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A universal transgene silencing method based on RNA interference

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ABSTRACT

Inducible gene expression systems have contributed significantly to the understanding of molecular regulatory networks. Here we describe a simple and powerful RNA interference-based method that can silence the expression of any transgene. We first used an IRES bicistronic lentiviral vector and showed that targeting the second cistron with a specific siRNA resulted in silencing of both transgenes. We then inserted a siRNA minimal target sequence in the 3′-untranslated region (3′-UTR) of a transgene and showed that the cognate siRNA delivered by a lentiviral vector led to the partial silencing of the transgene. The multimerization of this siRNA target sequence led to the highly efficient silencing of four different transgenes. This new method to silence transgene expression is more versatile than existing methods of conditional inactivation of gene expression, such as transcriptional switches or site-specific recombination. It is applicable to a wide variety of models including primary cells, terminally differentiated cells and transgenic animals.

INTRODUCTION

Among the different methods collectively referred to as reverse genetics, conditional gene expression systems have been widely used *in vitro* and *in vivo* to gain considerable knowledge on fundamental biological processes. Different systems have been designed that allow regulated expression of a given transgene, most of them relying on transcriptional regulation or site-specific recombination. However, several limitations are associated with these approaches, as they require the delivery of *trans*-acting factors (a transcription activator or a recombinase) engineered in such a way that their activities are drug dependent (1). Because these systems often require the construction of cell lines that stably express these *trans*-acting factors and the selection of highly responsive clones, they are not suitable for use in primary, quiescent or differentiated cells.

Based on an evolutionary conserved cellular process, RNA interference (RNAi) has provided a new approach to manipulate gene expression and elucidate gene function (2). The siRNA technology is based upon introduction inside cells of short (21–23 nt) double-stranded RNAs (siRNAs) that induce the specific degradation of their cognate target mRNAs. As many cell types are refractory to nucleic acid transfection methods, efforts have been made to design short hairpin RNAs (shRNAs) delivering viral vectors that strongly facilitate the expression in a wide range of cell types and that ensure long-term silencing.

We have developed a new and universal transgene silencing method based on RNA interference. The method relies on two lentiviral vectors, one directing the expression of a transgene, and the other directing the expression of a shRNA. The transgene expression vector directs the trancription of an mRNA harboring a multimerized minimal siRNA target sequence located within its 3′-untranslated region (3′-UTR). The silencing vector delivers a universal shRNA specific to the multimerized target region, resulting in efficient silencing of the transgene. The simplicity and versatility of this method should make it very useful, especially in those experimental systems where existing conditional gene expression technologies are not applicable.

MATERIALS AND METHODS

Constructs

All constructs described here were derived from GAE0, created by replacing the eGFP gene of pSIV-$_{RMES}$GAE (3,4) by a polylinker ligated between the AgeI and XhoI restriction sites. To create GAEts1 and GAEts3, we digested GAE0 with HindIII and XhoI and ligated the following hybridized oligodeoxynucleotides: for GAEts1, sense 5′-AGCTTGCTCGAGCAAGCTGACCCTGAAGTTCATCTGATGCATGG-3′, antisense 5′-TCGACCATGCATCAGATGAACTTCAGGGTCAGCTTGCTCGAGCA-3′; for GAEts3, sense 5′-AGCTTGCTCGAGCAAGCTGACCCTGAAGTTCATCTGCAAGCTGACCCTGAAGTTCATCTGCAAGCTGACCCTGAAGTTCATGCATGG-3′, antisense 5′-TCGACCATGCATGAACTTCAGGGTCAGCTTGCAGATGAACTTCAGGGTCAGCTTGCAGATGAACTTCAGGGTCAGCTTGCTCGAGCA-3′. These oligonucleotide duplexes abolished the 3′ XhoI site and created a new 5′ XhoI site. Sequences coding for ZEO^R, GFPμ, TRX, TRX-Ires-GFP and H-2K^k were cloned into AgeI/XhoI-cut GAE plasmids. To create SirGFP, we removed the CMV promoter and the polylinker from GAE0 by a BamHI/BglII digestion and we inserted the minimal human H1 promoter (positions −100 to −1) (5) in the 3′LTR between positions 9778 and 9928 (SIVmac-251

