdown target gene expression in a highly specific way.

In this paper we describe a single lentiviral vector capable of delivering tightly regulated Dox responsive knockdown of target genes as shown by the regulated knockdown of two different genes, FOXP3 and *MYB* using single short hairpin RNAs delivered into several different cell lines (HEK293T, Jurkat and MCF7). We also demonstrate regulated knockdown of endogenous FOXP3 in primary human T reg cells. In contrast with the findings of Brake *et al,* who observed a substantial loss of shRNA activity in transduced cells [6], we observed efficient knockdown of target genes in either transfected or transduced cells.

Throughout our experiments we observed significant knockdown of target genes (FOXP3 60-95%, β-catenin 80% and *MYB* ~80% knockdown respectively) indicating efficient expression and processing of the short hairpin cassettes. This suggests that sufficient shRNA molecules can be produced from a single copy of the tet-H1 promoter shRNA cassette in the viral backbone. We obtained high levels of FOXP3 knockdown using an MOI=1 in cell lines (MOI= 25 in primary human T regs) compared with others who have had to use high viral titres or isolated cell clones to obtain robust target knockdown by short hairpin RNAs (summarised in [7]) This compares favourably with results using multiple copies of the short hairpin to achieve durable target gene knockdown [5, 6]. Significantly, the short hairpin RNA delivery vector plv-T-sh described here appears not to be susceptible to inactivating mutations [5] as demonstrated by the finding that MCF7 transduced with a lentiviral construct encoding a validated *MYB* shRNA Gonda et al [35] retains the ability to knockdown *MYB* oncogene levels even after prolonged culture in the absence of Dox.

Importantly, in the absence of Dox the tet-H1 promoter appears to be tightly regulated by the repressor protein, with little evidence of leakiness. Not only was FOXP3 expression in Jurkat cells transduced with plv-T-sh-1355, when cultured in the absence of Dox, indistinguishable from that in parental cells expressing FOXP3 alone, but importantly plv-T-sh-1355 RNA was only detected (using the highly sensitive SL-RT-PCR technique) in cells treated with Dox. Similar observations were seen in MCF7 cells transduced with plv-T-shMyb and importantly in the primary human Treg cells transduced with plv-T-sh-1355 to knockdown endogenous FOXP3. This has been a hurdle in the development of inducible short hairpin RNA technologies where leakiness of shRNA expression has been observed in both transfected and transduced cells [8].

In experiments examining the kinetics of shRNAi induction, we show that this knockdown is both sensitive and rapid. For example, plv-T-sh-1355 RNA was induced using just 10ng/ml Dox and FOXP3 protein expression itself could be reduced in the presence of 10ng/ml Dox, 100x lower concentrations of Dox than routinely used, with ≈60% knockdown of FOXP3 obtained and maximal repression occurring from 25ng/ml of Dox onwards. This may be useful for *in vivo* experiments where systemic administration of Dox is required. Secondly, following induction by Dox, de-repression of the Tet-H1 promoter-shRNA cassette, initiation of transcription and processing of the short hairpin must be rapid, since we observe partial knockdown of FOXP3 protein as early as 12 hours (68% FOXP3 remaining) and maximal knockdown of FOXP3 by 24 hours (22 % FOXP3 remaining) compared with non-Dox treated cells. This compares favourably with other recently developed systems in which prolonged exposures to Dox (5 and 8 days) were required for maximal knockdown at the protein level [2, 8]. One of the major strengths of our system is that compared with those of others, our system is fully and quickly reversible even when using high concentrations of Dox.. Additionally wash out of the Dox resulted in loss of the plv-T-sh-1355 RNA with the

concomitant gain of FOXP3. Others show reversibility but this is often incomplete or relatively slow and insensitive [33, 34].

The vector described in this paper provides a simple, single vector system that is quickly and strongly inducible and additionally, fully reversible. We have carried out carefully analysed kinetics quantitating all of our results with respect to appropriate controls to show convincingly the system described here is an advance on systems currently available. This system offers not only a valuable tool to study gene function, but also as a tool for modelling human disease, and as a potential therapeutic modality.

