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Control of small inhibitory RNA levels and RNA interference by doxycycline induced activation of a minimal RNA polymerase III promoter

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ABSTRACT

RNA interference (RNAi) mediated by expression of short hairpin RNAs (shRNAs) is a powerful tool for efficiently suppressing target genes. The approach allows studies of the function of individual genes and may also be applied to human therapy. However, in many instances regulation of RNAi by administration of a small inducer molecule will be required. To date, the development of appropriate regulatory systems has been hampered by the few possibilities for modification within RNA polymerase III promoters capable of driving efficient expression of shRNAs. We have developed an inducible minimal RNA polymerase III promoter that is activated by a novel recombinant transactivator in the presence of doxycycline (Dox). The recombinant transactivator and the engineered promoter together form a system permitting regulation of RNAi by Dox-induced expression of shRNAs. Regulated RNAi was mediated by one single lentiviral vector, blocked the expression of green fluorescent protein (GFP) in a GFP-expressing HEK 293T derived cell line and suppressed endogenous p53 in wild-type HEK 293T, MCF-7 and A549 cells. RNA interference was induced in a dose- and time-dependent manner by administration of Dox, silenced the expression of both target genes by 90% and was in particular reversible after withdrawal of Dox.

INTRODUCTION

The efficient and specific suppression of genes by RNAi (1) constitutes a valuable new tool to study the physiological role of individual genes *in vitro* and *in vivo*. The method may also be applied to human therapy whenever genes involved in the respective pathology have to be inhibited. Silencing of gene expression by RNAi may be induced in target cells by expressing short hairpin RNAs (shRNAs) yielding small inhibitory RNAs (siRNAs) after *in situ* cleavage (2). Since long poly A tails strongly interfere with the silencing effect (3), shRNAs are appropriately expressed by RNA polymerase III which recognizes a simple run of T residues as a stop signal and therefore does not require a poly A sequence to terminate transcription. As a consequence, respective RNA polymerase III promoters, such as the H1 promoter (4,5) or the U6 promoter (6–8), are widely used to drive the production of shRNAs. Both the H1 and the U6 promoters are constitutively active, and therefore shRNAs can be expressed in a large variety of cells in order to study the consequences of the stable inhibition of target genes. However, constitutive gene silencing cannot be used in the context of transgenic 'knock-down' animals when genes essential for cell survival, cell cycle regulation and cell development are analyzed. Such studies require conditional gene silencing induced by administration or withdrawal of a small inducer molecule. Conditional suppression of genes will also be important for therapeutic applications by permitting termination of treatments at the onset of unwanted side effects.

Conditional RNAi can be obtained by expression of shRNAs from a modified RNA polymerase III promoter allowing external control of its activity. A further requirement for drug-induced transcriptional activity is the expression of a heterologous transcription factor that specifically interferes, in the presence or the absence of the inducer molecule, with the activity of the modified promoter but does not interact with the genome of the host cell. Because of their simple structural organization RNA polymerase III promoters offer only a few possibilities for modification. The U6 promoter (9) is composed of a TATA box, a proximal (PSE) and a distal

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sequence element (DSE) that are each located at fixed distances upstream from the transcription initiation site. The space between the individual elements are restricted, thereby limiting approaches based on steric interference with the transcription initiation complex. Modifications may be applied neither to the TATA box nor to the PSE since together they form the essential core unit of the promoter recruiting the transcription initiation complex.

In several studies (10–13) regulatory systems have been proposed that employ RNA polymerase III promoter constructs controlled by reversible steric inhibition of the formation of the transcription initiation complex. However, Lin *et al.* observed severe leakiness when using these systems (14). Another recent attempt has been based on a Krab-Tet repressor fusion protein which allows Dox-controlled inhibition of the expression of shRNAs from a H1 promoter juxtaposed with Tet-operon sequences (15). However, this approach may be limited by secondary effects due to the inhibitory activity of Krab on both RNA polymerase II and RNA polymerase III promoters over long distances (16). A third attempt has been based on the activation of an engineered U6 promoter by the recombinant transcription factor Gal4-Oct-2^{Q}(Q→A) that constitutively binds to four Gal-4 binding sites replacing the DSE sequence in the promoter construct (17). The transcription factor Gal4-Oct-2^{Q}(Q→A) comprises the DNA binding unit of the transactivator Gal-4 from yeast and an artificial transactivation domain referred to as the Oct-2^{Q}(Q→A) domain. This transactivation domain is composed of four copies of the peptide sequence Q^{18}III(Q→A) comprising the amino acid residues 143 to 160 of the human transcription factor Oct-2 in which all glutamine residues had been changed to alanine. Regulated expression of the transcription factor Gal4-Oct-2^{Q}(Q→A) under the control of the ecdysone dependent regulatory system ultimately allowed regulated production of shRNAs from the engineered U6 promoter (18). However, the usefulness of this indirectly regulated expression of shRNAs is limited since three vectors were necessary to mediate expression of all the components required.