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sequence, GenBank accession No. M19499). We digested the resulting construct with BglII and HindIII and ligated the following oligodeoxynucleotides coding for the SirGFP shRNA: sense 5′-GATCCCCGCAAGCTGACCCTGAAGTTCTTCAAGAGAGAACTTCAGGGTCAGCTTGCTTTTTGGAAA-3′, antisense 5′-AGCTTTTCCAAAAAGCAAGCTGACCCTGAAGTTCTCTCTTGAAGAACTTCAGGGTCAGCTTGCGGG-3′. The oligodeoxynucleotides coding for the SirGFPμ shRNA were sense 5′-GATCCCGCGGCAAACTCACACTCAAGTTTCAAGAGAACTTGAGTGTGAGTTTCCGTTTTTGGAAA-3′, antisense 5′-AGCTTTTCCAAAAACGGCAAACTCACACTCAAGTTCTCTTGAAACTTGAGTGTGAGTTTGCCGCGG-3′. To create SirCtl, we used the following oligodeoxynucleotides: sense 5′-GATCCCCAGAGTTGCTTTCTCCGTTCTTCAAGAGAGAACGGAGAAAGCAACTCTTTTTTGGAAA -3′, antisense 5′-AGCTTTTCCAAAAAAGAGTTGCTTTCTCCGTTCTCTCTTGAAGAACGGAGAAAGCAACTCTGGG-3′. To create the GFPμ coding sequence, we performed two PCR reactions on pSIV-$_{RMES}$GAE as a template, using the following pairs of oligodeoxynucleotides: (1) sense 5′-GGGAATTCTGGCTACCGGTCGCCACCAG-3′, (2) sense 5′-GGCAA**A**CT**C**A-C**A**CT**C**AAGTTT**T**AT**T**TGCACCACCGGC-3′, (3) antisense 5′-GCCGGTGGTGCAA**A**ATA**A**ACTT**G**AG**T**GT**G**AG**T**TTGCCGTAGG-3′, (4) antisense 5′-GCTACTCGAGGATCCTCACTTGTACAGCTCGTCCATGC-3′. Bold bases correspond to the mutations introduced in GFP. Two PCR reactions were performed with oligodeoxynucleotides (1) and (3), and (2) and (4), respectively. Amplification products were purified, mixed and a high fidelity PCR reaction was carried out with oligodeoxynucleotides (1) and (4) to generate GFPμ.

Lentiviral vector manipulations

SIV vectors were produced as previously described (3) upon cotransfection of helper constructs (coding for *gag*, *pol*, *tat*, *rev* and VSV-G) in 293T cells. Filtered supernatants were concentrated 100-fold by ultracentrifugation. To determine viral titers, we transduced 5×10^5 293T target cells with serial dilutions of vector preparation and we counted GFP-positive cells 72 h later with a flow cytometer. To measure exogenous reverse transcriptase activity, we incubated viral vector dilutions 1 h in 50 mM Tris–HCl (pH 8.3), 10 mM MgCl2, 0.2 M KCl, 0.5 mM [^{3}H]TTP, 0.2 mM polyA(dT)15, 0.05% v/v NP40 at 37°C. Samples were loaded onto DE81 filters and the DNA-incorporated radioactivity was quantified using a phosphoimager following washing.

Cell culture and detection of transgene expression

All cells were cultured at 37°C in Dubelcco's Modified Eagle's Medium (Invitrogen-Gibco) complemented with 10% v/v fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml). We detected H-2K^k expression using the MACS H-2K^k-FITC kit, according to the instructions of the manufacturer (Miltenyi Biotech). Zeocin (Invitrogen) was used at a final concentration of 200 μg/ml. For western blot analysis, we lysed the transduced cells at 4°C in 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% NP40 v/v for 1 h. HA-tagged-TRX was revealed with an HA antibody (Sigma).