References

1. Brummelkamp, TR, Bernards, R, and Agami, R (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**: 550-553.
2. Szulc, J, Wiznerowicz, M, Sauvain, MO, Trono, D, and Aebischer, P (2006). A versatile tool for conditional gene expression and knockdown. *Nature methods* **3**: 109-116.
3. An, DS, Xie, Y, Mao, SH, Morizono, K, Kung, SK, and Chen, IS (2003). Efficient lentiviral vectors for short hairpin RNA delivery into human cells. *Hum Gene Ther* **14**: 1207-1212.
4. Liu, B, Paton, JF, and Kasparov, S (2008). Viral vectors based on bidirectional cell-specific mammalian promoters and transcriptional amplification strategy for use in vitro and in vivo. *BMC Biotechnol* **8**: 49.
5. ter Brake, O, t Hooft, K, Liu, YP, Centlivre, M, von Eije, KJ, and Berkhout, B (2008). Lentiviral vector design for multiple shRNA expression and durable HIV-1 inhibition. *Mol Ther* **16**: 557-564.
6. ter Brake, O, and Berkhout, B (2007). Lentiviral vectors that carry anti-HIV shRNAs: problems and solutions. *J Gene Med* **9**: 743-750.
7. Pluta, K, Diehl, W, Zhang, XY, Kutner, R, Bialkowska, A, and Reiser, J (2007). Lentiviral vectors encoding tetracycline-dependent repressors and transactivators for reversible knockdown of gene expression: a comparative study. *BMC Biotechnol* **7**: 41.
8. Aagaard, L, *et al.* (2007). A facile lentiviral vector system for expression of doxycycline-inducible shRNAs: knockdown of the pre-miRNA processing enzyme Drosha. *Mol Ther* **15**: 938-945.
9. Ventura, A, *et al.* (2004). Cre-lox-regulated conditional RNA interference from transgenes. *Proc Natl Acad Sci U S A* **101**: 10380-10385.
10. Naldini, L, *et al.* (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272**: 263-267.
11. Naldini, L, Blomer, U, Gage, FH, Trono, D, and Verma, IM (1996). Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci U S A* **93**: 11382-11388.
12. Barry, SC, Harder, B, Brzezinski, M, Flint, LY, Seppen, J, and Osborne, WR (2001). Lentivirus vectors encoding both central polypurine tract and posttranscriptional regulatory element provide enhanced transduction and transgene expression. *Hum Gene Ther* **12**: 1103-1108.
13. Salmon, P, Kindler, V, Ducrey, O, Chapuis, B, Zubler, RH, and Trono, D (2000). High-level transgene expression in human hematopoietic progenitors and differentiated blood lineages after transduction with improved lentiviral vectors. *Blood* **96**: 3392-3398.
14. May, C, *et al.* (2000). Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin. *Nature* **406**: 82-86.
15. Seppen, J, Barry, SC, Harder, B, and Osborne, WR (2001). Lentivirus administration to rat muscle provides efficient sustained expression of erythropoietin. *Blood* **98**: 594-596.
16. Tang, Q, and Bluestone, JA (2008). The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* **9**: 239-244.
17. Bennett, CL, *et al.* (2001). The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* **27**: 20-21.

18. Chang, X, *et al.* (2005). The Scurfy mutation of FoxP3 in the thymus stroma leads to defective thymopoiesis. *J Exp Med* **202**: 1141-1151.
19. Yagi, H, *et al.* (2004). Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. *Int Immunol* **16**: 1643-1656.
20. Fontenot, JD, Gavin, MA, and Rudensky, AY (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* **4**: 330-336.
21. Hori, S, Nomura, T, and Sakaguchi, S (2003). Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**: 1057-1061.
22. Li, B, and Greene, MI (2008). Special regulatory T-cell review: FOXP3 biochemistry in regulatory T cells--how diverse signals regulate suppression. *Immunology* **123**: 17-19.
23. Westin, EH, *et al.* (1982). Differential expression of the amv gene in human hematopoietic cells. *Proc Natl Acad Sci U S A* **79**: 2194-2198.
24. Gonda, TJ, Sheiness, DK, and Bishop, JM (1982). Transcripts from the cellular homologs of retroviral oncogenes: distribution among chicken tissues. *Mol Cell Biol* **2**: 617-624.
25. Mucenski, ML, *et al.* (1991). A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. *Cell* **65**: 677-689.
26. Anfossi, G, Gewirtz, AM, and Calabretta, B (1989). An oligomer complementary to c-myb-encoded mRNA inhibits proliferation of human myeloid leukemia cell lines. *Proc Natl Acad Sci U S A* **86**: 3379-3383.
27. Ramsay, RG, and Gonda, TJ (2008). MYB function in normal and cancer cells. *Nature reviews* **8**: 523-534.
28. Torelli, G, *et al.* (1987). Expression of c-myb protooncogene and other cell cycle-related genes in normal and neoplastic human colonic mucosa. *Cancer Res* **47**: 5266-5269.
29. Guerin, M, Sheng, ZM, Andrieu, N, and Riou, G (1990). Strong association between c-myb and oestrogen-receptor expression in human breast cancer. *Oncogene* **5**: 131-135.
30. Biroccio, A, *et al.* (2001). c-Myb and Bcl-x overexpression predicts poor prognosis in colorectal cancer: clinical and experimental findings. *Am J Pathol* **158**: 1289-1299.
31. Thompson, MA, Flegg, R, Westin, EH, and Ramsay, RG (1997). Microsatellite deletions in the c-myb transcriptional attenuator region associated with over-expression in colon tumour cell lines. *Oncogene* **14**: 1715-1723.
32. Hugo, H, *et al.* (2006). Mutations in the MYB intron I regulatory sequence increase transcription in colon cancers. *Genes Chromosomes Cancer* **45**: 1143-1154.
33. Herold, MJ, van den Brandt, J, Seibler, J, and Reichardt, HM (2008). Inducible and reversible gene silencing by stable integration of an shRNA-encoding lentivirus in transgenic rats. *Proc Natl Acad Sci U S A* **105**: 18507-18512.
34. Seibler, J, Kleinridders, A, Kuter-Luks, B, Niehaves, S, Bruning, JC, and Schwenk, F (2007). Reversible gene knockdown in mice using a tight, inducible shRNA expression system. *Nucleic Acids Res* **35**: e54.
35. Drabsch, Y, *et al.* (2007). Mechanism of and requirement for estrogen-regulated MYB expression in estrogen-receptor-positive breast cancer cells. *Proc Natl Acad Sci U S A* **104**: 13762-13767.
36. Bresatz, S, Sadlon, T, Millard, D, Zola, H, and Barry, SC (2007). Isolation, propagation and characterization of cord blood derived CD4+ CD25+ regulatory T cells. *J Immunol Methods* **327**: 53-62.

