

Inducible microRNA expression by an all-in-one episomal vector system

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ABSTRACT

Here we describe an episomal, one-vector system which allows the generation of cell populations displaying homogenous, inducible gene inactivation by RNA interference in a one step procedure. A dual tet-repressor/activator system tightly controls a bi-directional promoter, which simultaneously drives expression of microRNAs and a fluorescent marker protein. We demonstrate the effectiveness of this vector by knockdown of p53 expression in a human cell line which resulted in the expected loss of G₁-arrest after DNA damage. The generation of a cell pool homogeneously expressing the ectopic microRNAs was achieved in 1 week without the need for viral infections. Induction of microRNA expression did not elicit an interferon response. Furthermore, the vector was adapted for convenient ligation-free transfer of microRNA cassettes from public libraries. This conditional knockdown-system should prove useful for many research and gene therapeutic applications.

INTRODUCTION

RNA interference is a process which leads to degradation of endogenous mRNA via formation of duplexes with small non-coding RNAs. The application of RNA interference has revolutionized the functional analysis of genes and may also facilitate gene therapeutic approaches (1–5). Several different strategies have been devised to achieve RNA interference in cells. For example, synthetic short interfering RNAs (siRNA) of 21 nt can be efficiently transfected into cells. Alternatively, transfection of plasmids encoding shRNAs (short hairpin), which are processed to siRNAs by the enzyme DROSHA, is commonly used. It is possible to achieve up to 90% reduction in expression of the respective protein by RNA interference. Transiently introduced siRNAs are effective for up to 5 days as they are degraded with time and diluted during cell divisions. For permanent gene

inactivation it is necessary to stably integrate plasmids driving the expression of shRNAs into the genome of the respective target cell. Initially, RNA polymerase III (Pol III) promoters were used to mediate permanent shRNA expression (6). Recently, Pol II promoters have been employed to drive the expression of precursor microRNAs (7). The constitutive expression of siRNAs may however compete with endogenous microRNAs, leading to toxic side effects.

For the biochemical and functional analysis of essential genes conditional approaches are necessary. Furthermore, the analysis of isogenic cell populations which only differ in the activation state of a conditional allele has advantages when compared to the analysis of cells permanently expressing a knock-down construct. In the latter case multiple clones obtained by tedious single cell cloning and expansion have to be analyzed to avoid clonal variations. In addition, the long-term down-regulation of proteins by stable knock-down strategies may lead to compensatory activation of parallel pathways, obscuring the initial effect of the gene-specific inactivation. Therefore, the conditional activation of microRNA expression provides significant advantages.

Although a number of systems for conditional gene expression have been developed, the Tet-repressor system was most widely applied in recent years (8). In this system elements of the bacterial *tet* operon were transferred into mammalian cells: the tet-repressor was fused to the VP16 transactivation domain (tTA). In the presence of tetracycline or its derivative doxycycline (DOX) this fusion protein will not bind a promoter which harbors a tet-repressor binding site. Later a mutant tet-VP16 fusion was developed which only binds to DNA in the presence of DOX (rtTA, reverse tetracycline controlled transactivator) (9). In many cases the leakiness of the regulated promoters has presented a problem. This can be avoided by the simultaneous expression of a tet-repressor fused to the KRAB protein (tTS^{KRAB}), which keeps the inducible gene inactive in the absence of doxycycline (10). Bornkamm *et al.* (11) combined recently the rtTA and tTS^{KRAB} in the episomal pRTS-1 vector, which allows conditional expression of cDNAs in mammalian cells. Here we demonstrate that the pRTS-1 vector can be used to conditionally regulate the expression of microRNAs mediating RNA-interference,

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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thereby providing a convenient tool to determine gene functions.

MATERIALS AND METHODS

Cell lines/culture and reagents

U2OS osteosarcoma cells were maintained in DMEM containing 10% fetal bovine serum and penicillin (100 IE)/streptomycin (100 µg/ml). Doxycycline (Sigma) was resolved in water (1 mg/ml). Etoposide was resolved in DMSO (40 mg/ml) and used at a final concentration of 20 µg/ml. Poly(I:C) (Sigma) was resolved in water (10 mg/ml) and used at a final concentration of 10 µg/ml.

Generation of plasmids

For generation of the shuttle vector pSHUMI the oligos pUC19linker-fw 5'-AATTGGGCCTCACTGGCCACCGGAGATCTGTTCGACGGACGCGTACCGGTG-3' and pUC19linker-rv 5'-TCGACACCGGTACGCGTCCGTCGACAGATCTCCGGTGGCCAGTGAGGCC-3' were annealed and inserted into the EcoRI/XhoI sites of pUC19 resulting in pUC19m. A BglII/AgeI fragment containing miR30 sequences from the LMP plasmid (12) was inserted into the BglII/AgeI sites of pUC19m. The resulting pSHUMI plasmid can be used to subclone short hairpin sequences using the XhoI and EcoRI restriction sites.

