Conditional Suppression of Cellular Genes: Lentivirus Vector-Mediated Drug-Inducible RNA Interference

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RNA interference has emerged as a powerful technique to downregulate the expression of specific genes in cells and in animals, thus opening new perspectives in fields ranging from developmental genetics to molecular therapeutics. Here, we describe a method that significantly expands the potential of RNA interference by permitting the conditional suppression of genes in mammalian cells. Within a lentivirus vector background, we subjected the polymerase III promoter-dependent production of small interfering RNAs to doxycycline-controllable transcriptional repression. The resulting system can achieve the highly efficient and completely drug-inducible knockdown of cellular genes. As lentivirus vectors can stably transduce a wide variety of targets both in vitro and in vivo and can be used to generate transgenic animals, the present system should have broad applications.

The externally controllable expression of exogenous cDNAs can be readily obtained in cells or in animals owing to techniques pioneered more than a decade ago (2, 8). Recently, it was demonstrated that the knockdown of endogenous genes could be achieved by RNA interference, and plasmid- or viral vector-based delivery systems for the stable expression of small interfering RNAs (siRNAs) were rapidly created (1, 3, 5, 7, 9, 17). In many situations, however, it is desirable to suppress genes in a regulated fashion, for instance, to study cellular factors that play essential roles during differentiation or development. On the basis of this premise, we created a lentivirus vector-based system for drug-inducible production of siRNAs in stably transduced mammalian cells.

MATERIALS AND METHODS

Vector construction. Vectors were constructed by using standard cloning procedures. The pSUPER and pSUPER-p53 constructs were described previously (5). pSUPER-siLamin was a gift from R. Oggi (Lausanne, Switzerland). pLV-H was constructed by inserting the H1 promoter from pSUPER into the 5′ long terminal repeat (LTR) of pWPXL (http://www.tronolab.unige.ch/). To construct pLV-TH, the tetrO cassette was excised from pUHD13-3 (obtained from H. Bujard, Heidelberg, Germany) and cloned into pLV-H, upstream of the H1 promoter. Finally, the H1 promoter cassette in pLV-H and pLV-TH was replaced by the H1-siRNA cassette excised from pSUPER-siRNA, generating pLV-HsiRNA and pLV-THsiRNA, respectively. The sequence encoding tTR-KRAB (kindly provided by P. Lorenz and H.-J. Thiesen, Rostock, Germany) was cloned into pWPXL, replacing the green fluorescent protein (GFP) marker (pLV-tTR-KRAB), or as part of a bicistronic unit also encoding Discosoma sp. Red, using the eucaryophilic yeasts vector 5′ internal ribosome entry site.

The lentivirus vectors described here are available upon request (www.tronolab.unige.ch).

Cell culture and transduction with lentivirus vectors. The 293T, HeLa, and MCF-7 cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. All recombinant lentiviruses were produced by transient transfection of 293T cells according to standard protocols (21). Briefly, subconfluent 293T cells were cotransfected with 20 μg of a plasmid vector, 15 μg of pCMV-ΔR8.91, and 5 μg of pMD2G-VSVG by calcium phosphate precipitation. After 16 h medium was changed, and recombinant lentivirus vectors were harvested 24 h later.

To analyze the regulation of GFP, a HeLa cell clone carrying a single copy of the WPXL-GFP provirus (HeLa-GFP) was used. For transduction, HeLa-GFP, MCF-7, or HeLa cells were plated on 24-well plate (20 × 10^4 cells/well), and after 16 h medium containing recombinant lentivirus vectors was added. Following 16 h of incubation, the cells were washed and split, and doxycycline (DOX) was added to half of the transduced cells at a final concentration of 5 μg/ml. Five days later the cells were harvested and analyzed by fluorescence-activated cell sorting (FACS).

Western blotting. Cell extracts were prepared in radioimmunoprecipitation assay lysis buffer (25 mM Tris [pH 7.5], 1% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA, 150 mM NaCl) containing a cocktail of protease inhibitors (Sigma). The protein samples (10 μg) were separated on sodium dodecyl sulfate–10% gradient polyacrylamide gels, electroblotted to polyvinylidene difluoride membranes (Perkin-Elmer), and exposed to antibodies against p53 (Santa Cruz Biotechnology), lamin A/C (Santa Cruz Biotechnology), and actin (Calbiochem). Antibodies conjugated with horseradish peroxidase (Amersham) and enhanced chemiluminescence (Amersham) were used for detection.