In the present study, we set out to develop a regulatory system that (i) allows efficient regulation of RNAi, (ii) does not cause secondary effects and (iii) can be delivered to target cells by one single lentiviral vector. We based our approach on a heterologous transactivator that conditionally binds in the presence of a small inducer molecule to a minimal U6 promoter thereby activating transcription of shRNAs. We investigated whether the Oct-2^{Q}(Q→A) domain can be conditionally and functionally linked to a minimal U6 promoter construct via the conditional DNA binding domain of the transactivator rtTA2-M2 that derives from the *Escherichia coli* Tet-repressor protein and mediates dimerization and Doxycycline (Dox)-induced binding to *tet* operator sequences with high affinity (19). We replaced the three minimal VP 16-derived activation domains in rtTA2-M2 (20) by the Oct-2^{Q}(Q→A) domain. For conditional binding to an inducible minimal U6 promoter, the functional recognition sites for Staf and Oct-1 within the DSE of the human U6 promoter (21) were replaced by seven *tet* operator sequences. The modified promoter and the engineered transcription factor were capable of together forming a regulatory system allowing conditional RNAi by Dox-dependent expression of shRNAs. The regulatory system was delivered to target cells by one single lentiviral vector.

MATERIALS AND METHODS

Plasmid constructions

The plasmids pUHR 10-3 and pUHRT 62-1, which contain the components of the Tet regulatory system, were kindly provided by H. Bujard (Zentrum für Molekulare Biologie, Heidelberg, Germany). The plasmid pcDNA-Δ that allows the use of BbsI in subsequent cloning experiments was generated by self-ligation of the vector fragment obtained by PstI digestion of the plasmid pcDNA 3 (Invitrogen, Cergy Pontoise, France). The core unit of the human U6 promoter that did not contain the functional binding sites for the transcription factors Staf and Oct-1 (21) was amplified by PCR from genomic DNA of HEK293T cells. The oligonucleotides 5′-CGACGCGTTGCAGAGCTCGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAG-3′ and 5′-CGGGATCCAGAAGACCACGGTGTTTCGTCCTTTCCACAAGAT-3′ (Eurogentec, Angers, France) were the sense and antisense primers respectively, and the DNA fragment amplified contained both a MluI and a SacI site upstream, and a BbsI and a BamHI site downstream from the truncated U6 promoter. The fragment was inserted between the MluI and BamHI sites of pcDNA-Δ yielding the plasmid pcDNA-ΔU6t. A MluI–SacI fragment containing seven *tet* operator sequences was amplified by PCR from pUHR 10-3 and inserted between the MluI and BamHI sites of pcDNA-ΔU6t to give pcDNA-ΔU6min. The DNA fragment encoding shRNAs designed to silence expression of green fluorescent protein (shGFP) was generated by annealing the oligonucleotides 5′-ACCGCAAGCTGACCCTGAAGTTCTTCAAGAGAGAACTTCAGGGTCAGCTTGCTTTTTCTCGAGG-3′ and 5′-GATCCCTCGAGAAAAAGCAAGCTGACCCTGAAGTTCTCTCTTGAAGAACTTCAGGGTCAGCTTG-3′. Annealing of the oligonucleotides 5′-ACCGACTCCAGTGGTAATCTACTTCAAGAGAGTAGATTACCACTGGAGTCTTTTTCTCGAGG-3′ and 5′-GATCCCTCGAGAAAAAGACTCCAGTGGTAATCTACTCTCTTGAAGTAGATTACCACTGGAGT-3′ yielded the DNA fragment encoding shRNAs designed to silence expression of p53 (shp53). Both DNA fragments encoding shRNAs were inserted into pcDNA-ΔU6min linearized by BbsI–BamHI digestion. The resulting plasmids were named pcDNA-ΔU6min-shGFP and pcDNA-ΔU6min-shp53, respectively.