37. Chen, C, *et al.* (2005). Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* **33:** .
38. Tang, F, Hajkova, P, Barton, S, Lao, K, and Surani, M (2006). MicroRNA expression profiling of single whole embryonic stem cells. *Nucleic Acids Res* **34**.
39. Rasmussen, R (2001). Rapid Cycle Real-Time PCR: Methods and Applications. In: Meuer, S, C Wittwer and K Nakagawara eds). *Quantification on the LightCycler instrument*. Springer Press: Heidelberg. pp 21-34
40. van de Wetering, M, *et al.* (2003). Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. *EMBO Rep* **4**: 609-615.
41. Seidler, B, *et al.* (2008). A Cre-loxP-based mouse model for conditional somatic gene expression and knockdown in vivo by using avian retroviral vectors. *Proc Natl Acad Sci U S A* **105**: 10137-10142.
42. Bauer, G, *et al.* (2008). In vivo biosafety model to assess the risk of adverse events from retroviral and lentiviral vectors. *Mol Ther* **16**: 1308-1315.
43. Salmon, P, and Trono, D (2007). Production and titration of lentiviral vectors. *Curr Protoc Hum Genet* **Chapter 12**: Unit 12 10.
44. Dull, T, *et al.* (1998). A third-generation lentivirus vector with a conditional packaging system. *J Virol* **72**: 8463-8471.
45. Taher, TE, Tulone, C, Fatah, R, D'Acquisto, F, Gould, DJ, and Mageed, RA (2008). Repopulation of B-lymphocytes with restricted gene expression using haematopoietic stem cells engineered with lentiviral vectors. *Gene Ther* **15**: 998-1006.
46. Barry, S, Brzezinski, M, Yanay, O, Seppen, JE, and Osborne, WR (2005). Sustained elevation of neutrophils in rats induced by lentivirus-mediated G-CSF delivery. *J Gene Med* **7**: 1510-1516.
47. Brzezinski, M, Yanay, O, Waldron, L, Barry, SC, and Osborne, WR (2007). G-CSF-lentivirus administration in rats provided sustained elevated neutrophil counts and subsequent EPO-lentivirus administration increased hematocrits. *J Gene Med* **9**: 571-578.
48. Yanay, O, *et al.* (2003). Treatment of canine cyclic neutropenia by lentivirus-mediated G-CSF delivery. *Blood* **102**: 2046-2052.

Figure Legends.

Figure1.

A single lentiviral expression vector for drug-inducible knockdown of FOXP3.

(1**a**) A diagrammatic representation of the lentiviral vector design. plvEIG lentiviral vector provides the backbone for this system. The short hairpin expression cassette (Tetracycline mini operon fused to the H1 promoter followed by the target stem loop and a terminator, pT) is inserted via Gateway recombination downstream of the packaging signal. An EF1a promoter drives transcription of the TRex™ repression cassette and an IRES is inserted upstream of a GFP marker. (1**b**) Schematic representation of the PCR cloning strategy used to generate the short hairpin cassettes which are then recombined into the gateway vectors.

Figure 2.

A short hairpin sequence is able to specifically and inducibly knockdown expression of FOXP3.

(**2a**) HEK293T cells were transiently co-transfected with pDONR vectors containing one of five short hairpin sequences (sh-1355, sh-485, sh-1289, sh-767, sh-981) or control short hairpin; sh- ß catenin (sh-ß cat) and also with the FOXP3 reporter vector (▨) or ß-catenin (ß cat) control reporter (□). (**2b**) HEK293T cells were transfected with ß-catenin control reporter vector and transduced with plvtsh-βcat (□), or with plvTsh-1355(⊡) or transfected with the FOXP3 reporter vector and transduced with plvtsh-1355 (▨). Cells were then incubated – or + Dox. Chart labels denote the vector with which the cells are transfected (abbreviated to FOXP3 or ß-cat), followed by the lentiviral vector (abbreviated to sh-1355 or sh- ß-cat). Results are mean +SD expressed relative to the firefly luciferase control (n=3).