RESULTS

We routinely direct the stable expression of thioredoxin (TRX)-based peptide aptamer libraries in various mammalian cell types and we set out to develop a facile RNAi-based method that could silence efficiently the expression of every library member in every cell type. To this end, we designed pSIV-TRX-i-GFP and SirGFP, two SIV-derived lentiviral vectors encoding a TRX-IRES-GFP bicistronic mRNA and a shRNA directed against GFP, respectively (Figure 1A). We detected a strong expression of both TRX and GFP in HeLa cells transduced with pSIV-TRX-i-GFP and we silenced the expression of both proteins by transducing SirGFP (Figure 1B and C). We observed a similar silencing of expression of a peptide aptamer library (data not shown). As a control, we introduced six mutations in the shRNA coding sequence of SirGFP to create SirGFPμ and we showed that the transduction of this vector did not affect the expression of both TRX and GFP (Figure 1B and C). Since we were successful in silencing the expression of both cistrons by solely targeting the second cistron of the bicistronic messenger, we hypothesized that inserting a minimum SirGFP target sequence within the 3′-UTR of an mRNA would direct its degradation by the cognate siRNA. To test this idea, we designed two SIV-derived vectors, GAEts1-H2K and GAE-H2K (Figure 2A), that direct the expression of the mouse MHC protein H-2K^k, with or without a single SirGFP minimal target sequence positioned in the 3′-UTR of the transgene, respectively. The transduction of these two vectors in 293T cells directed a similar expression level of H-2K^k. The transduction of SirGFP did not affect H-2K^k expression directed by GAE-H2K but produced a 34% inhibition of H-2K^k expression directed by GAEts1-H2K (Figure 2B). This silencing was significantly weaker than that observed with the bicistronic mRNA when the second GFP cistron was targeted. This lower silencing could be due to a difference in the local accessibility of the target sequence, a key determinant of RNAi efficacy (2). We reasoned that multiple copies of the minimal target sequence might improve the silencing, as reported recently (6). This should increase the probability that at least one siRNA base pairs with the mRNA and this might also enhance the accessibility of the target sequences by modifying local secondary structures or perhaps local occupancy by RNA binding proteins. We thus introduced three copies of the target sequence in the 3′-UTR of the transgene to create GAEts3-H2K (Figure 2A), and upon transduction with SirGFP, we observed a 97% inhibition of H2K^k expression directed by this new vector (Figure 2B). Such dramatic improvement prompted us to generalize this observation to other transgenes. To obtain a GFP transgene whose expression is insensitive to SirGFP, we introduced six silent mutations localized precisely within the siRNA target sequence. We then cloned this mutated sequence, referred to as GFPμ, into GAEts3. Fluorescence of HeLa cells transduced by GAEts3-GFPμ was greatly decreased upon transduction of SirGFP (Figure 3A), thereby confirming the silencing observed on H2K^k. Similarly, when directed by GAEts3, TRX expression was silenced down to an undetectable level upon transduction with SirGFP, but was unaffected upon transduction with SirGFPμ. In addition, we observed a dose response effect on the extent of TRX silencing as we increased the transduced amount of SirGFP