An EcoRI–BamHI fragment encoding the conditional DNA binding domain of rtTA2-M2 (19) was amplified by PCR from pUHRT 62-1 using the oligonucleotides 5′-CGGAATTCACCATGTCTAGACTGGACAAGAGCAAAG-3′ and 5′-CGGGATCCTGAAGACTACGGTCCGCCGCTTTCGCACTTTAGCTGT-3′ as the sense and antisense primers, respectively. Upstream from the BamHI site the fragment contained a stop codon and a BbsI site allowing extension with a fragment encoding additional amino acid residues. Insertion of the fragment between the EcoRI–BamHI sites of pcDNA-Δ yielded the plasmid pcDNA-Δ/rtTA2-M2trunc. The DNA fragment coding the peptide sequence Q^{18}III(Q→A) was generated by annealing the oligonucleotides 5′-ACCGAACCTGTTCGCTCTCCCCGCTGCAACAGCGGGAGCCCTACTGACAT-

CAGCACCGTAGTCTTCG-3′ and 5′-GATCCGAAGACTACGGTGCTGATGTCAGTAGGGCTCCCGCTGTTGCAGCGGGGAGAGCGAACAGGTT-3′ and was inserted into pcDNA-Δ/rtTA2-M2trunc linearized by BbsI–BamHI digestion. The resulting plasmid contained again a stop codon and a BbsI site upstream from the BamHI site allowing further rounds of extension with the same fragment. Extension with the fragment encoding Q^{18}III(Q→A) was repeated three times yielding the plasmid containing the rtTA2-Oct2 cDNA. The sequence encoding rtTA2-Oct2 was recovered by EcoRI–BamHI digestion and inserted into pΔ500rtTA2-M2-WPRE (22) from which rtTA2-M2 had been removed by EcoRI–BamHI digestion. A SalI–EcoRI fragment containing the phosphoglyerate kinase (PGK) promoter was amplified by PCR and inserted between the SalI–EcoRI sites upstream from rtTA2-Oct2 yielding pΔ500PGK-rtTA2-Oct2-WPRE.

The cassettes allowing shRNA expression were recovered from pcDNA-ΔU6min-shGFP and pcDNA-ΔU6min-shp53 by MluI–SpeI digestion and inserted into the lentivector precursor plasmid pTrip-CMVmin-WPRE (22) from which the element CMVmin had been removed by MluI–SpeI digestion. The WPRE sequence was removed from the resulting plasmids (pTrip-U6min-shGFP-WPRE and pTrip-U6min-shp53-WPRE) by SpeI–KpnI digestion and replaced by the rtTA2-Oct2 expression cassette recovered from pΔ500PGK-rtTA2-Oct2-WPRE by NheI–KpnI digestion. The resulting plasmids, pTrip-U6min-shGFP-PGK-rtTA2-Oct2-WPRE and pTrip-U6min-shp53-PGK-rtTA2-Oct2-WPRE, were used for the production of lentivirus vector particles.

The DNA fragment encoding the riboprobe for the detection of the GFP silencing siRNAs was generated by annealing the oligonucleotides 5′-GATCCGCAAGCTGACCCTGAAGTTCTTCAAGAGAGAACG-3′ and 5′-AATTCGTTCTCTCTTGAAGAACTTCAGGGTCAGCTTGCG-3′ and was inserted between the BamHI–EcoRI sites of pcDNA 3. All plasmid constructs were verified by sequencing using a ABI-PRISM 13100 DNA sequencer (Applied Biosystems, Courtabeuf, France).

Cell culture, lentiviral transductions and selection of transduced cells

The HEK 293T, MCF-7 and A549 cell lines were cultivated at 37°C under a humidified atmosphere of 5% CO2 / 95% air in DMEM supplemented with 10% fetal calf serum (FCS), 20 U/ml penicillin G and 20 μg/ml streptomycin sulfate. Lentivirus vector particles were produced by transient cotransfection of HEK 293T cells by the vector plasmid, an encapsidation plasmid (p8.7), and a VSV expression plasmid (pHCMV-G) as described (23). Vector stocks were titered by determination of the amount of the p24 capsid protein using an HIV-1 core profile enzyme linked immunosorbent assay (ELISA) (Beckman Coulter, Roissy, France). For transduction HEK 293T GFP cells were incubated overnight with vector in the presence of 10 μg/ml DEAE dextran (Sigma-Aldrich, St. Quentin Fallavier, France). Transduced cells were selected after 5 days of cultivation in the presence of 6 μg/ml Dox using a FACSVantage SE cell-sorting instrument (Becton Dickinson, Rungis, France). Selected clones were expanded and analyzed by fluorescence microscopy and FACS.

