Genes & Genomics (2019) 41:483–490
https://doi.org/10.1007/s13258-019-00784-z

Online ISSN 2092-9293
Print ISSN 1976-9571

RESEARCH ARTICLE

Promoter cross-talk affects the inducible expression of intronic shRNAs from the tetracycline response element

Seong Kyun Park[1] · Byung Joon Hwang[1] · Yun Kee[2]

Received: 22 November 2018 / Accepted: 3 January 2019 / Published online: 17 January 2019
© The Genetics Society of Korea 2019

Abstract

Background RNA interference (RNAi), defined as double-stranded, RNA-mediated gene silencing, is a useful tool for functional genomic studies. Along with increasing information about genomic sequences due to the innovative development of genome-sequencing technologies, functional genomic technologies are needed to annotate the genome and determine the processes by which each gene is regulated. Lentiviral vectors have been used to efficiently deliver reagents, such as small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs), into cells and tissues for functional genomic analyses.

Objective We developed a lentiviral vector that efficiently expresses intronic shRNA from the tetracycline regulatory element (TRE) promoter in a doxycycline-dependent manner.

Methods We developed a lentiviral vector system that contains reverse tetracycline-controlled transactivator 3 (rtTA3) and the TRE promoter, which are necessary for the doxycycline-inducible expression of shRNAs that are expressed as intronic miR-30a precursors. We then measured the cross-talk between the cytomegalovirus (CMV) and TRE promoters in the vector.

Results We found that nearby promoters influence each other and that the TRE promoter should be located far from other promoters, such as the CMV promoter, in a vector. The orientation of a promoter with respect to other promoters also influences its transcriptional activity. A head-to-head orientation of the CMV and TRE promoters maintains the lowest level of transcription from TRE in the absence of doxycycline, compared to the tail-to-tail and head-to-tail orientations.

Conclusion Based on these findings, we were able to construct a lentiviral vector that faithfully expresses intronic miR-30a shRNA precursors in a doxycycline-inducible manner.

Keywords Promoter cross-talk · Tetracycline-inducible promoter · Lentiviral vector · ShRNA

Introduction

In most eukaryotes, RNA interference (RNAi) can mediate post-transcriptional gene silencing in a nucleotide sequence-specific manner. To initiate RNAi, double-stranded RNA is delivered to cells or tissues in one of two different forms: small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) (Fire et al. 1998; Hammond et al. 2000; Paddison et al. 2002; Zou et al. 2003). Gene silencing by siRNA and shRNAs is faster and cheaper than a gene knockout mediated by homologous recombination. Chemically synthesized double-stranded siRNAs of about 21 bp are generally introduced into cells where they either induce the degradation of the target mRNA or inhibit its translation. To deliver shRNAs into cells, vectors containing RNA polymerase III promoters (e.g., U6 and H1 promoters) are used to express shRNAs that are processed into siRNAs inside the cell (Xia et al. 2003; Borchert et al. 2006; Pan et al. 2006; Yuan et al. 2006a, b).

Since vector systems that use RNA polymerase III promoters are not able to express shRNAs in a tissue-specific manner, RNA polymerase II promoter-based expression systems have been deployed to express shRNAs more specifically (Silva et al. 2005; Zhou et al. 2005; Yuan et al. 2006a, b; Giering et al. 2008). In such systems, shRNA is expressed in the form of a microRNA precursor in an intron, which is first processed into a mature shRNA and then into an siRNA inside the cell. Thus, shRNAs can be expressed

✉ Yun Kee
yunkee@kangwon.ac.kr

[1] Department of Molecular Bioscience, College of Biomedical Science, Kangwon National University, Chuncheon, Kangwon-do, Republic of Korea

[2] Division of Biomedical Convergence, College of Biomedical Science, Kangwon National University, Chuncheon, Kangwon-do, Republic of Korea

in a tissue-specific manner, which is usually marked by the expression of a fluorescent protein. Several vectors, including retroviral and lentiviral vectors, have been developed to efficiently and robustly express intronic shRNA precursors that have different hairpin loops and promoters (Lin et al. 2006; Noguchi et al. 2007, 2011; Ying and Lin 2009). In most cases, constitutively active promoters are involved in these vector systems, although temporally inducible promoters such as the tetracycline response element (TRE) can be used to carry out more controlled experiments (Czauderna et al. 2003; Strebhardt et al. 2007; Rangasamy et al. 2008; Wiederschain et al. 2009; MATSUSHITA et al. 2013).