For generation of the pEMI vector, regions containing 5'miR30 and homology region 2 (HR2) were amplified from pSM2c (7) using the primers 5'miR30-fw 5'-CGAGATCTTGTTTGAATGAGGCTTCAGTAC-3' and 5'miR30-rev 5'-GCACCGGTGCGGCCCGCTCGAGCCTTCTGTTGGGT-TAACC-3' and HR2-fw 5'-CGCTCGAGATCCATGGCA-TATGGGATCCAAGGCAGTTATTGGTGCCCTTAAAC-3' HR2-rev 5'-GCACCGGTTTCAGATCCTCTTCGGAGATCAG-3' and inserted into the pTOPO vector (Invitrogen). The 5'miR30 part was subcloned into pSHUMI using BglII/AgeI restriction sites and the HR2 region was introduced using XhoI/AgeI restriction sites. The PheS expressing cassette was released from pBSPheS (13) using NcoI/BglII and ligated between the 5'miR30 and HR2 sequences into the NcoI/BamHI sites. From the resulting vector a fragment containing the 5'miR30/PheS/HR2 region was released by SfiI and inserted into the SfiI sites of pRTS-1 resulting in pEMI (plasmid for episomal microRNA expression).

Restriction mediated microRNA transfer

A p53-specific hairpin was released from pSM2c (Oligo ID: v2HS_93615, current accession: NM_000546) (7) using XhoI/EcoRI and inserted into pSHUMI. A MAD2-specific microRNA was generated in a two-step PCR: the primers forward 5'-tgctgtgacagtgagcgCTGGGAAGAGTCGGGAC-CACAGtagtgaagccacagatg-3' and reverse 5'-tccgagcagtagcaATGGGAAGAGTCGGGACCACAGtacatctgtgcttcac-3' were annealed, extended by PCR and amplified using universal miR30XhoI/EcoRI primers (miR30Xho I Fw, 5'-CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAG-TGAGCG-3'; miR30EcoR I Rv, 5'-CTAAAGTAGCCCCCTT-GAATTCCGAGGCAGTAGGCA-3'). The resulting fragment was cut with XhoI and EcoRI and inserted into pSHUMI.

The SfiI fragments from pSHUMI containing the microRNA cassettes were inserted into pRTS-1.

Mating-assisted genetically integrated cloning (MAGIC)

Ligation-free gene transfer using the MAGIC system was done essentially as described (13). In brief, the donor bacterial strain (DH10βF'DOT sbcC, PIR1 positive) was transformed with a p53-specific pSM2c vector and grown on kanamycin (30 µg/ml) containing Luria-Bertani (LB) plates at 37°C. The recipient strain (BW287051/pML300) was transformed with the recipient vector pEMI and grown in the presence of 100 µg/ml carbenicillin, 50 µg/ml spectinomycin and 0.2% (w/v) glucose on LB plates at 30°C. Donor and recipient colonies were used to inoculate overnight liquid cultures. The recipient bacteria were washed twice with LB. Both donor and recipient bacteria were diluted 1:50 with LB/0.2% (w/v) rhamnose and grown at 30°C until an OD₆₀₀ of 0.15–0.25. The bacteria were mixed for conjugation in the presence of 0.2% (w/v) L-arabinose and incubated at 37°C for 2 h without and for 2 h with agitation. Recombinant bacteria were plated on a selection plate containing of chloramphenicol (30 µg/ml), carbenicillin (100 µg/ml) and 10 mM DL-p-chlorophenylalanine and incubated at 42°C overnight. Recombination events were detected by colony PCR using the primers [CmR-fw, 5'-CCGTTTGTGATGGCTTCCA-TGTC-3' (corresponding to the chloramphenicol resistance) and pEMI-rev 5'-AATCAAGGGTCCCCAAACTC-3' (matching to pEMI)].

Generation of cell pools

U2OS osteosarcoma cells were transfected by lipofection with pEMI-plasmids using FuGene reagents (Roche) according to the manufacturer's instructions. After 48 h, cells were selected in media containing 150 µg/ml hygromycin for 7 days. Homogeneity of the selected cell pools was tested by addition of 100 ng/ml doxycycline for 24 h and monomeric red fluorescent protein (mRFP)-fluorescence detection.

DNA content analysis by FACS

U2OS (5×10^4) were plated into T25 cell culture flasks. Floating cells and trypsinized cells were collected by centrifugation at 1700 r.p.m. (600 g) for 7 min, cells were fixed with ice-cold 70% ethanol and stored over night on ice. After washing with phosphate-buffered saline (PBS), 1 ml FACS solution [PBS, 0.1% Triton X-100, 60 µg/ml propidium iodide (PI) and 0.5 mg/ml DNase free RNase] was added per sample and incubated at room temperature for 30 min. DNA content was determined by propidium iodide staining (FACSCalibur, Becton-Dickinson).

Western blot analysis

Cells were lysed in RIPA lysis buffer [50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1% NP40, 0.5% (w/v) sodium deoxycholate, 0.1% SDS and complete mini protease inhibitors (Roche)]. Lysates were sonicated and centrifuged at 13 000 r.p.m. (16 060 g) for 15 min at 4°C. Per lane 30 µg of whole cell lysate was loaded on 12% SDS-acrylamide gels, separated and transferred on Immobilon PVDF membranes (Millipore corporation, MA). For immunodetection

membranes were incubated with antibodies specific for p53 (DO-1; Santa Cruz), p21 (Ab-11; NeoMarkers), Mad2 (Clone 48, BD Biosciences) and β -actin (A-2066; Sigma). HRP (horse-radish peroxidase)-coupled secondary antibodies were detected by enhanced chemiluminescence (Perkin Elmer Life Sciences, Boston).