Northern blot analysis

A ^{32}P-labeled riboprobe was transcribed from the plasmid encoding the riboprobe using [α-^{32}P]ATP (Amersham Biosciences, Orsay, France) and the Riboprobe System–T7 (Promega, Charbonnières, France). Small RNAs were isolated from aliquots of 10^7 cells with the *mir*Vana™ PARIS™ Kit (Ambion, Huntingdon, UK). Samples containing 3.3 μg of small RNAs were denatured by heating at 95°C for 5 min in the presence of 50% formamide. After electrophoresis on a 15% polyacrylamide gel in the presence of 8 M urea the RNA was stained with ethidium bromide and examined on a transilluminator. The RNA was then transferred by electroblotting to a BrightStar-Plus Nylon membrane (Ambion), fixed by ultraviolet (UV) crosslinking and hybridized to the probe. The resulting ^{32}P-labeled RNA–RNA hybrids were detected by autoradiography using Hyperfilm™ MP (Amersham Biosciences).

Western blot analysis

Cell extracts were prepared in lysis buffer [25 mM Tris–HCl (pH 7.5), 1% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA, 150 mM NaCl] containing a cocktail of protease inhibitors (Roche, Meylan, France). The protein samples (30 μg) were separated on SDS–9% polyacrylamide gels and then transferred to Protan nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) in an electroblotting apparatus, using standard procedures (24). Immunodetection was performed as described previously (25), using a monoclonal anti-p53 antibody (BD Biosciences, Erembodegem, Belgium), a monoclonal anti-actin antibody (Chemicon, Hampshire, UK) and an anti-mouse Ig-horseradish peroxidase (HRP) conjugate (Amersham Biosciences).

RESULTS AND DISCUSSION

To achieve regulated RNAi, we engineered a novel Tet-dependent transactivator (Figure 1A) by linking the conditional DNA binding domain of the tetracycline-dependent transactivator rtTA2-M2 (19) to the Oct-2^Q(Q→A) domain (17) which is capable of specifically activating a minimal U6 promoter (Figure 1A). An inducible minimal U6 promoter was constructed by replacing the functional binding sites (21) for the transcription factors Staf-1 and Oct-1 within the DSE by seven *tet* operator sequences (Figure 1B). In the absence of Dox the recombinant Tet-dependent transactivator will not bind to the minimal U6 promoter (Figure 1C) and as a consequence the shRNA coding sequence will not be transcribed. In contrast, the Tet-dependent transactivator will bind to the minimal U6 promoter in the presence of Dox (Figure 1D), thereby activating the expression of shRNAs.

As a delivery system we designed a single lentivirus vector by inserting two expression cassettes into its backbone (Figure 2A). The first cassette contained the minimal U6 promoter and was used to produce shRNAs. The second cassette was employed to express the engineered transcription factor rtTA2-Oct2 composed of the conditional DNA binding domain of rtTA2-M2 and the Oct-2^Q(Q→A) activation domain. The transcription factor was constitutively transcribed from the PGK promoter; and the polyA sequence of the vector in the 3′ long terminal repeat (LTR) was used for