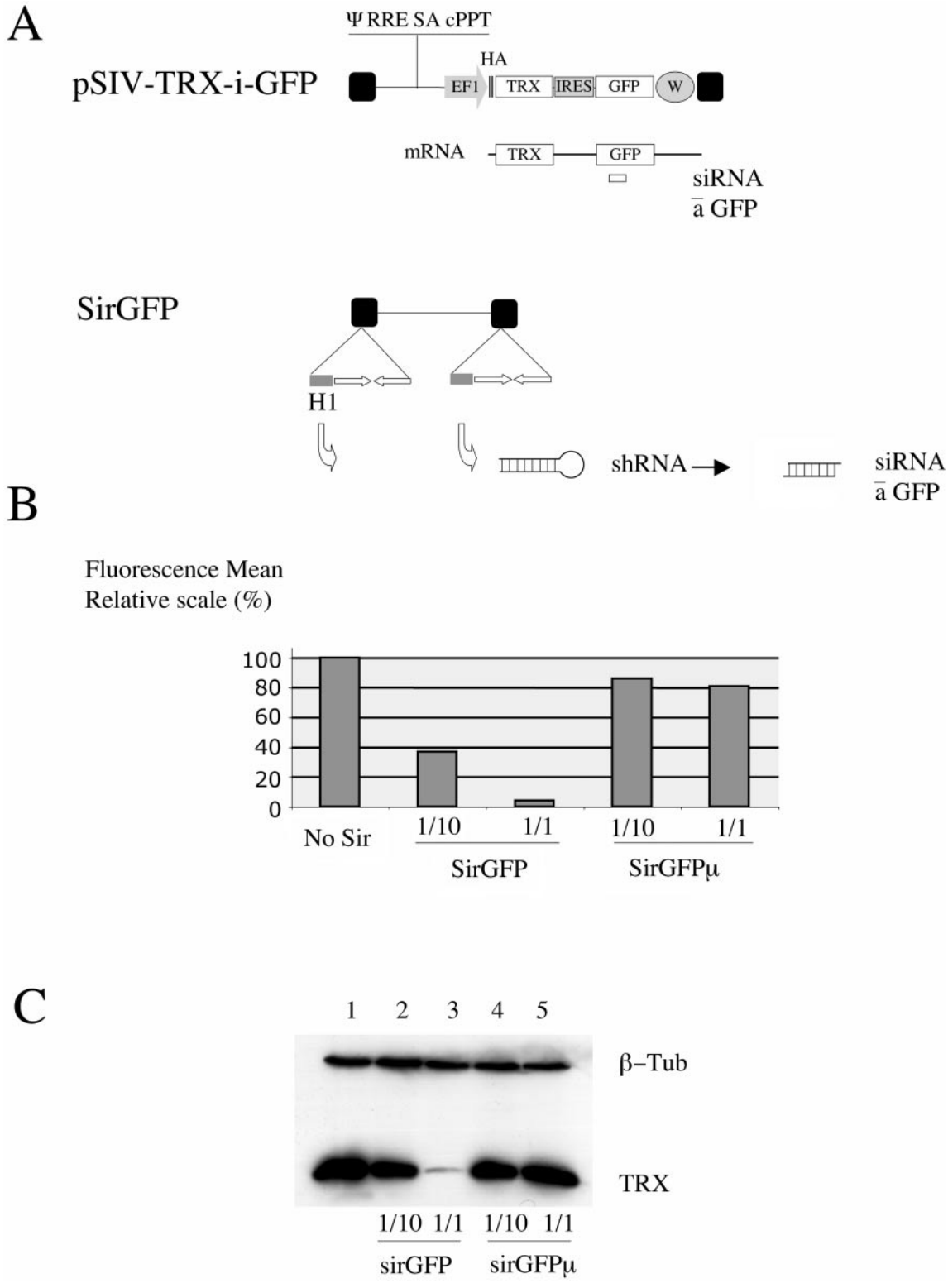

Figure 1. A siRNA directed against the second transgene of a bicistronic vector silences the expression of both transgenes. (**A**) Schematic representation of integrated proviral SIV vectors. (**B**) Silencing of GFP. 2×10^5 HeLa cells were transduced with pSIV-TRX-i-GFP (MOI 15: 100% of transduction) and 24 h later, with two doses of SirGFP or a control construct SirGFPμ. RT activities of all viral preparations were measured to normalize doses of SirGFPs. The RT activity ratio between SirGFPs versus pSIV-TRX-i-GFP is indicated by 1/10 and 1/1. The mean fluorescence intensity was measured by flow cytometry, 72 h after transduction of SirGFPs. Results are given as percentages of residual fluorescence intensity in the global cell population. (**C**) Silencing of TRX. The experiment is the same as in (B). Cell lysates were subjected to a SDS–PAGE followed by a western blot analysis with antibodies against β-tubulin and the HA epitope tag. Black boxes: U3-deleted SIN LTR, gray boxes: Expression regulators (EF1, short elongation factor 1 promoter; W, woodchuck virus post-transcriptional regulatory element; H1, RNA polymerase III H1 promoter). The Ψ RRE SA region includes SIV *cis*-active minimal elements essential for packaging and processing of the SIV vector RNA (11). cPPT: DNA flap (14). GFP: enhanced green fluorescent protein. TRX: human thioredoxin. SirGFP codes for a small hairpin RNA targeting GFP.