Quantitative real-time PCR (qPCR)

After treatment of U2OS with 100 ng/ml doxycycline or transfection with poly(I:C) (Sigma), total RNA was isolated (Total RNA Isolation System; Promega, Madison, USA). cDNA was generated from 1 μ g total RNA per sample using anchored oligo-dT primers (Reverse-iT First Strand Synthesis; ABgene). qPCR was performed by using the LightCycler (Roche) and the FastStart DNA Master SYBR

Green 1 kit (Roche Applied Science). The following primer sets were used: *IFIT1*, 5'-GCCATTTTCTTTGCTTCCCCTA-3' and 5'-TGCCCTTTTGTAGCCTCCTTG-3'; β -actin, 5'-tgac-attaaggagaagctgtgctac-3' and 5'-gagttgaaggtagttcgtggatg-3'; Mad2-specific-microRNA, 5'-GATGTACTGTGGTCCCGA-CTCT-3' and 5'-TCAAAGAGATAGCAAGGTATTCACT-3'.

The generation of specific PCR products was confirmed by melting curve analysis and gel electrophoresis (data not shown). Fold inductions were calculated as described previously (14) with standardization using β -actin.

Microscopy

mRFP and phase contrast images of living cells were obtained on an inverted Axiovert 200M microscope (Zeiss,