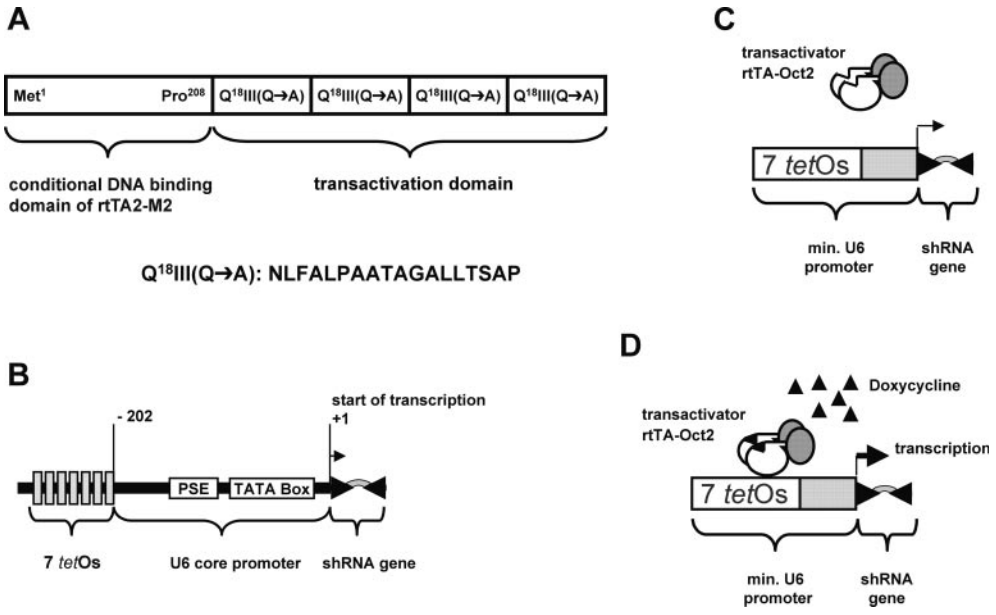

Figure 1. Schematic diagrams illustrating the regulatory system allowing Dox-induced RNAi. (**A**) Primary structure of the transactivator rtTA-Oct2 composed of the conditional DNA binding domain of rtTA2-M2, and the Oct-2^{Q}(Q→A) domain mediating specific induction of a minimal RNA polymerase III promoter (17). (**B**) Structure of the minimal U6 promoter: The 202 bp sequence upstream from the transcription start site was derived from the human U6 promoter and contains the PSE and the TATA box. Upstream from this sequence, seven *tet* operator sequences (*tet*Os) have been inserted to allow conditional binding of the transactivator. (**C**) In the absence of Dox (off state), rtTA-Oct2 does not bind to the operator sequences and hence shRNAs are not synthesized. (**D**) In the presence of Dox (on state), the transactivator binds and thereby activates the expression of shRNAs designed to induce the degradation of the respective target mRNAs.

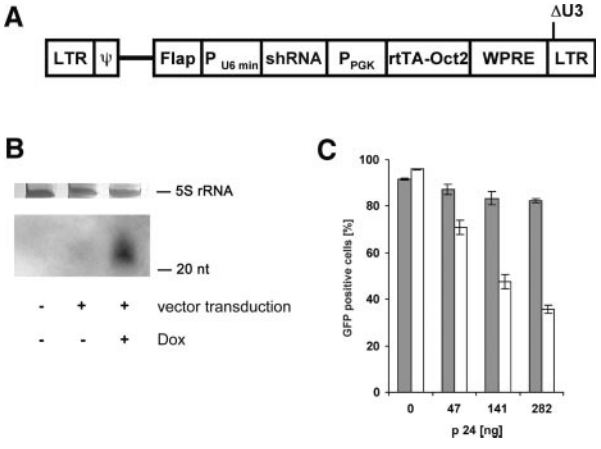

Figure 2. A single lentiviral vector mediates Dox-regulated RNAi. (**A**) Design of the vector: LTR, ψ and Flap are sequences derived from HIV-1 (the LTRs, the packaging sequence and the central Flap element, respectively). $P_{U6\,min}$ and P_{PGK} are the Tet-regulated minimal U6 promoter and the phosphoglycerate kinase promoter; WPRE is the Woodchuck hepatitis virus responsive element; rtTA-Oct2, the cDNA encoding the transcription factor rTA-Oct2; and shRNA, the sequence encoding shRNAs. (**B** and **C**) Experimental validation of regulated RNAi using a vector that expresses shRNAs designed to silence the expression of GFP. (B) 'Northern blot' analysis of Dox-regulated expression of siRNAs from the vector. HEK 293T GFP cells (1×10^5) were incubated for 24 h with and without vector corresponding to 141 ng of protein p24, and cultivated in the presence and absence of 6 μg/ml Dox for 7 days. Then, small RNAs were isolated from the cells and probed for siRNAs designed to silence the expression of GFP. 5S-rRNA detected by ethidium bromide staining of the polyacrylamide gel served as an internal control to show equal loading. (C) Experimental validation of RNAi-mediated silencing of GFP. HEK 293T GFP cells (8×10^4) were incubated overnight with various quantities of vector expressed as ng of protein p24, and cultivated in the absence (grey bars) and in the presence (white bars) of 6 μg/ml Dox for 5 days prior to FACS analysis. Values are averages of percentages of GFP-positive cells ± SE, $n = 3$.

polyadenylation. The vector contained a WPRE sequence (26) to enhance the expression of rtTA2-Oct2 and to stabilize the RNA genome of the vector during the production of vector particles in transiently transfected HEK 293T cells. A Flap sequence was also included to improve transduction of non-dividing cells (23). For safety reasons the U3 promoter region was deleted from the 3′ LTR so that the vector was self-inactivating (27).