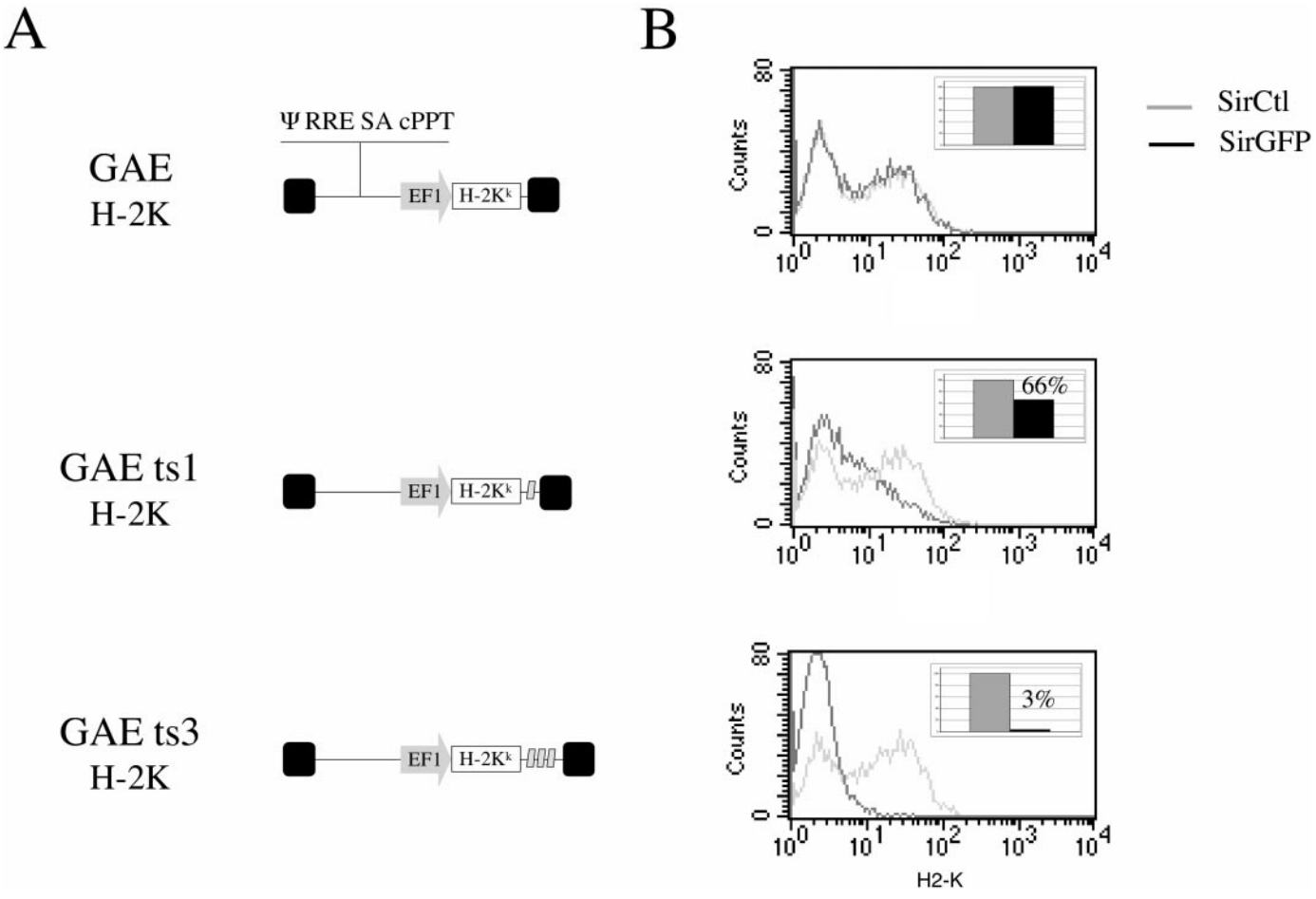

Figure 2. H-2K^k transgene silencing by an siRNA directed against a 3′-UTR minimal target sequence. (**A**) Schematic representation of integrated proviral SIV vectors. The gray boxes depict the GFP siRNA minimal target sequence. (**B**) Silencing of H-2K^k expression. 3×10^5 293T cells were plated and transduced 24 h later by RT-normalized doses of H-2K^k expression vectors. Transductions with SirGFP vectors were performed 24 h later and H-2K^k expression was measured 4 days later by flow cytometry. Gray and black lines or bars depict control and SirGFP-treated cells, respectively. Percentages of H-2K^k positive cells are indicated for each condition.

vector (Figure 3B). Finally, to be able to monitor SirGFP transduction, we endowed this vector with a GFPμ expression cassette to create SirGFP Flu (Figure 3C). We transduced 293T cells with SirGFP Flu at a low multiplicity of infection so as to obtain 20% GFP-positive cells. We then transduced this resulting heterologous population with GAE or GAEts3 vectors directing the expression of a zeocin resistance gene (Zeo^R). We cultivated both transduced cell populations for 20 days in the presence of zeocin and we measured the GFP-positive sub-population in each case. While 20% of the cells transduced with GAE-Zeo^R remained GFP-positive, the percentage of GFP-positive cells within the population transduced with GAEts3-Zeo^R dramatically dropped below 2% (Figure 3C). This result extends our observations to yet another transgene whose silencing produced long-term functional effects.