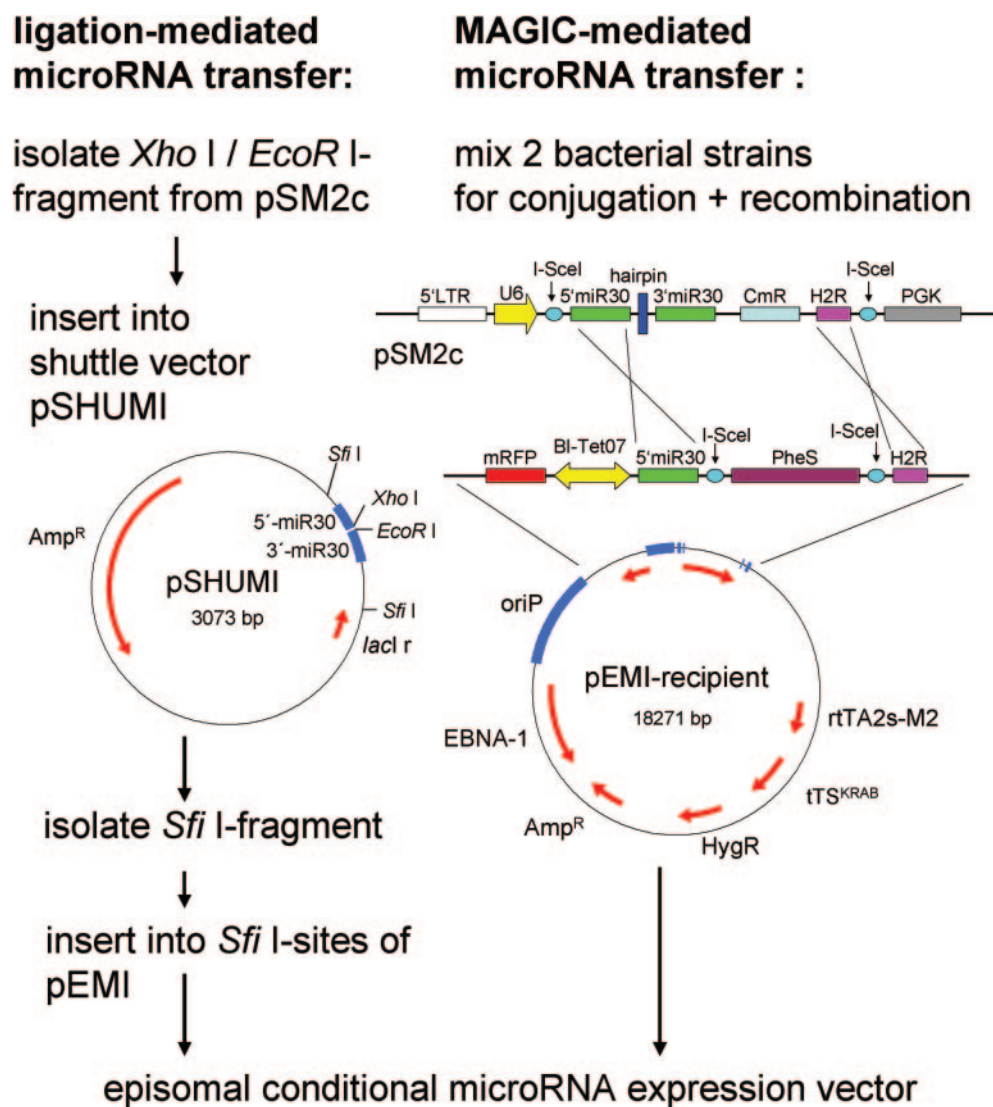


Figure 1. Generation of episomal vectors for conditional expression of microRNAs. Flow chart showing the steps necessary to generate pEMI-vectors harboring microRNA cassettes. Amp^R, ampicillin resistance; CmR, chloramphenicol resistance; EBNA-1, EBV nuclear antigen required for Ori P function; EBV, Epstein-Bar virus; H2R, homology 2 region; Hyg B, hygromycin B; LTR, long-terminal repeats; MAGIC, mating-assisted genetically integrated cloning; miR-30, precursor microRNA; mRFP, monomeric red fluorescent protein; OriP, EBV origin of replication; pEMI, plasmid for episomal microRNA expression; pheS, phenylalanine synthase relaxed-specificity allele Gly294; P_{PGK}, PGK-promoter; pSHUMI, plasmid for shuttling of microRNAs; pSM2c, pSHAG-MAGIC 2c retroviral vector for microRNA expression; P_{tet}-bi-1, bidirectional tet-responsive promoter; rtTA2^S-M2, reverse tetracycline controlled transactivator; tTS^{KRAB}, tetracycline repressed silencer.

Oberkochem, Germany) using Metamorph software (Universal Imaging).

RESULTS

Construction of episomal vectors for RNAi

Recently, comprehensive libraries of microRNAs which were designed to facilitate the RNA interference mediated down-regulation of all human or mouse genes have been described (7). These microRNAs are publicly available and are provided in the pSHAG-MAGIC 2c (pSM2c) retroviral vector (<http://cgap.nci.nih.gov/RNAi>), which provides constitutive expression driven by a long-terminal repeat (LTR). Here, we

have chosen several microRNA cassettes from the human library for transfer from pSM2c vectors to the pRTS-1 vector. Since pRTS is a relatively large vector (~18 kb) the intermediate pUC19-based shuttle vector pSHUMI (plasmid for shuttling microRNAs) is necessary for the transfer procedure (Figure 1). For a faster transfer of microRNAs we also adapted the pRTS vector to the ligation-free MAGIC technique (mating-assisted genetically integrated cloning) (13). The resulting pEMI (plasmid for episomal microRNA expression; Figure 1) harbors a *pheS* Gly294 allele encoding a tRNA synthase for phenylalanine with relaxed specificity, which incorporates toxic chloro-phenylalanine and thereby facilitates selection against non-recombinant clones. Bacteria