A first vector contained a shRNA encoding sequence which was designed to silence the expression of GFP as described (5). A HEK 293T GFP cell-clone that stably expresses GFP as a transgene was transduced with the vector construct. Cells were cultivated in the presence and absence of Dox (6 μg/ml) prior to isolating small RNAs from the cultures as well as from controls (non-transduced HEK 293T GFP cells). 'Northern Blot' analysis of the RNA samples revealed that siRNAs designed to silence GFP were expressed in transduced cells cultivated in the presence of Dox (Figure 2B). The siRNAs were not detected in non-transduced cells. In transduced cells cultivated without Dox no signal exceeding the detection threshold was observed. 'Northern Blotting' did not allow detection of shRNAs probably because of their rapid cleavage into siRNAs by Dicer nuclease.

Subsequently, HEK 293T GFP cells were transduced with various amounts of vector and incubated in the presence and absence of Dox (6 μg/ml). Incubation with Dox reduced the number of GFP-expressing cells by up to 60% as was determined by FACS analysis (Figure 2C). The decrease in GFP-positive cells correlated with the amount of vector applied. The number of GFP-positive cells among transduced cells incubated in the absence of Dox was 10–15% lower than among non-transduced cells. This difference also correlated with the amount of vector applied and may have been caused

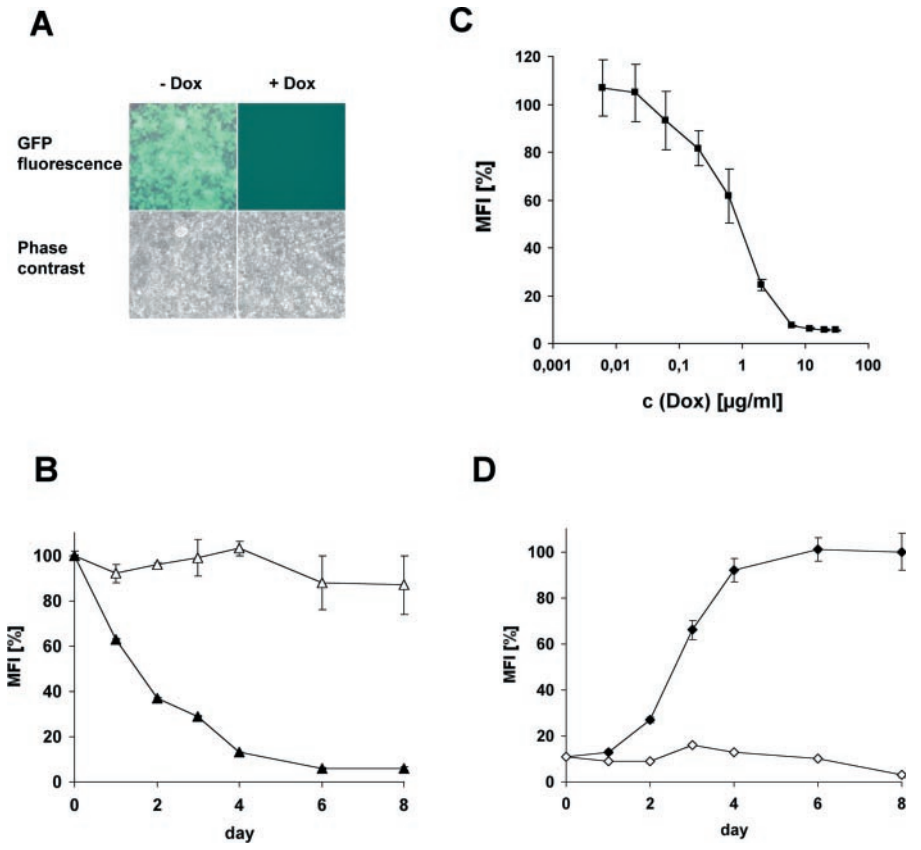

Figure 3. Characterization of Dox-regulated RNAi in a representative cell-clone (C9): (**A**) Microscopic analysis of cells incubated in the presence or in the absence of 6 µg/ml Dox at 72 h after induction. (**B**) Time course of Dox-induced RNAi: RNAi was induced or not induced at day 0 by administration of 6 µg/ml Dox and mean intensities of GFP fluorescence were measured by FACS analysis at various times after induction. Closed triangles represent intensities of cells incubated with Dox, open triangles give those of untreated cells. The fluorescence intensity observed at day 0 was defined as 100%, values are means ± SE, $n = 3$. (**C**) Mean intensities (± SE, $n = 3$) of GFP fluorescence obtained by FACS analysis of cells cultivated for 5 days in the presence of various concentrations of Dox. The fluorescence intensity in untreated cells was defined as 100%. (**D**) Reappearance of GFP fluorescence after withdrawal of Dox: prior to the analysis, cells were cultivated for 5 days in the presence of 6 µg/ml Dox. At day 0, Dox was withdrawn or not withdrawn and the mean fluorescence intensity was followed by FACS analysis. Closed rhomboids represent values from cells that were not treated with Dox from day 0, open rhomboids give values from cells incubated with 6 µg/ml Dox throughout the experiment. The fluorescence intensity measured 8 days after removal of Dox was defined as 100%, values are means ± SE, $n = 3$.

by leakage expression of shRNAs in cells containing multiple copies of the vector genome.