Figure 3 Isolation of Jurkat cell lines expressing plvEIG-FOXP3 and plv-T-sh1135.

(**3a)** Jurkat cells were transduced with plvEIG-FOXP3 viruses and sorted based on the intensity of GFP fluorescence. Frequency histogram shows the top 5% GFP^+ (– –) isolated from the pooled population of GFP^+ cells (▬) and GFP^- cells (──). (**3b)** Frequency histogram shows the FOXP3 expression in the same sorted populations of GFP^+ cells. (**3c**) Frequency histogram shows the GFP levels in untransduced Jurkat cells (**....**), Top5% FOXP3 expressing cells (– –) and FOXP3 +ve cells super-infected with plv-T-sh1135 (▬). Since both of these lentiviral vectors express GFP, cells double transduced with plv-T-sh1355 and plvEIF-FOXP3 were isolated by gating on bright fluorescent GFP^+ signal, indicating the presence of both GFP^+ lentiviral vectors.

Figure 4 Conditional regulation of FOXP3 *expression in Jurkat cells and human Treg.*

(**4a**) Non-transduced Jurkat cells, cells expressing plvEIG-FOXP3 alone (top 5%) and cells co-expressing plvEIG-FOXP3 and plv-T-sh-1355 were cultured in the absence or in the presence of Dox (1 µg/ml) for 48hr. The frequency histogram shows intracellular FOXP3 expression in un-transduced Jurkat cells (....), cells expressing plvEIG-FOXP3 (top 5%) (▬) and cells expressing plvEIG-FOXP3 and plv-T-sh1355, in the presence (▪▪▪▪) or absence (▬) of Dox (1µg/ml). (**4b**) FOXP3 mRNA expression levels were determined by RT PCR showing FOXP3 expression in non-transduced Jurkat cells, cells expressing plvEIG-FOXP3 (top 5%) and cells expressing plvEIG-FOXP3 and plv-T-sh-1355, in the presence (▨) or absence (▨) of Dox (1µg/ml). (**4c**) plv-T-sh-1355 RNA expression was determined using SL-RT-PCR. Amplicons from semi-quantitative SL-RT-PCR were separated using 3% agarose. plv-T-sh-1355 and Hprt-1 RNA expression in non-transduced Jurkat cells, cells expressing plvEIG-FOXP3 and cells expressing plvEIG-FOXP3 and plv-T-sh-1355, in the presence and absence of Dox are shown. (**4d**) Isolated human T regs transduced with plv-T-

sh-1355 were cultured in the presence (▪▪▪) or absence (——) of Dox for 48 hr. Cells were stained intracellularly with PEα hu FOXP3 antibody.

Figure 5. Activation of the short hairpin is sensitive, rapid and reversible.

(**5a**) Jurkat cells transduced with both plvEIG-FOXP3 and plv-T-sh-1355 were cultured in the presence of increasing concentrations of Dox for 48hr, and intracellular FOXP3 expression determined by flow cytometry. (**5b**) plv-T-sh-1355 and control Hprt-1 RNA expression were determined using SL-RT-PCR. Amplicons from semi-quantitative SL-RT-PCR were separated using 3% agarose. (**5c**) Jurkat cells transduced with both plvEIG-FOXP3 and plv-T-sh-1355 were cultured in the presence of Dox (1μg/ml) and FOXP3 expression levels analysed over time. (**5d**) Jurkat cells transduced with both plvEIG-FOXP3 (top5%) and plv-T-sh-1355 were cultured in the presence or absence of Dox (1μg/ml) for 48hr. After FOXP3 measurement, the remaining cells were washed 2 times and returned to culture (labelled 0hr). At the times indicated cells were removed for quantification. FOXP3 expression in cells transduced with plvT-sh1355 was determined by expressing MFI from cells in the absence of Dox relative to MFI in cells cultured in the presence of Dox. (**5e**) Relative FOXP3 mRNA expression for the cells was determined by calculating FOXP3 mRNA expression from cells cultured in the presence of Dox, relative to that in cells cultured in the absence of Dox. (**5f**) plv-T-sh-1355 RNA expression was determined using SL-RT-PCR. Ct values for plv-T-sh-1355 RNA were normalised with respect to Ct values for control Hprt-1 and plotted. (**5g**) Amplicons from semi-quantitative SL-RT-PCR were separated using 3% agarose.

Figure 6.