Lentiviral vectors have been used as the prime tool for the expression of shRNAs, particularly in cells and tissues that do not rapidly divide and in which plasmid transfection is not successful (Manjunath et al. 2009; Moore et al. 2010). Since lentiviral vectors integrate into the host genome after infection, long-term expression of shRNAs is another advantageous feature of lentiviral vectors used in gene delivery. Efforts to improve the efficiency and safety of gene delivery have resulted in several vector modifications, such as reducing the chance of generating replication-competent viral particles (Naldini et al. 1996; Kordower et al. 2000; Lois et al. 2002; Levine et al. 2006; Montini et al. 2006; Pluta and Kacprzak 2009; MD et al. 2014).

In this study, we developed a lentiviral vector system that contains both reverse tetracycline-controlled transactivator 3 (rtTA3) and the TRE promoter, which are necessary for the doxycycline-inducible expression of shRNAs. In this system, shRNAs are expressed as intronic miR-30a precursors. We found that cross-talk between the cytomegalovirus (CMV) and TRE promoters affects their activities. The relative orientation and distance between these promoters affects their strength. The maximal level of doxycycline-induced gene expression and the lowest level of transcription in the absence of doxycycline were achieved using the head-to-head orientation of both promoters and locating them far from one another. Based on these findings, we were able to construct a lentiviral vector that faithfully expresses intronic miR-30a shRNA precursors in a doxycycline-inducible manner, which could be used to temporally control shRNA expression and thus similarly regulate overall gene expression.

Materials and methods

Cell culture and transfection

HEK 293T and CHO-K1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (CORNING, Corning, USA) supplemented with 10% FBS (Lonza, Basel, Switzerland), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37 °C and 5% CO_2. To generate a stable cell line expressing the murine EGFP-p53 fusion protein, CHO-K1 cells were transfected using the JetPRIME transfection reagent (PolyPlus, New York, USA) and selected in media containing 100 µg/mL hygromycin B. A homogeneous cell population expressing GFP was enriched by fluorescence-activated cell sorting (FACS). The CHO-K1 stable cell line expressing EGFP-p53 was maintained in DMEM containing 10% FBS, 1% penicillin/streptomycin, and 50 µg/mL hygromycin B.

For transient expression of shRNAs, 5×10^5 cells were seeded into six-well plates 24 h before transfection and transfected with plasmids expressing various forms of shRNA (2 µg) using PolyPlus following the manufacturer's instructions. Doxycycline was added 6 h after transfection at a final concentration of 1 µg/mL, and fresh medium containing doxycycline (1 µg/mL) was added every 24 h.

Plasmids

Vectors containing the miR-30a shRNA precursor sequence (5′ end: 122 bp; shRNA-3′ end: 127 bp) and genes for different fluorescent proteins (TagBFP, EGFP, and RFP_{670}) were prepared by the sequential ligation of PCR products and amplification of the ligation mixtures. Different combinations of PCR products were cloned between the cPPT and 3′ LTR of the pGreenPuro lentiviral vector (SBI, Palo alto, USA) as follows: pHAB267: cPPT-CMV promoter-TagBFP-T2A-rtTA3-WPRE-TRE promoter-RFP_{670}-EGFP-3′ LTR; pHAB271: cPPT-TRE promoter-RFP_{670}-EGFP-CMV promoter-TagBFP-T2A-rtTA3-WPRE; pHAB371: cPPT-CMV promoter-TagBFP-T2A-rtTA3-WPRE-EGFPreverse-RFP_{670}reverse-TRE promoterreverse-3′ LTR; and pHAB372: cPPT-WPRE-rtTA3reverse-T2A-TagBFPreverse-CMV promoterreverse-TRE promoter-RFP_{670}-EGFP-3′ LTR. In these constructs, EGFP was flanked by the 5′ and 3′ ends of the miR-30a precursor. To assemble the intronic miR-30a shRNA precursor sequences, shRNA sequences against target genes were cloned between the 5′ and 3′ ends of the precursor after removing EGFP by *Xho*I (NEB, Ipswich, USA) and *Eco*RI (NEB, Ipswich, USA) digestion.