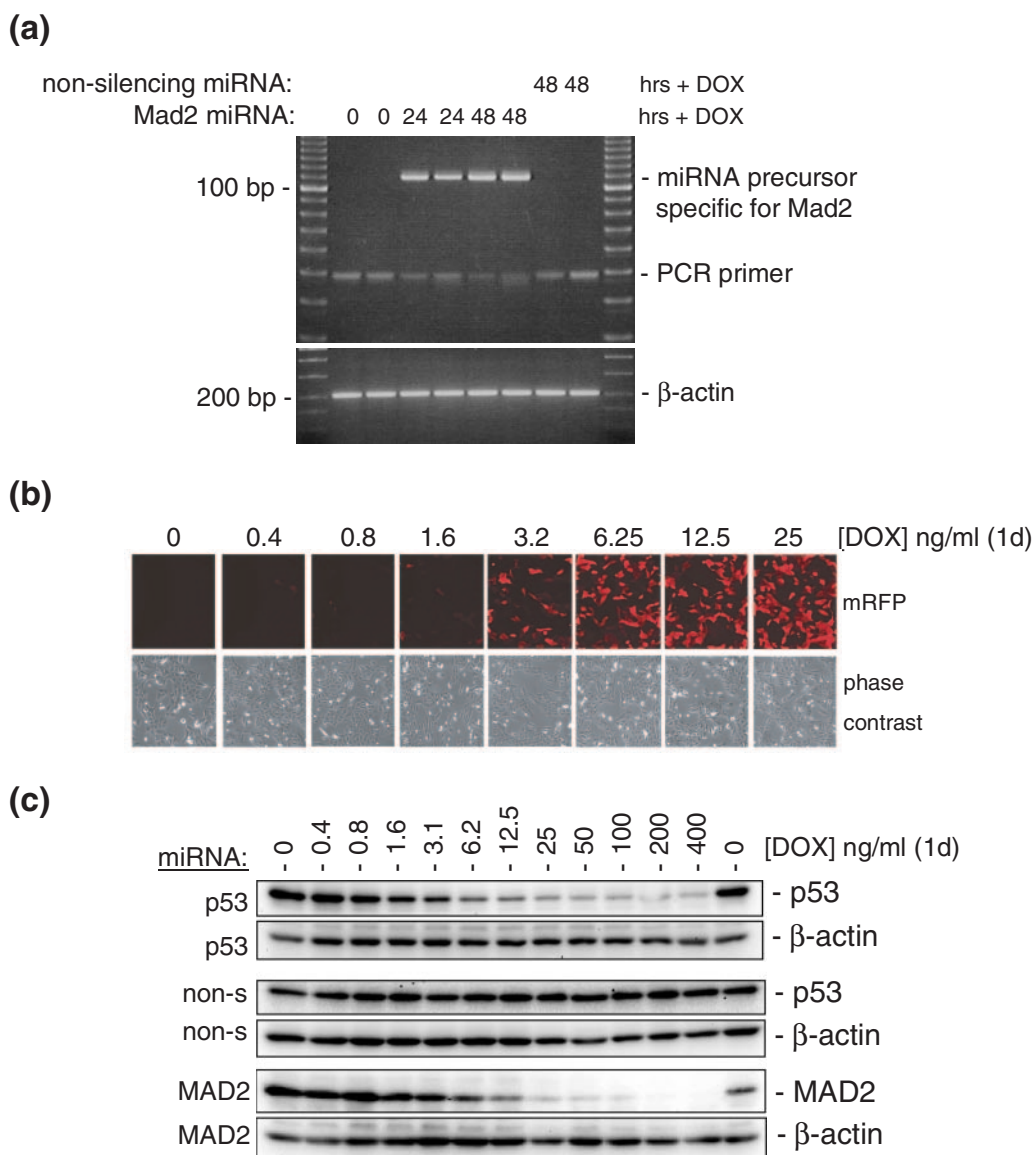


Figure 2. Conditional microRNA expression mediated by pEMI. (a) RT-PCR analysis of microRNA expression. U2OS cells stably transfected with pEMI encoding a Mad2-specific or non-silencing microRNA (miRNA) were treated with 200 ng/ml DOX for 24 or 48 h. The analysis was performed in duplicates. After 30 cycles of PCR DNA fragments were separated either on 15% polyacrylamide gels (for detection of the Mad2-specific miRNA precursor) or 2% agarose gel (for β-actin). DNA markers in outer lanes: 10 bp ladder (upper panel) and 100 bp ladder (lower panel). (b) Detection of mRFP expression 24 h after addition of the indicated doxycycline (DOX) concentrations to U2OS cells transfected with pEMI vectors encoding p53-specific miRNAs. Exposure times: 500 ms for mRFP, 50 ms for phase contrast. (c) Doxycycline (DOX) dose-reponse of p53 conditional knockdown: U2OS cell pools stably transfected with pEMI-p53miRNA plasmid were treated with the indicated DOX concentrations for 24 h (upper panel). Control cells with pEMI-non-silencing (non-s) microRNA expression are also shown (middle panel). pEMI-MAD2miRNA-mediated down-regulation of MAD2 expression (lower panel).

containing the pEMI-recipient vector were conjugated with bacteria containing a pSM2c vector encoding a p53-specific microRNA. Of 80 resulting bacterial colonies 79 (98.7%) harbored pEMI vectors containing the p53-microRNA as determined by colony PCR (data not shown). Successful recombination was also confirmed by restriction and sequence analysis (data not shown).

Functional evaluation of pEMI vectors

The human osteosarcoma cell line U2OS was transfected with pEMI vectors encoding either p53- or MAD2-specific or non-silencing microRNAs, which do not recognize any human mRNA. Selection for cells containing the pEMI vectors with hygromycin B was completed within 7 days. The resulting pools of resistant cells were analyzed for RT-PCR analysis to determine the expression of the ectopic microRNA after addition of doxycycline (DOX) (Figure 2a). In the absence of DOX no MAD2-specific microRNA was detected after 30 PCR cycles. However, 24 h after addition of DOX the microRNA was expressed. By 48 h the expression increased further as determined by quantitative PCR (data not shown). As no microRNA expression was detected in the absence of DOX, these results show that the pEMI vectors mediate an extremely stringent control over microRNA expression. In line with this observation the cell pools were consistently devoid of mRFP expression in the absence of DOX as determined by live cell fluorescence microscopy (Figure 2b). Within 24 h after addition of DOX approximately half of the cells were positive for mRFP at 3.2 ng/ml DOX and virtually all cells were positive at 25 ng/ml (Figure 2b). Even at only 0.8 ng/ml DOX a decrease in p53 protein levels was observed within 24 h (Figure 2c). At a DOX concentration of 25 ng/ml the degree of p53 down-regulation and frequency of mRFP-positive cells reached its maximum and p53 protein was hardly detectable. Similar

results were obtained with microRNAs specific for *MAD2*, although the maximal down-regulation of *MAD2* was reached at higher DOX-concentrations, presumably due to the different half-lives of p53 and *MAD2* or varying efficiencies of the respective microRNAs. Induction of a non-silencing microRNA did not affect the levels of p53 protein. In a time course analysis the level of p53 protein began to decrease one day after induction of the p53 microRNA (Figure 3a). At 5 ng/ml DOX the maximum knock-down was reached after 2–3 days. After a 48 h exposure to DOX, its removal led to complete restoration of p53 expression within 6 days which was paralleled by the disappearance of mRFP as determined by fluorescence microscopy (Figure 3b).