Validation of reversible gene silencing using an endogenous target gene, MYB

(**6a**) The Myb dependant growth of the MCF7 cell line was confirmed by lentiviral delivery of a validated shMyb construct (black lines). Cells were cultured –Dox (–○–), + Dox (–●–), after a single washout (–◆–), or washout and re-addition of dox (···■···). Cells transduced with a scrambled sh control (grey dashed lines) were also treated – Dox (–▼- -), or + Dox (–▲– ·). (**6b)** The on target effects of the lentiviral shMyb were confirmed by western blot (upper panel), showing cells -, + Dox, after washout and after add back of Dox. 1ug/ml Dox used for induction. Controls with the scrambled sh and an empty vector are shown. Loading controls are shown by blotting with anti β-tubulin antibody (lower panel).

Figure 1a)

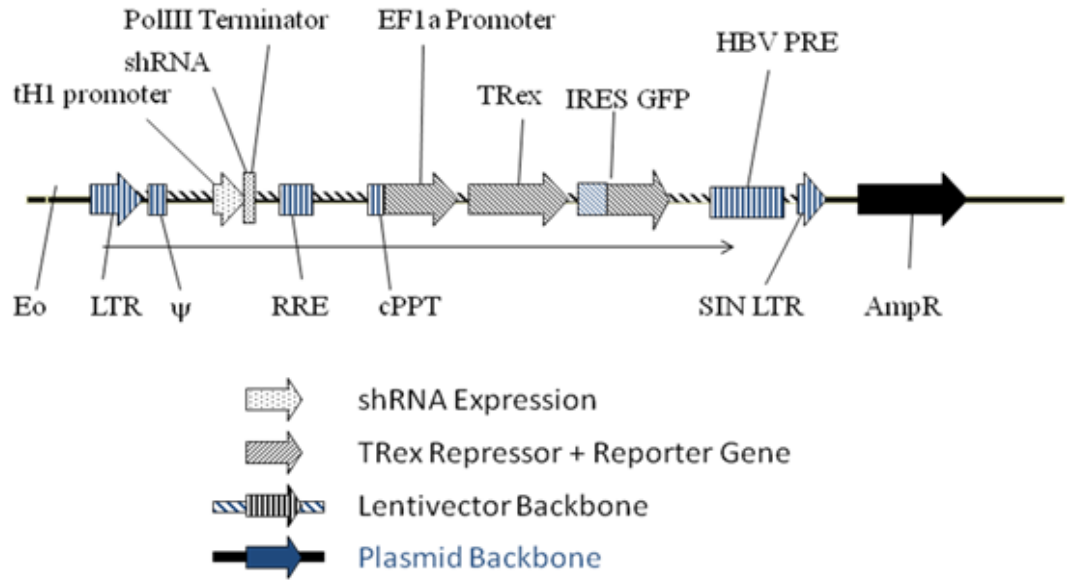
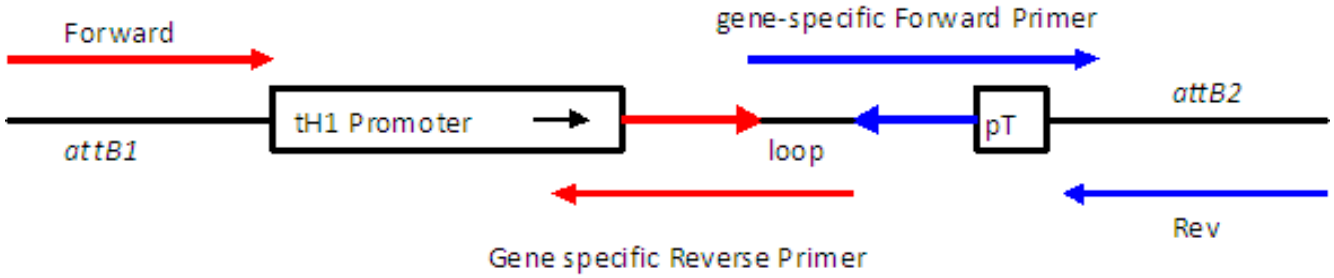


Figure 1b)