Intronic miR30a shRNA precursor

The following sequence represents the intronic miR-30a shRNA precursor; the sense and antisense strands are represented by underlined, lowercase letters and the hairpin loop by italic, bold capital letters: TTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCTGGGATTACTTCTTCAGGTTAACCCAACAGAAGG**CTCGAG**AAGGTATATTGCTGTTGACAGTGAGCGAccctgtcatcttttgtcccttt***TAGTGAAGCCACAGATGTA***aagggacaaaagatgacagggGTGCCTACTGCCTCG**GAATTC**AAGGGGCTACTTTAGGAGCAATTATCTTGTTTACTAAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAAATGGTATAAATTAAATCACTTT. This shRNA precursor is located in the intron after the RFP_{670} ORF.

For experiments to reduce the expression of EGF-p53 using p53-specific shRNAs, the following shRNA sequences, which were shown to knock down the expression of p53, were replaced by the *Xho*I (CTCGAG)/*Eco*RI (GAA TTC) fragment in the miR-30a shRNA precursor.

p53-393 shRNA

CTCGAGAAGGTATATTGCTGTTGACAGTGAGCGAccctgtcatcttttgtccctt***TAGTGAAGCCACAGATGTA***aagggacaaaagatgacagggGTGCCTACTGCCTCG**GAATTC**.

p53-814 shRNA

CTCGAGAAGGTATATTGCTGTTGACAGTGAGCGCccactacaagtacatgtgtaa***TAGTGAAGCCACAGATGTA***ttacacatgtacttgtagtggATGCCTACTGCCTCG**GAATTC**.

Negative control shRNA

CTCGAGAAGGTATATTGCTGTTGACAGTGAGCGAggatgtttcaccaaggaaata***TAGTGAAGCCACAGATGTA***tatttccttggtgaaacatccGTGCCTACTGCCTCG**GAATTC**.

The above oligonucleotide templates were amplified by PCR using the primers (5′- GTGCAATGCTCGAGAAGG TATATTG-3′ and 5′- TAAGCGATGAATTCCGAGGCA GTAG-3′). Then *Xho*I/*Eco*RI digests of the PCR products were cloned into the same restriction enzyme (RE) sites in the vector containing the miR-30a shRNA precursor.

Flow cytometric analysis

To quantitate the expression of fluorescence proteins (TagBFP, EGFP, and RFP_{670}) by flow cytometry, HEK 293T cells were transiently transfected with pHAB267, pHAB271, pHAB371, or pHAB372. After treatment with doxycycline, cells were treated with trypsin (0.25%), briefly washed with 1 × PBS, and resuspended in the FACS buffer (1 × PBS, 2% FBS) before harvest.

A Moflo XDP flow cytometer (Beckman Coulter, Brea, USA) was used for the analysis. The instrument settings were as follows: log forward scatter (FSC) and log side scatter (SSC): 440 V. TagBFP was excited at 405 nm. EGFP was excited at 488 nm, and RFP_{670} was excited at 633 nm. TagBFP, EGFP, and RFP_{670} fluorescence was collected using 450/15 nm, 529/28 nm, and 670/30 nm bandpass filters, respectively. The singlet cell population was gated on FSC and SSC. The typical sampling rate was 500 cells per second, and the typical sample size was 50,000 cells per measurement unless otherwise stated. The data were analyzed using Kaluza software (Beckman Coulter, Brea, USA).

Results and discussion

A lentiviral vector for the expression of shRNAs from a doxycycline-inducible TRE promoter

To understand how the regulatory elements in lentiviral vectors affect the expression of intronic shRNAs, we constructed a stable cell line in which the EGFP-p53 fusion protein was produced from the UBC promoter. Most cells expressed a uniform level of GFP, as measured by fluorescence microscopy and flow cytometry.