DISCUSSION

We have developed an extremely easy and versatile transgene silencing method. The only tools required are a transgene expression vector and a universal shRNA expression vector. Although the method is based on RNA interference, most classical concerns about this mechanism are either irrelevant or, at least, addressable. Because a single, validated siRNA target sequence is used for every transgene, the recurrent concern of designing an efficient siRNA against every target does not apply. Recent studies have shown that some siRNAs could induce a non-specific interferon response (7,8) and may cross-react with targets showing limited sequence similarity (9). The present system makes use of a unique GFP siRNA whose specificity and by-standard effects have been already assessed *in vivo* (10). However, to rule out any potential undesired effects, an interferon response could be examined and a transcriptome analysis could be undertaken. Other siRNA/target sequence pairs could also be explored should it prove necessary.

This method circumvents some limitations of existing conditional gene inactivation systems. In contrast with transcriptional switches such as the tetracycline or ecdysone regulatory systems, it does not require the expression of a transactivator in host cells and is therefore more versatile. Some concerns associated with site-specific recombination systems such as

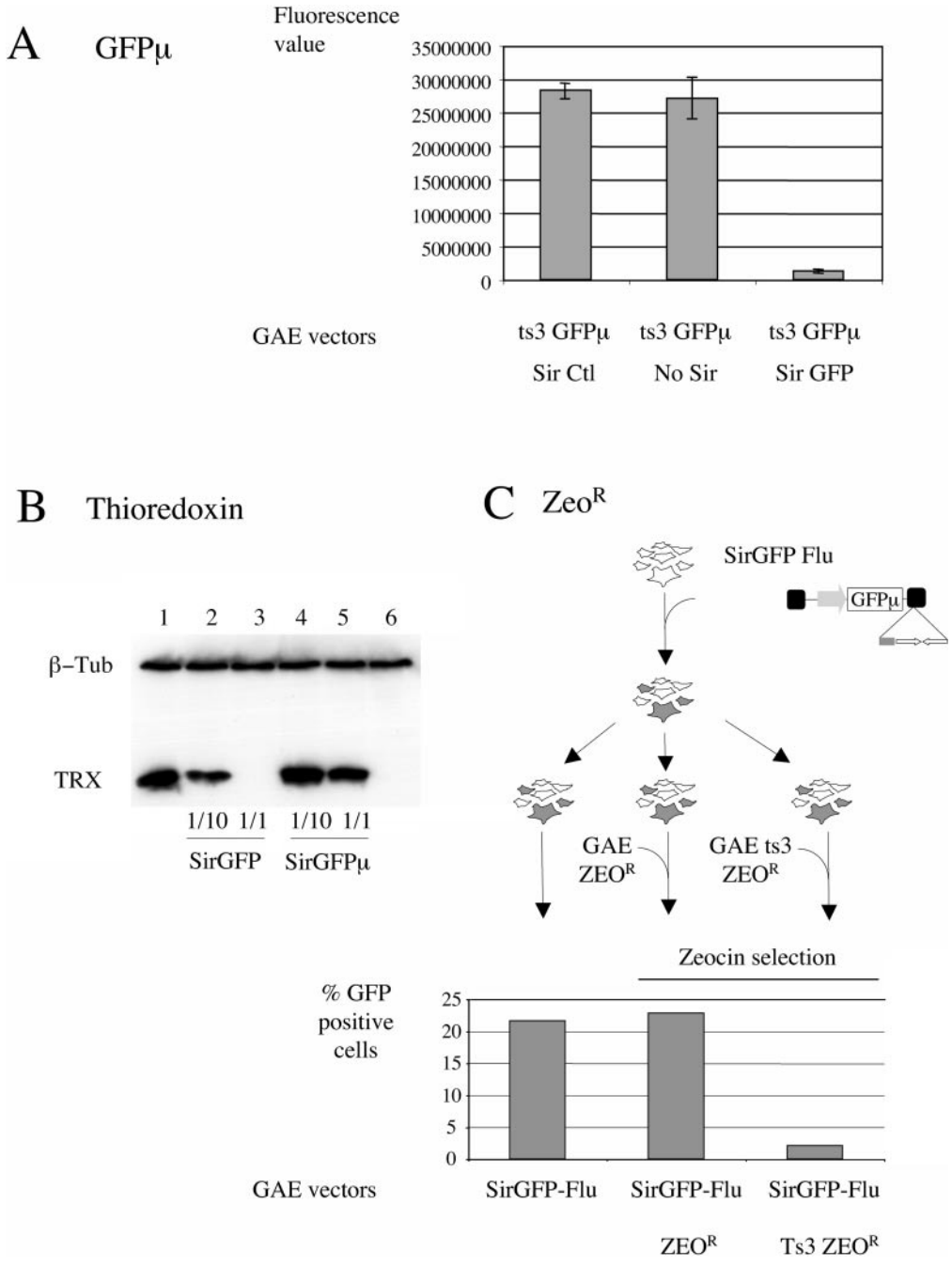

Figure 3. Transgene silencing by a universal siRNA directed against a multimerized minimal target sequence. (**A**) Silencing of GFPμ. 293T cells were transduced with GAEts3-GFPμ. Cells were transduced with SirGFP or SirCtl, 24 h later. We checked that shRNAs coded by SirGFP did not affect GAE-directed expression of GFPμ (data not shown). Fluorescence signals were measured 72 h later by a FITC plate reader (EnVision-Perkin Elmer). (**B**) Silencing of TRX in HeLa cells (see Figure 1C). (**C**) Silencing of ZEO^R. 293T cells were transduced at low MOI with SirGFP-Flu to obtain 25% of GFP-positive cells, 72 h after transduction. Cells were then split and transduced with normalized doses of GAE-ZEO^R and GAEts3-ZEO^R (MOI 10). Zeocin was added to the culture medium 24 h later and maintained for 15 days. The silencing of ZEO^R, resulting in cell death, was monitored by determining the percentage of GFP-positive cells (i.e. the percentage of cells transduced with SirGFP-Flu) before and after the 20 day ZEO selection.