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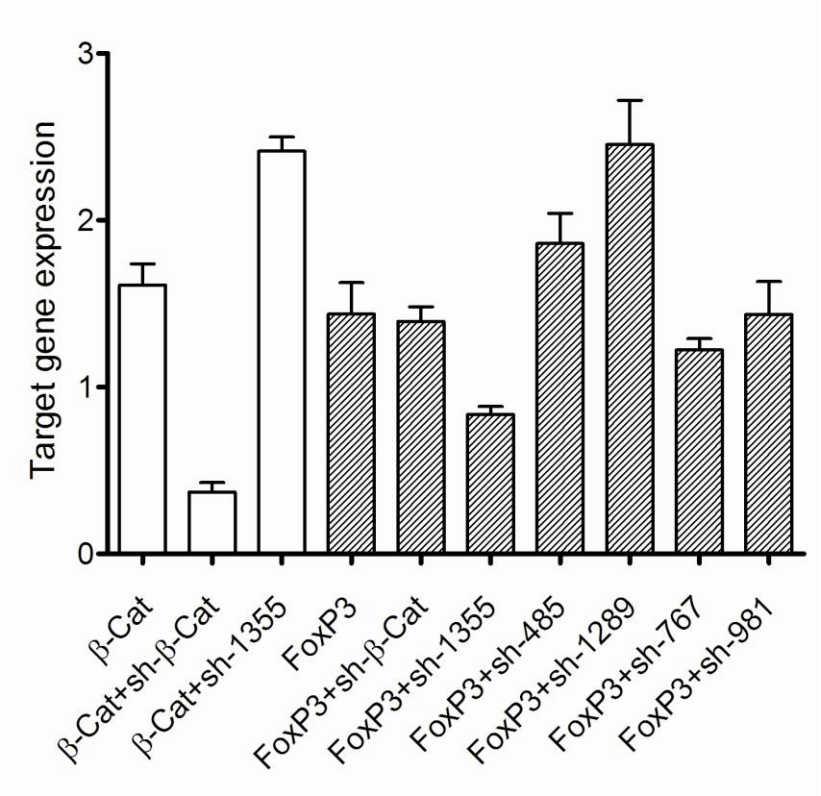

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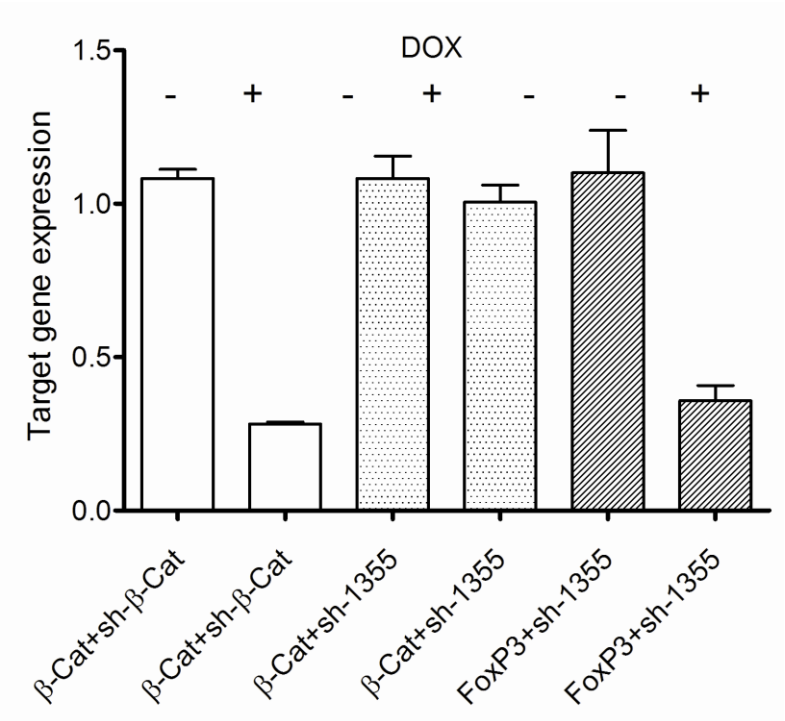

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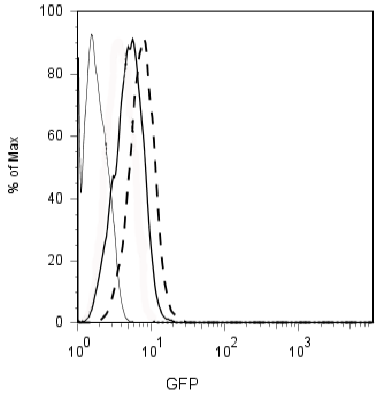

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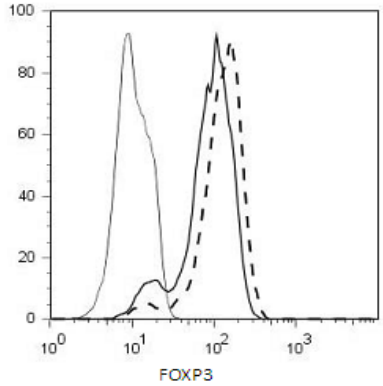

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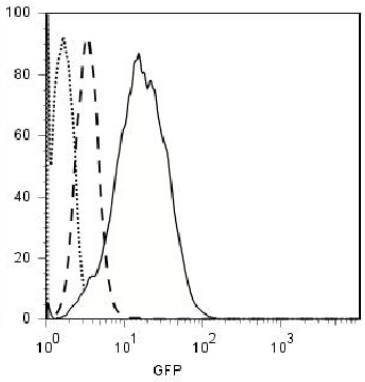