We constructed a lentiviral vector to express shRNAs from a doxycycline-inducible TRE promoter: TagBFP and rtTA3 were constitutively expressed from the CMV promoter, and RFP_{670} and intronic miR-30a shRNA were expressed from the doxycycline-inducible TRE promoter (Fig. 1). We also generated a vector with the EGFP ORF that contained the region between the 5′ and 3′ ends of the miR-30a precursor, flanked by *Xho*I and *Eco*RI RE sites

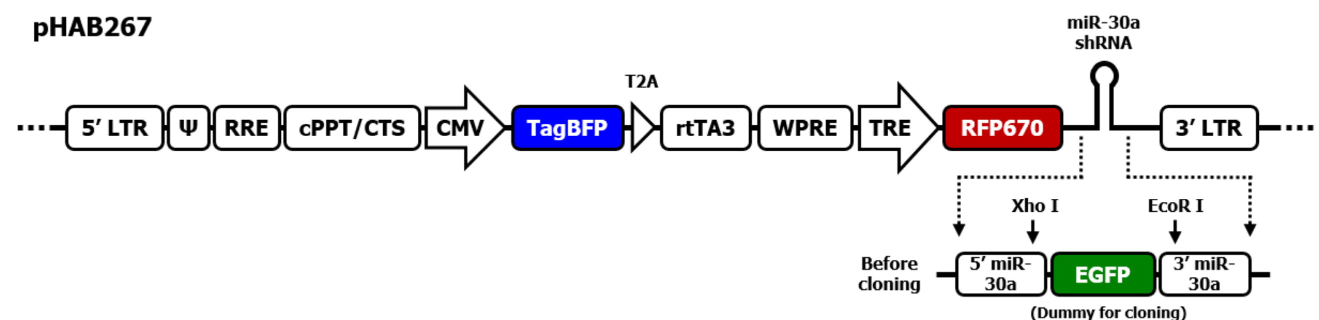

Fig. 1 A lentiviral vector is used to express shRNAs from a doxycycline-inducible promoter. Both TagBFP and rtTA3 are expressed as a single RNA transcript from the CMV promoter and translated independently due to the presence of the T2A peptide between them. During translation, a stalled ribosome on the T2A sequence falls off and reinitiates translation from an ATG codon in rtTA3. TagBFP marks the cells infected with the lentiviral vector. The rtTA3 protein is the transactivator necessary for transcription from the TRE promoter. The shRNAs is expressed in the form of an intronic miR-30a precursor. For enzymatic cloning, an shRNA precursor sequence is cleaved into 5′ and 3′ segments with the EGFP ORF cloned between them. When necessary, the shRNA is cloned into the precursor region using *Eco*RI and *Xho*I digestion

(Fig. 1). We chose the intronic miR-30a precursor because it is known to efficiently proceed to a mature shRNA after transcription by the RNA polymerase II promoter (Lee et al. 2004). The vector also contains the rtTA3 transactivator that is necessary for doxycycline-inducible transcription from the TRE promoter (Strebhardt et al. 2007).