Cre/loxP are also circumvented, such as irreversible off-target effects on degenerate recombination sites in mammalian genomes or the partial delivery of the recombinase.

Conceivably, this method can be evolved through different ways to achieve a reversible silencing of transgenes, e.g. by using an inducible Pol III promoter (11,12). Alternatively, transient silencing could also be achieved through the transfection of target-specific synthetic siRNAs. However, this approach would then be restricted to cells for which an efficient transfection procedure is available. Another most interesting promise of this method is to achieve a fine quantitative control on transgene expression levels by varying the copy number of siRNA target sequences in the 3′-UTR of the transgene, as shown in Figure 2, or by tuning the amount of

delivered shRNAs, e.g. by varying the amount of the corresponding viral particles.

Conditional transgene silencing methods help gain useful knowledge on gene function. For example, they allow the determination of whether the continuous expression of a differentiation factor is required to maintain the cells in their differentiated state, or whether the said factor exerts yet another function once the cells are differentiated. Our method is particularly well suited to the specific silencing of exogenously expressed mutants or isoforms of a specific gene, as the expression of the endogenous wild-type gene is not affected. Furthermore, it is well adapted to large phenotypic screens of various expression libraries (cDNA, antisense or combinatorial libraries) expressed from retroviral vectors, in order to establish that the observed phenotype is caused by the expression of a library member and not by a molecular perturbation induced by the insertion of the retroviral DNA into the cell host's genome. Finally, with the use of VSVG envelope-pseudotyped lentivectors delivering both transgenes and siRNA, a wide range of mammalian cell lines and types including primary, quiescent or fully differentiated cells is now easily accessible for conditional gene expression experiments. Present and future advances in vector pseudotyping might also authorize transgene silencing in specific target cells or tissues in the whole animal (13). In conclusion, the simplicity, power and versatility of this new transgene silencing method should complement usefully the existing reverse genetics methodology.

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