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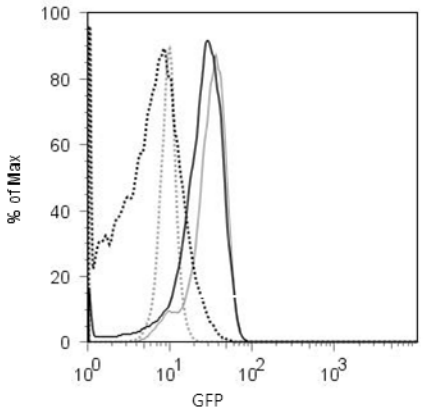

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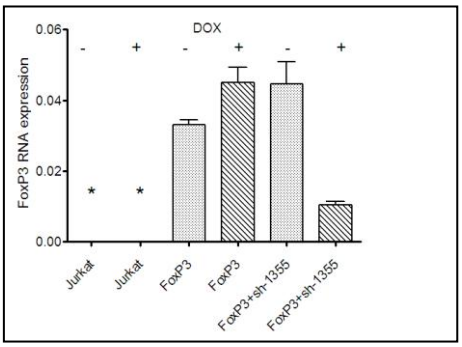

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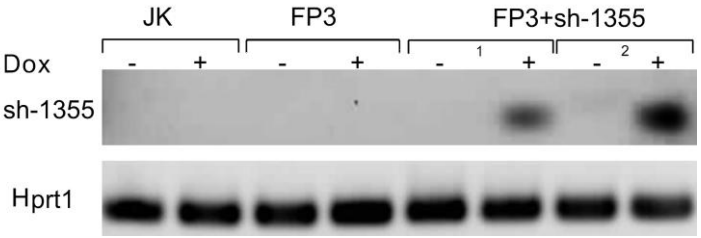

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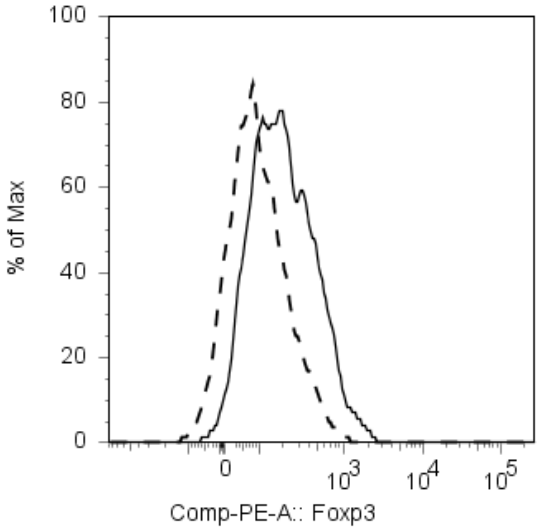

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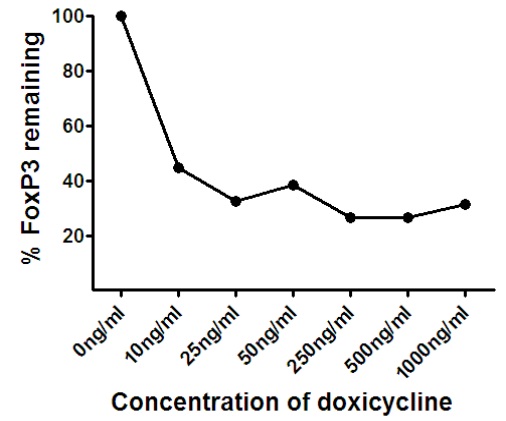


Figure 5b)

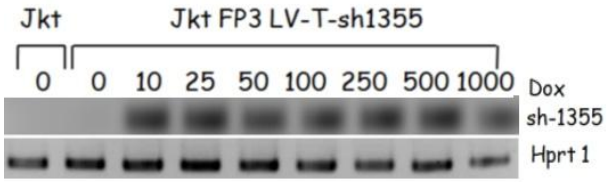


Figure 5c)

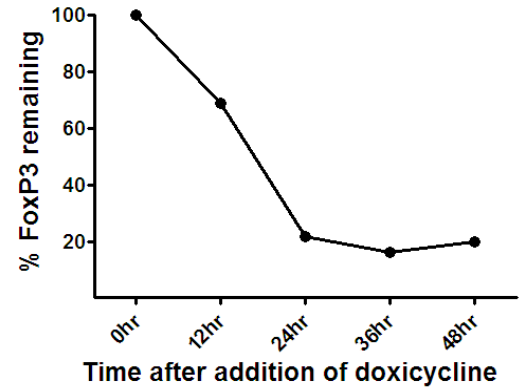


Figure 5d)