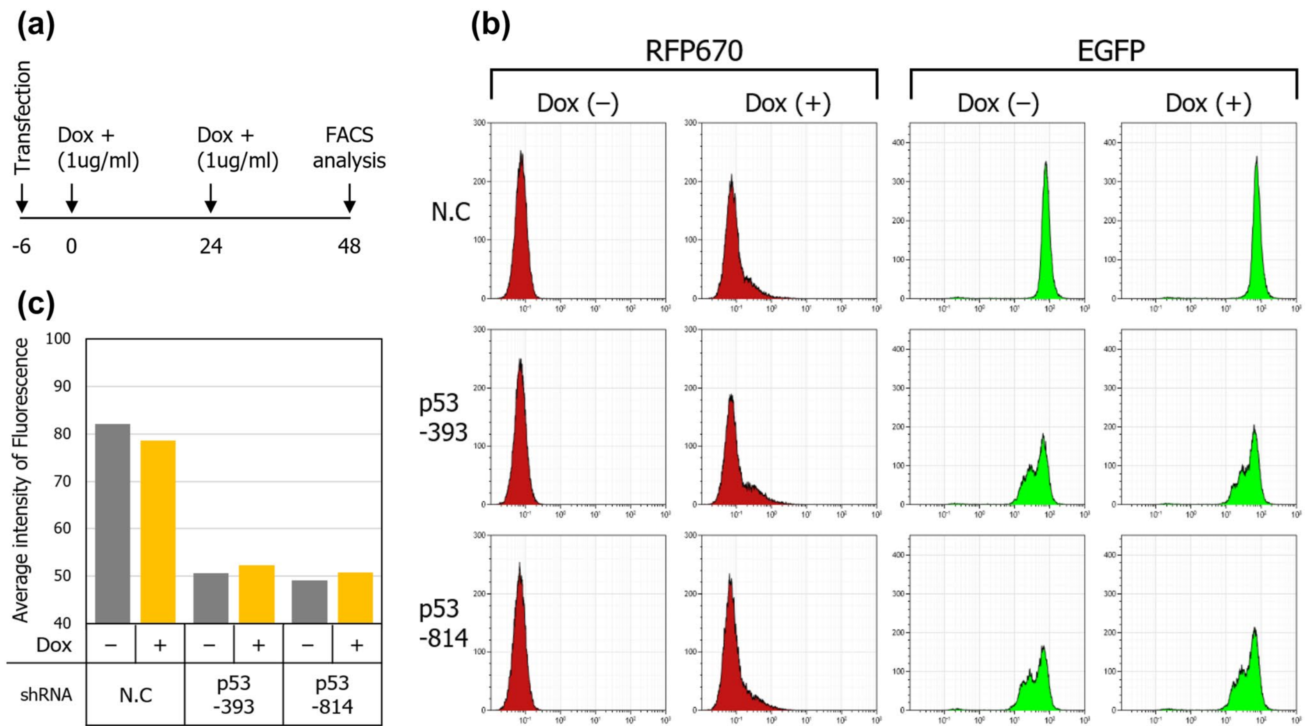

Fig. 2 shRNAs against p53 knock down p53 expression independent of doxycycline. **a** Timeline of doxycycline treatment and FACS analysis. **b** Flow cytometric analysis of the CHO-K1 stable cell line expressing EGFP-p53; 1.0×10^4 cells were counted for each FACS experiment. **c** The EGFP intensity in each cell was divided by the number of cells analyzed to obtain the average EGFP signal and EGFP-p53 level

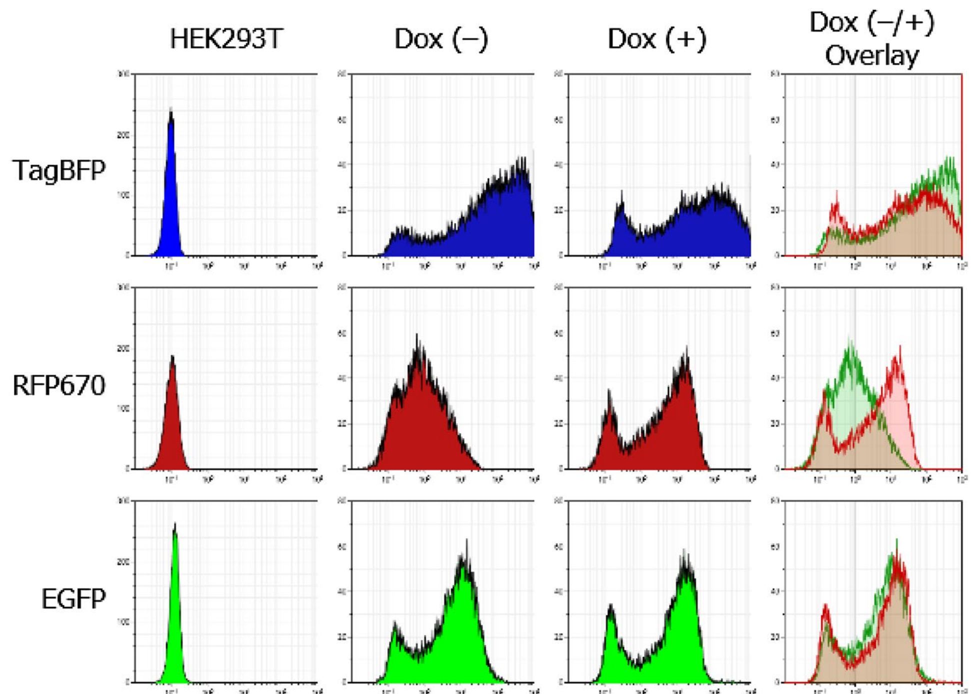

Fig. 3 EGFP in the RFP_{670} intron is strongly expressed in the absence of doxycycline. HEK 293T cells were transfected with pHAB267 to measure the transcriptional activity of the CMV and TRE promoters. TagBFP is directly downstream of the CMV promoter, RFP_{670} is downstream of the TRE promoter, and EGFP is located in the RFP_{670} intron, flanked by the 5′ and 3′ ends of the miR-30a precursor; 1.0×10^4 cells were counted for each FACS experiment. Dox (−): without doxycycline, Dox (+): with doxycycline