FACS analysis. Harvested HeLa-GFP cells transduced with lentivirus vectors carrying ΔNGFR cDNA were incubated with monoclonal antibody specific for human nerve growth factor receptor NGFR (Becton Dickinson PharMingen) labeled with phycoerythrin, washed twice, and analyzed with a FACSscan (Becton Dickinson) for green (GFP) and red (NGFR-phycoerythrin) fluorescence. MCF-7 and HeLa cells cotransduced with LV-THis/p53 or LV-THis/lamin and pLV-tTR-KRAB-Red and cultured in presence or absence of DOX were harvested and analyzed with a FACSscan for green and red (dsRed) fluorescence.

Immunofluorescence. MCF-7 and HeLa cells cotransduced with LV-THis/p53 or LV-THis/lamin and pLV-tTR-KRAB-Red and cultured for 5 days in the presence or absence of DOX were fixed with methanol (10 min, −20°C), blocked with phosphate-buffered saline−1% bovine serum albumin, and stained with antibodies against p53 (Santa Cruz Biotechnology) or lamin A/C (Santa Cruz Biotechnology), using secondary antibodies conjugated with Alexa 633 (Molecular Probes) for detection. Images were acquired by using three-color confocal microscopy (LSM 510; Carl Zeiss) and analyzed with Zeiss software.

RESULTS AND DISCUSSION

We took advantage of a tetracycline-controlled hybrid protein, tTR-KRAB, in which the tetracycline repressor (tTR) from Escherichia coli Tn10 is fused to the KRAB domain of
human Kox1 (6, 8). KRAB is an approximately 75-amino-acid transcriptional repression module found in many zinc finger-containing proteins, which can suppress, in an orientation-independent manner, both polymerase II- and polymerase III-mediated transcription within a distance of up to 3 kb from its binding site, presumably by triggering the formation of heterochromatin (4, 6, 12, 14, 19). When linked to the DNA-binding domain of tTR, KRAB can modulate transcription from an integrated promoter juxtaposed with tet operator (tetO) sequences (6). In the absence of DOX, tTR-KRAB binds specifically to tetO and suppresses the activity of the nearby promoter. Conversely, in the presence of DOX, tTR-KRAB is sequestered away from tetO, thus permitting gene expression (6).

We used human immunodeficiency virus type 1-derived lentivirus vectors (designated LV) as delivery vehicles because we aimed for a system that would be easy to apply to a broad variety of cellular targets, both ex vivo (cell lines, primary cells...
including stem cells, fertilized oocytes, and blastocysts) and in vivo (e.g., brain and liver) (9, 13, 15, 16–18, 20), and because tetO-linked transcriptional units are repressed by tTR-KRAB only when integrated in the genome. The tTR-KRAB cDNA was expressed from the ubiquitously active EF1-α promoter as part of a bicistronic transcript also producing the dsRed marker (Fig. 1A, LV-tTR-KRAB). The regulated siRNA vectors were constructed by inserting a tetO-H1 promoter-siRNA cassette into the U3 region of the 3′ LTR of a self-inactivating lentivirus vector (Fig. 1A, LV-THsi). During reverse transcription, the vector RNA 3′ U3 region serves as the template for the synthesis of its 5′ DNA homologue, so that the tetO-H1-siRNA cassette is duplicated in the integrated provirus (Fig. 1B). We chose this double-copy configuration to obtain higher rates of siRNA synthesis. Sequences encoding siRNA hairpin precursors were designed as described previously (5). Control vectors carried either a constitutively active H1-siRNA cassette (LV-Hsi) or the H1- or tetO-H1 transcriptional elements without downstream siRNA-coding sequence (LV-H and LV-TH, respectively). All siRNA and control vectors also carried a marker gene downstream of an internal EF1-α promoter. We predicted (Fig. 2A) that cells cotransduced with LV-THsi and LV-tTR-KRAB would normally express the gene targeted by the siRNA when maintained in the absence of DOX, owing to tTR-KRAB-mediated suppression of siRNA synthesis. In contrast, addition of the drug would relieve this inhibition and allow for target gene downregulation (Fig. 2B). Expression of the internal marker gene would also be subjected to conditional tTR-KRAB repression, thus providing an internal monitoring device.