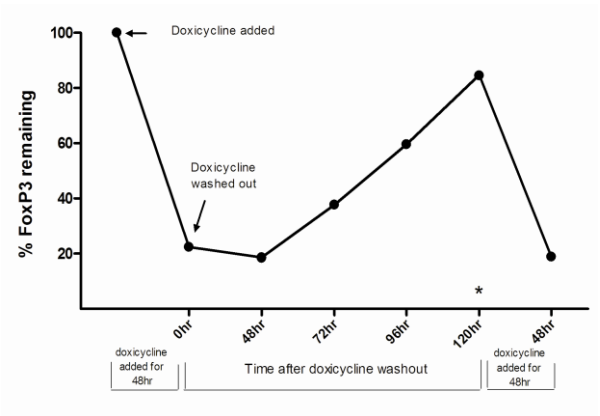


Figure 5e)

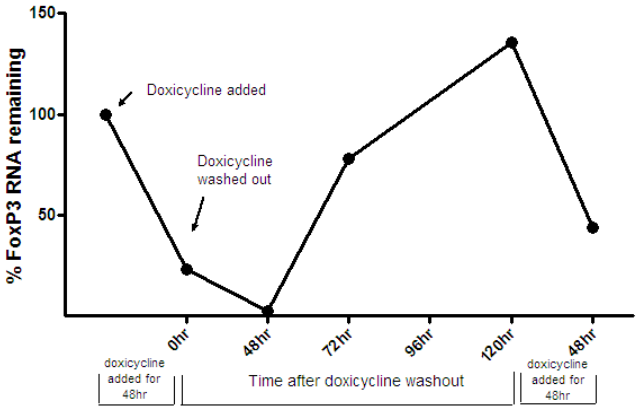
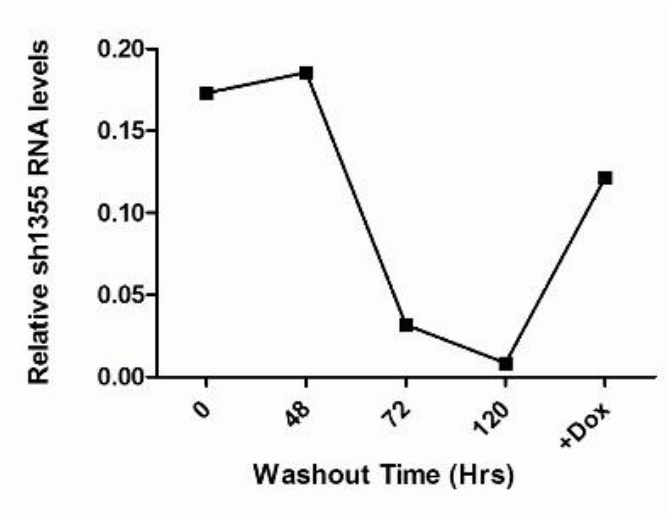


Figure 5f,g


Relative sh1355 RNA levels
0.20
0.15
0.10
0.05
0.00
0
48
72
120
+Dox
Washout Time (Hrs)

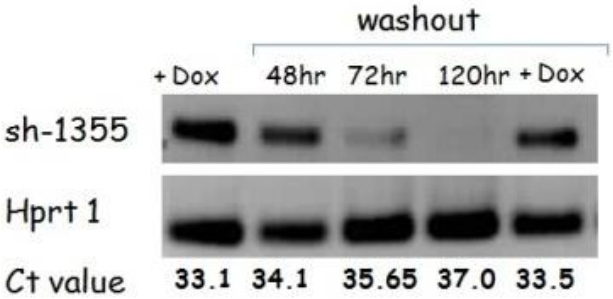

washout
+ Dox
48hr
72hr
120hr
+ Dox
sh-1355
Hprt 1
Ct value
33.1
34.1
35.65
37.0
33.5

Figure 6a,

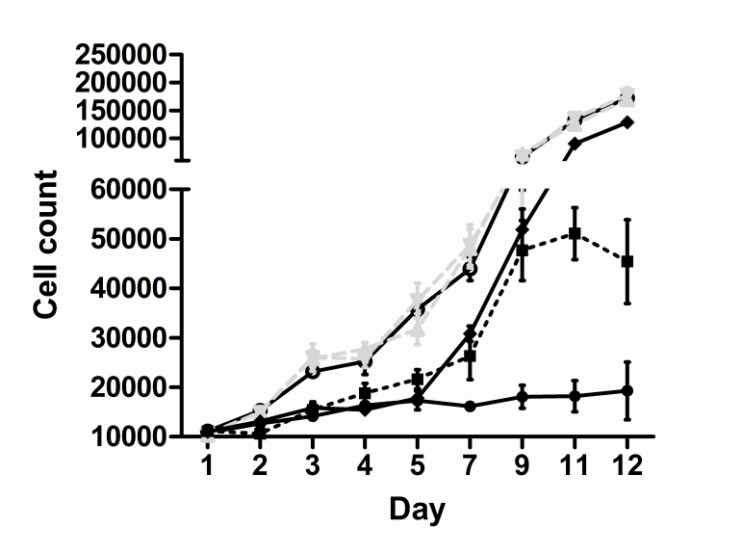


Figure 6b)