In the pHAB267vector, TagBFR-T2A-rtTA3 was transcribed as a single transcript but translated as independent polypeptides: TagBFP and rtTA3. Thus, the cells that contained this vector were expected to express TagBFP and support the doxycycline-inducible expression of RFP_{670} and the intronic miR-30a precursor as a single transcript. During splicing, the mature miR-30a shRNA was produced from its intronic precursor. As described in the materials and methods, different shRNA sequences can be cloned into the vector as precursor forms using *Eco*RI and *Xho*I RE sites (Fig. 1).

shRNAs against p53 block its expression regardless of doxycycline treatment

When two different shRNAs (mp53-393 and mp53-814) that specifically target p53 were transiently expressed in the CHO-K1 stable cell line expressing EGFP-p53, GFP expression was significantly reduced, as measured by flow cytometry (Fig. 2). A negative control shRNA that does not target p53 did not cause any reduction of the GFP signal (Fig. 2). Thus, it appears that intronic shRNA precursor RNAs are transcribed from the transiently transfected plasmid and then processed into mature shRNAs. Interestingly, the specific knockdown of GFP-p53 expression by p53-specific shRNAs was observed even without doxycycline treatment, suggesting the existence of other mechanism(s) that express intronic shRNA precursors other than the doxycycline-inducible TRE promoter (Fig. 2).

To discern whether doxycycline-independent transcription is caused by the vector itself or by the CHO-K1 cell line expressing EGFP-p53, we transiently transfected HEK 293T cells with the pHAB267 vector, which has the EGFP ORF in place of the miR-30a shRNA. As shown in Fig. 3,