In a first series of experiments, we probed the ability of this system to regulate the production of GFP in HeLa cells stably expressing this fluorophore (Fig. 3A). Vectors were used at a multiplicity of infection of 10 to ensure good rates of (co)transduction. HeLa-GFP cells transduced with the empty LV-TH vector remained strongly GFP positive irrespective of their culture conditions. In contrast, cells transduced with the constitutively active LV-Hsi/GFP vector exhibited a strong downregulation of the marker. In cells transduced with the controllable LV-THsi/GFP vector, GFP expression was observed only in the presence of tTR-KRAB and in the absence of DOX (Fig. 3A). Correspondingly, in the absence of drug, tTR-KRAB suppressed the expression of the vector’s ΔNGFR internal reporter gene (Fig. 3B). As expected, the tTR-KRAB-mediated suppression of siRNA production was equally efficient whether tetO was inserted in the sense or antisense orientation and upstream or downstream of the H1 promoter (data not shown).

Next, we tested our system for the regulation of truly endogenous genes. We chose p53 and lamin genes as targets because highly effective siRNAs directed against these genes were previously identified and well characterized (5, 7). MCF-7 breast cancer cells were used as substrates for p53 downregulation studies (Fig. 4, left panels). Cells cotransduced with LV-tTR-KRAB and LV-THsi/p53 produced wild-type levels of p53 when cultured in the absence of DOX, indicating full repression of siRNA synthesis (lower blot, lane 7). This repression was mediated by TTR-KRAB, since p53 was undetectable in cells transduced only with LV-THsi/p53, whether or not DOX was present in the culture medium (upper blot, lanes 7 and 8). In contrast, addition of the drug to the dually transduced cells resulted in rates of p53 downmodulation as robust as those observed in cells containing the constitutively active LV-Hsi/p53 vector (compare lane 8 in the lower blot with lanes 5 and 6 in both blots). Similar results were obtained for lamin in HeLa cells transduced with the corresponding siRNA lentivirus vectors (Fig. 4, right panels). It is noteworthy that in both settings the drug-induced production of the siRNAs, and hence the suppression of the p53 or lamin target gene, correlated with the expression of the lentivirus vector internal GFP.
marker, whether examined by Western blotting (Fig. 4) or by FACS or confocal microscopy (data not shown).

Taken together, these results indicate that the tTR-KRAB-regulated, lentivirus vector-mediated delivery of siRNAs allows for the controllable suppression of cellular genes both with a high degree of efficacy and without significant leakiness. To complete the characterization of this system, we defined its kinetics and DOX dose responsiveness (Fig. 5). We chose p53 as target for these analyses because the half-life of this protein is relatively short, around 12 h. In MCF-7 cells dually transduced with the LV-THsi/p53 and LV-tTR-KRAB vectors, p53 steady-state levels started to decrease as early as 12 h after addition of 5 μg of DOX per ml to the culture medium and became undetectable by Western blotting within 36 h (Fig. 5).
5A). This suggests that RNA interference was fully effective in less than 24 h, implying that the DOX-mediated sequestration of tTR-KRAB rapidly unleashes high rates of siRNA production from the integrated H1 promoters. A dose-response analysis further revealed an extreme sensitivity to DOX control, while pointing to the possibility of some tuning of the gene suppression. Indeed, whereas p53 downregulation was already apparent at the low DOX concentration of 0.004 μg/ml, full-blown suppression was achieved only at a dose of 0.25 μg/ml (Fig. 5B). The anti-p53 siRNA used in this experiment being very efficient, a greater range of DOX concentrations may allow for a modulation of the degree of gene knockdown with siRNAs of lower specific activity.

In summary, we provide a system for the conditional suppression of genes in mammalian cells. The versatility of its mode of delivery suggests very broad uses, as lentivirus vectors can transduce a wide range of targets, including stem cells, and can be used for generating transgenic animals from several species. In the latter setting, the system described here should offer significant advantages over currently available conditional knockout techniques, among which are its reversibility and simplicity of use. While the lentivirus vector-mediated delivery of drug-inducible RNA interference may thus be of particular interest for the study genes involved in development and differentiation, it is likely to be useful in many other areas of biology as well.

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