When p53 was activated by treatment with the DNA damaging agent etoposide, an increase in the levels of p53 and its target p21 was observed within 3.5 h (Figure 4a). Induction of the p53-specific microRNA 2 days before etoposide addition resulted in strong suppression of p53 and prevented any significant increase in p53 and p21 protein after DNA damage. A control cell population expressing a non-silencing microRNA showed normal stabilization of p53 and p21 induction after addition of etoposide (Figure 4a). Furthermore, a flow cytometric analysis revealed that suppression of p53 inhibited arrest in the G₁ phase upon etoposide treatment, whereas in the absence of p53-specific microRNAs, or upon expression of the non-silencing microRNA cells arrested normally (Figure 4b).

As an example for the inactivation of an essential gene by the system introduced here we conditionally down-regulated the expression of the *MAD2* protein, which was shown to result in mitotic failure and extensive cell death when permanently inactivated (15,16). After introduction of the pEMI-plasmid encoding a *MAD2*-specific microRNA we observed no effect on the viability and cell cycle distribution. Only when *MAD2* was down-regulated by addition of DOX an

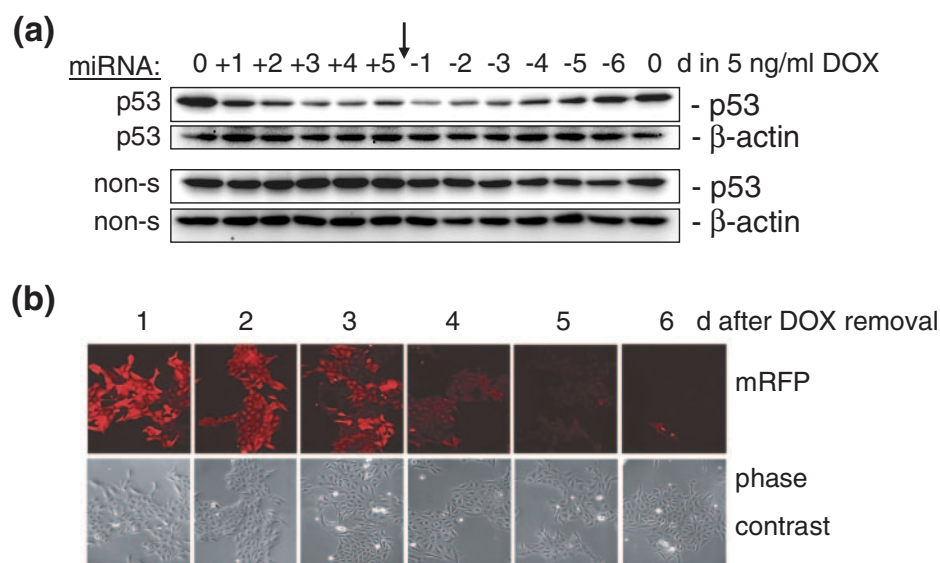


Figure 3. Reversibility of microRNA expression mediated by pEMI. (a) Time course and reversibility of pEMI-mediated p53 knockdown. After stable transfection with pEMI-p53miRNA or controls, U2OS cells were treated with 5 ng/ml DOX for the indicated periods. For knockdown reversal, 5 ng/ml DOX was removed after 2 days of microRNA induction and cell lysates were harvested 1–6 days later (–1 to –6). Control cells were treated identically (lower panel). (b) Detection of mRFP expression after removal of DOX for the indicated number of days. Exposure times: 500 ms for mRFP, 50 ms for phase contrast.