To establish uniform conditions for precise characterization of the regulatory system, cell clones were amplified from individual transduced cells. Several clones were obtained that displayed Dox-regulated expression of GFP (see Supplementary Table). Fluorescence microscopy of a representative clone (C9) demonstrated that GFP was only expressed in the absence of Dox (Figure 3A). We then used FACS analysis to study the effect of Dox on the expression of GFP. The addition of Dox to the cells was followed by a significant decrease in GFP fluorescence within 24 h; after 5–6 days the reduction of GFP fluorescence was 90% (Figure 3B). In the absence of Dox there was no change in GFP fluorescence during the incubation. To determine the minimal concentration of Dox required to induce RNAi, cells of the clone C9 were incubated with various concentrations of Dox (Figure 3C). A concentration of 6 µg/ml was required to induce a 90% suppression of GFP within 5 days. Lower concentrations of Dox were either ineffective or caused incomplete or delayed RNAi. To test inducible RNAi for reversibility, cells of the clone C9 were cultivated for 5 days in the presence of Dox. Then Dox was removed, and the expression of GFP was followed. GFP fluorescence had increased significantly 48 h after the removal of Dox (Figure 3D); however, incubation without Dox for 5–6 days was required to restore maximal expression of GFP. No increase in GFP fluorescence was detected in cells incubated with Dox throughout the experiment.

The next step was to investigate whether the regulation system can be employed for the silencing of other target genes. As a target we chose the p53 gene because of detectable expression in mammalian cells, availability of reliable antibodies to monitor levels of the protein, and the existence of an efficient shRNA (2). Moreover, in a recent study genetic deletion of p53 suppressed neurodegeneration in animal models of Huntington's disease (28). Thus, local and regulated down-regulation of p53 may potentially constitute a novel gene therapy approach for the treatment of Huntington disease patients. We constructed a second vector, which contained a shRNA encoding sequence designed to silence expression of human p53 as described (2). HEK 293T cells, MCF-7 cells and A549 cells were transduced with various amounts of vector and incubated in the presence and absence of Dox (6 µg/ml) for 5–7 days before protein was extracted from the cultures as well as from non-tansduced controls. 'Western blot' analysis of protein samples containing identical amounts of protein

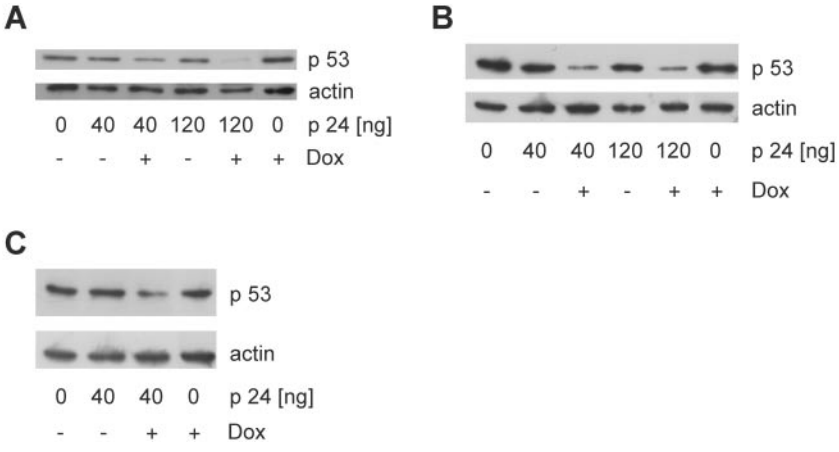

Figure 4. 'Western blot' analysis demonstrating silencing of p53 by Dox-regulated RNAi in (**A**) HEK 293T cells, (**B**) MCF-7 cells and (**C**) A549 cells. Cells (1×10^5) were incubated overnight with indicated quantities of vector, expressed as ng of protein p24, and then cultivated in the absence and in the presence of 6 μg/ml Dox. After a 5 day (MCF-7 and A549 cells) and a 7 day cultivation (HEK 293T cells), protein was extracted from the cells and analyzed by immunoblotting. Both p53 and actin were detected; the latter served as a control to demonstrate equal loading.

revealed that p53 levels were efficiently reduced when transduced cells were incubated in the presence of Dox (Figure 4). An up to 90% inhibition of the expression of p53 was observed in Dox treated cultures of transduced cells as assessed by densitometric analysis of the Blot data. No down-regulation of p53, or at best some minimal silencing because of leakage expression of shRNAs, was obtained when transduced cells were cultivated in the absence of Dox. The expression of p53 was not reduced when non-transduced cells were incubated in the presence of Dox (6 μg/ml).