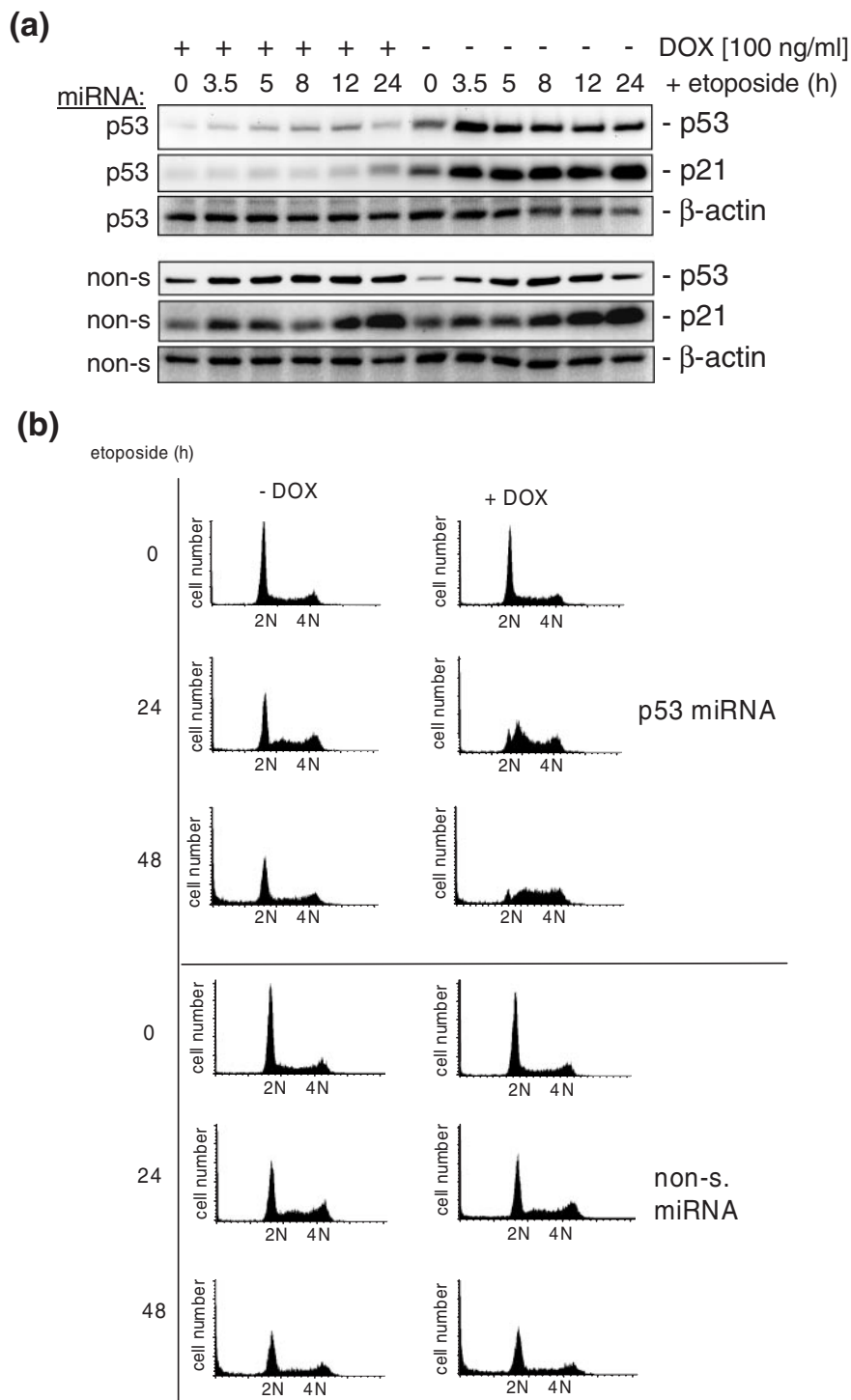


Figure 4. DNA-damage response is impaired after pEMI-mediated knockdown of p53. (a) Western blot analysis with U2OS cells stably transfected with pEMI-p53miRNA plasmids. Cells were cultured in 100 ng/ml DOX for 2 days and 20 μg/ml etoposide added for the indicated periods (upper panel). U2OS cells stably transfected with a non-silencing microRNA encoding pEMI plasmid served as a control (lower panel). (b) Fluorescence activated cell sorting (FACS) analysis of U2OS cells stably transfected with pEMI-p53miRNA or pEMI non-silencing-miRNA plasmids. Cells were cultured in 100 ng/ml DOX for 2 days and etoposide was added for the indicated time periods (20 μg/ml).

increased fraction of apoptotic cells and altered cell cycle distribution was observed (data not shown). These results show that the expression of microRNAs can be tightly controlled using pEMI vectors, which are therefore useful for studying essential genes.

To rule out toxic side effects mediated by the pEMI vector-driven microRNA expression, we analyzed the expression of the *IFIT1* (interferon-induced protein with tetratricopeptide repeats 1) gene by qPCR. Others have shown that expression of *IFIT1* mRNA is rapidly induced upon IFN treatment (17).

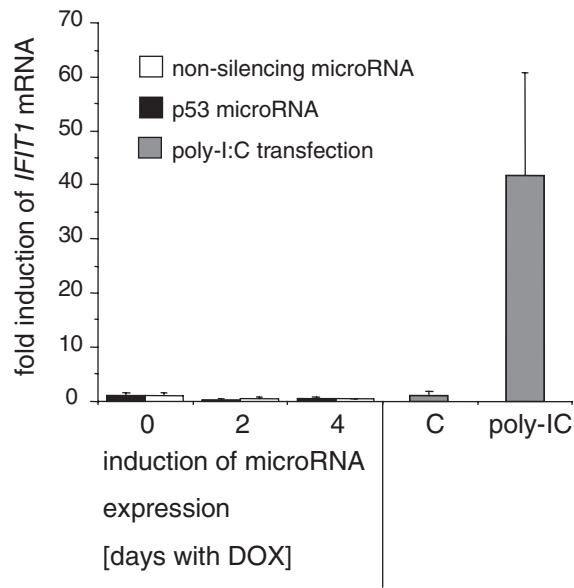


Figure 5. pEMI-driven microRNA expression does not elicit a dsRNA response. qPCR analysis of *IFIT1* (interferon-induced protein with tetratricopeptide repeats 1) expression after induction of microRNAs or treatment with poly(I:C). U2OS pools were treated with 100 ng/ml DOX for the indicated time periods to induce non-silencing or a p53-specific microRNA driven by pEMI vector. As a positive control for *IFIT1* gene induction, U2OS cells were transfected with poly(I:C) dsRNA (IC) or subjected to a mock transfection (C) for 18 h. Shown are the relative expression levels of *IFIT1* normalized to β -actin expression. *IFIT1* expression levels in untreated or MOCK transfected cells were set to 1. All experiments were performed in triplicates. Error bars indicate standard deviations.