Considered together, our findings indicate that the engineered minimal U6 promoter was conditionally reactivated by Dox-controlled binding of rtTA2-Oct2 containing the Oct-2^Q(Q→A) domain for transactivation. The minimal U6 promoter and the recombinant transcription factor together formed a regulatory system allowing conditional RNAi by Dox-controlled production of shRNAs. In particular, the system allowed the expression of the reporter transgene GFP as well as the expression of the endogenous gene p53 to be rendered under external regulation by means of administration of Dox. To fully induce RNAi a Dox concentration of 6 μg/ml was necessary which is more than 10-fold the concentration required to activate a minimal (cytomegalovirus) CMV promoter by the transactivator rtTA2-M2 (19). Indeed, the responsiveness to Dox was similar to that of the Tet-dependent regulatory system which, by using rtTA as a transactivator, requires a Dox concentration of 1 μg/ml to induce the expression of transgenes *in vitro* (29). That system was successfully applied in the context of transgenic mice (30). Taken into account that an at least 70% induction of RNAi was already observed in the presence of 1 μg/ml of Dox (Figure 3C), the regulation system presented here holds promise to be also applicable to animal studies. However, further studies using transgenic mice are required to examine the efficiency of Dox-regulated RNAi *in vivo*.

Nevertheless the finding that 6 μg/ml of Dox are necessary to fully induce RNAi in cultured cells is an unexpected result. It may be caused by the Oct-2^Q(Q→A) transactivation domain which may affect the affinity for Dox by interference with either the accessibility or the structure of the Dox binding site. Secondary intramolecular effects of the transactivation domain on characteristics of the conditional DNA binding domain can in particular not be ruled out because of the overall hydrophobicity of the Oct-2^Q(Q→A) domain.

The Oct-2^Q(Q→A) domain is composed of four copies of the peptide sequence Q^{18}III(Q→A) which comprises 18 amino acid residues (17). Fourteen out of the 18 amino acid residues are non-polar and hydrophobic. Four amino acid residues are polar, and charged amino acid side chains are lacking (Figure 1A). Because of these characteristics the Oct-2^Q(Q→A) domain probably forms a hydrophobic patch which facilitates the formation of the transcription initiation complex after binding in a correct steric orientation to the minimal U6 promoter. The peptide sequence Q^{18}III(Q→A) corresponds to the amino acid residues 143 to 160 of the human transcription factor Oct-2 in which all glutamine residues have been changed to alanine. Since the mutations change 6 amino acid residues out of 18, the Q^{18}III(Q→A) sequence may almost be considered as an individual synthetic peptide sequence. In particular, it is noteworthy that the Q→A mutations remove the capacity of transactivating RNA polymerase II promoters (17). For this reason and because we use the weak PGK promoter to drive expression of the transactivator, the regulatory system reported in the present study should not cause secondary effects by transactivation of promoters in the vicinity of the vector integration site.

The regulatory system that we developed requires expression of only one heterologous transactivator and can therefore be delivered to target cells by one single lentiviral vector. It is by far more complicated to establish conditional RNAi by ecdysone-regulated expression of the Gal4-Oct-2^Q(Q→A) transcription factor that in turn activates a minimal U6 promoter construct by constitutive binding. This indirect regulatory approach required expression of additional heterologuos components, and as a consequence three vectors were necessary to deliver regulated RNAi to target cells (18). The delivery of our regulatory system by one single lentiviral vector will significantly facilitate the application of conditional RNAi in many instances.

In summary, we have developed a regulatory system allowing Dox-controlled expression of shRNAs and demonstrated inducible, reversible and stable RNAi in mammalian cells.

The system may allow the expression of any gene to be rendered under external control by means of administration of Dox. Inducible RNAi will find applications in genetic studies using transgenic animals and will also open the door for novel gene therapy approaches such as the treatment of Huntington's disease by local and regulated silencing of p53.

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