Besides interferon, double-stranded RNA (dsRNA) and viral infection have been shown to increase the expression of *IFIT1* (18). pEMI-driven expression of a non-silencing microRNA for 2 and 4 days did not provoke an increased *IFIT1* expression (Figure 5). Also expression of a p53-specific microRNA did not lead to any increase in *IFIT1* expression, whereas transfection of U2OS cells with a synthetic dsRNA [poly(I:C)] led to ~40-fold increase in *IFIT1* mRNA expression within 18 h after transfection. Incubation of U2OS cells in media containing poly(I:C) also increased the level of *IFIT1* up to ~7-fold (data not shown) indicating this cell line is in principle very sensitive towards the presence of dsRNA. Furthermore, proliferation assays showed no significant anti-proliferative effect of pEMI-driven microRNA over-expression and cells were viable for several weeks when expressing microRNAs not targeting essential genes, whereas treatment with poly(I:C) led to an apoptotic response 2 days post transfection presumably by activating the dsRNA response (data not shown). Taken together, expression of ectopic microRNA by pEMI vectors does not lead to a dsRNA response or other toxic side effects.

DISCUSSION

A number of different conditional systems for RNA interference have been described previously (12, 19–26). They utilize tetracycline regulation or CRE-recombinase mediated activation of shRNAs or microRNAs). However, they rely on several separate plasmids providing the necessary regulators *in trans*. One major advantage of the system described in

this manuscript lies in the possibility to establish cells exhibiting inducible knock-down of the gene/protein of interest in one step as all components have been integrated on a single vector. This feature facilitates the analysis of cells which are characterized by a low transfection efficiency or a limited life span which presumably applies to most primary cells. With this vector system we could generate cell pools which homogeneously express the microRNA of interest in an inducible manner in only one week. Furthermore, the analysis is not restricted to a cell clone expressing the trans-regulator (tTA or CRE) at the appropriate level. Instead, a pool of different cells expressing the pEMI vector can be analyzed. Obviously, this provides significant advantages and protects against misleading clonal effects.

The vector system employed here is episomal and therefore microRNA expression levels are not dependent on the site of integration. Furthermore, the combination of a repressor and an activator acting through the tet-operon prevents expression in the off-state and allows precise titration of the expression level of the respective microRNA. Our determination of ectopic microRNA expression validate this point, as we were unable to detect expression in the uninduced state. The pEMI vector allows the conditional knockdown of proteins within a short period without the need for viral infection procedures or repeated transfections of several plasmids encoding the different components of the system. The expression of a fluorescent protein from the bidirectional promoter allows transfected cells to be sorted by flow cytometry, circumventing single cell cloning. Furthermore, cells exhibiting RNA interference can be traced in a heterogeneous population of cells in cell culture or in animals. In addition, the fluorescent protein marker facilitates the detection of any potential loss of microRNA expression in a cell population. Therefore, laborious, parallel analyses to confirm microRNA expression or its effects on the targeted gene are not necessary for every experiment. A further useful feature of this system is the possibility to reverse the knockdown and hence the induced phenotype. Thereby the causal link between the knockdown of any gene and the observed phenotype can be strengthened as non-reversible phenotypes may represent secondary effects.

We observed different degrees of inducibility over time in different cell lines. Whereas U2OS and H1299 cells showed a decrease of inducibility after 4 weeks of passaging, MCF7 cells did not show any decrease in the frequency of inducible (mRFP-positive) cells after several months of propagation. Cells which show loss of expression after continued passaging should therefore be conserved by freezing at early passages.

Mammalian cells discriminate between microRNAs that are endogenous DICER products and non-self dsRNAs, e.g. by-products of viral replication (27). The microRNAs which were used here are embedded in an authentic human miRNA miR30 (28). This human microRNA has been shown to be efficiently recognized and processed by the Drosha-DGCR8 complex and DICER in mammalian cells (28) and therefore should not induce non-specific effects that are triggered by viral dsRNAs, as, for example, activation of the interferon pathway. A commonly used and sensitive marker for the activation of these dsRNA signaling pathways leading to IRF-3 and IFN induction is the increased expression of the

dsRNA induced protein 56K (p56) encoded by the *IFIT1* (interferon-induced protein with tetratricopeptide repeats 1) gene (18,29–31). Expression of p56 has furthermore been closely linked to the general toxicity of distinct siRNA duplexes (27). We found that neither non-silencing microRNA nor a p53-specific microRNA expressed by the episomal pEMI vector triggered a dsRNA pathway response.

The controlled inactivation of genes by the system introduced here may be useful in certain therapeutic regimes and prevent the potential toxicity or immunogenicity which has been discussed for therapeutic applications of ectopic RNA interference. Using the bi-directional promoter it may be possible to express an RNA interference resistant wild-type protein to substitute for a mutant protein, which is simultaneously down-regulated by the ectopic microRNA. A similar approach has been recently described for the treatment of sickle cell anemia (32). As described above the pEMI vector is compatible with recently generated microRNA libraries and will therefore presumably become a widely used tool for conditional RNA interference (7).

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Conflict of interest statement. None declared.

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