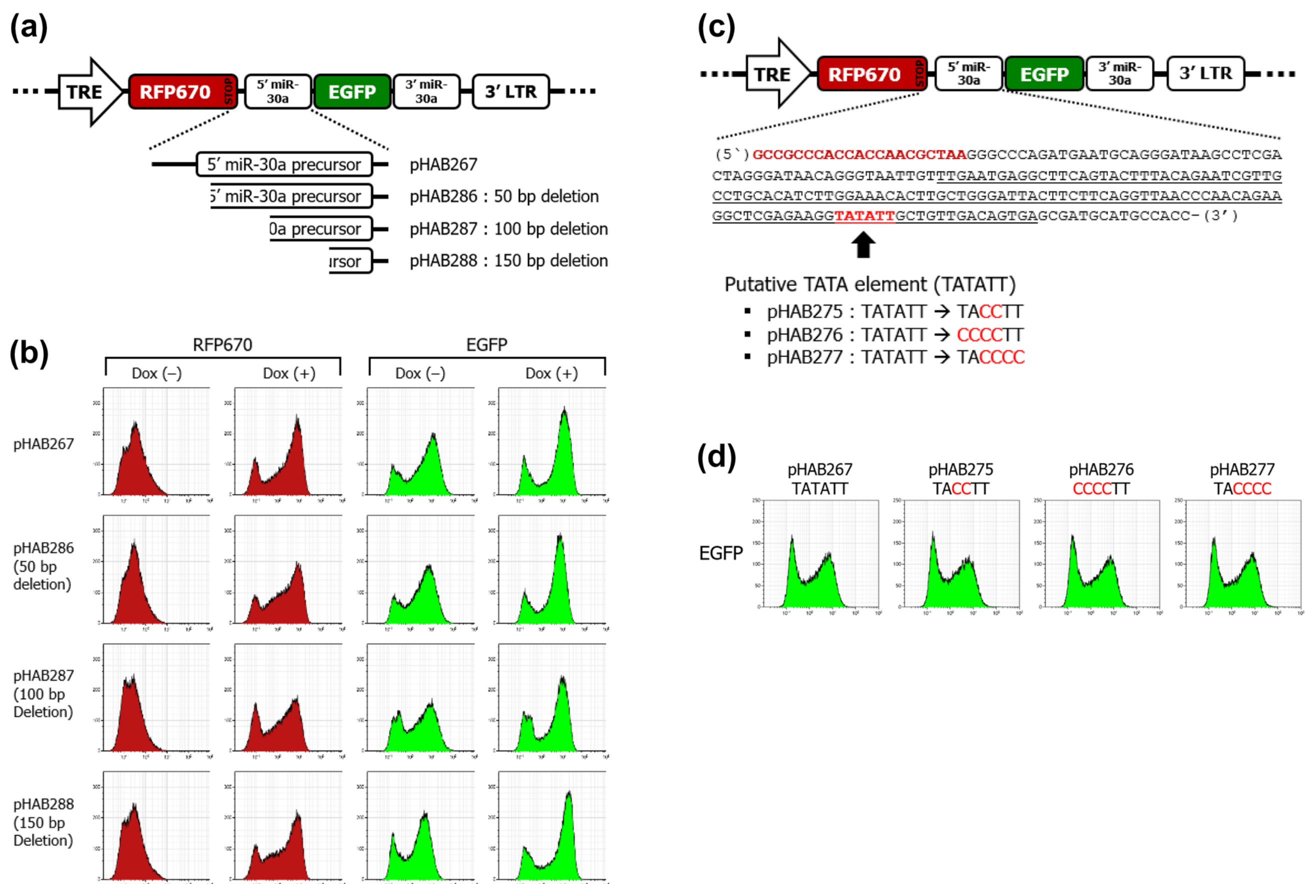

Fig. 4 Mutagenesis of the intronic 5′ miR-30a precursor region to look for a cryptic promoter. **a** Deletion mutants in which 50-bp regions were deleted in the intronic 5′ miR-30a precursor region (50-bp deletion in pHAB286, 100-bp deletion in pHAB287, and 150-bp deletion in pHAB288) in the pHAB267 vector were analyzed for RFP_{670} and EGFP expression using flow cytometry. **b** HEK 293T cells were transfected with the pHAB267, pHAB286, pHAB287, or pHAB288 vector and treated with doxycycline, and the expression levels of RFP_{670} and EGFP were analyzed by FACS. **c** Site-directed mutagenesis of a putative TATA element in the 5′ region of the miR-30a precursor. 'TAT ATTG' in pHAB267 was modified to 'TACCTTG' (pHAB275), 'CCC CTTG' (pHAB276), and 'TACCCCG' (pHAB277). **d** HEK 293T cells were transfected with each site-directed mutant construct, and EGFP expression was analyzed by FACS

TagBFP was constitutively expressed from the CMV promoter, with or without doxycycline. RFP_{670} expression was induced by doxycycline, as seen in the previous experiment, but RFP_{670} expression without doxycycline treatment was significantly higher than the control. The same amount of EGFP was produced regardless of doxycycline treatment (Fig. 3).

The doxycycline-independent expression of shRNAs or EGFP suggests the existence of a cryptic promoter in the 200-bp intronic region containing the 5′ end of the miR-30a precursor. Of note, the RFP_{670} ORF before the intron contains an in-frame stop codon, ruling out a possible RFP_{670}-GFP fusion protein. Different expression patterns of RFP_{670} with GFP also preclude the existence of a fusion protein (Fig. 3).

To identify the putative cryptic promoter, we carried out deletion and site-directed mutagenesis studies of the 200-bp region between RFP_{670} and EGFP. As shown in Fig. 4, a deletion of the majority of the 200-bp region and site-directed mutagenesis of a putative TATA element did not change the expression patterns of RFP_{670} and EGFP, or the doxycycline independence of EGFP expression. Based on these results, we conclude that the aberrant expression of EGFP is not due to the presence of a cryptic promoter.

The effects of promoter combinations on doxycycline-inducible TRE transcription

To determine whether different combinations of the CMV and TRE promoters cause different expression patterns of RFP_{670} and EGFP, we constructed different vectors in which the position and orientation of the promoters differed from each other. The pHAB267 vector contains a head-to-tail orientation of CMV-TRE, pHAB271 contains a head-to-tail orientation of TRE-CMV, pHAB371 contains a head-to-head orientation of CMV-TRE, and pHAB372 contains a tail-to-tail orientation of CMV-TRE (Fig. 5a). When each vector was transiently transfected into HEK 293T cells, doxycycline-inducible expression from the TRE promoter was only evident in the pHAB371 vector, as measured by the expression levels of EGFP and RFP_{670} using flow cytometry (Fig. 5b, c). When both promoters were

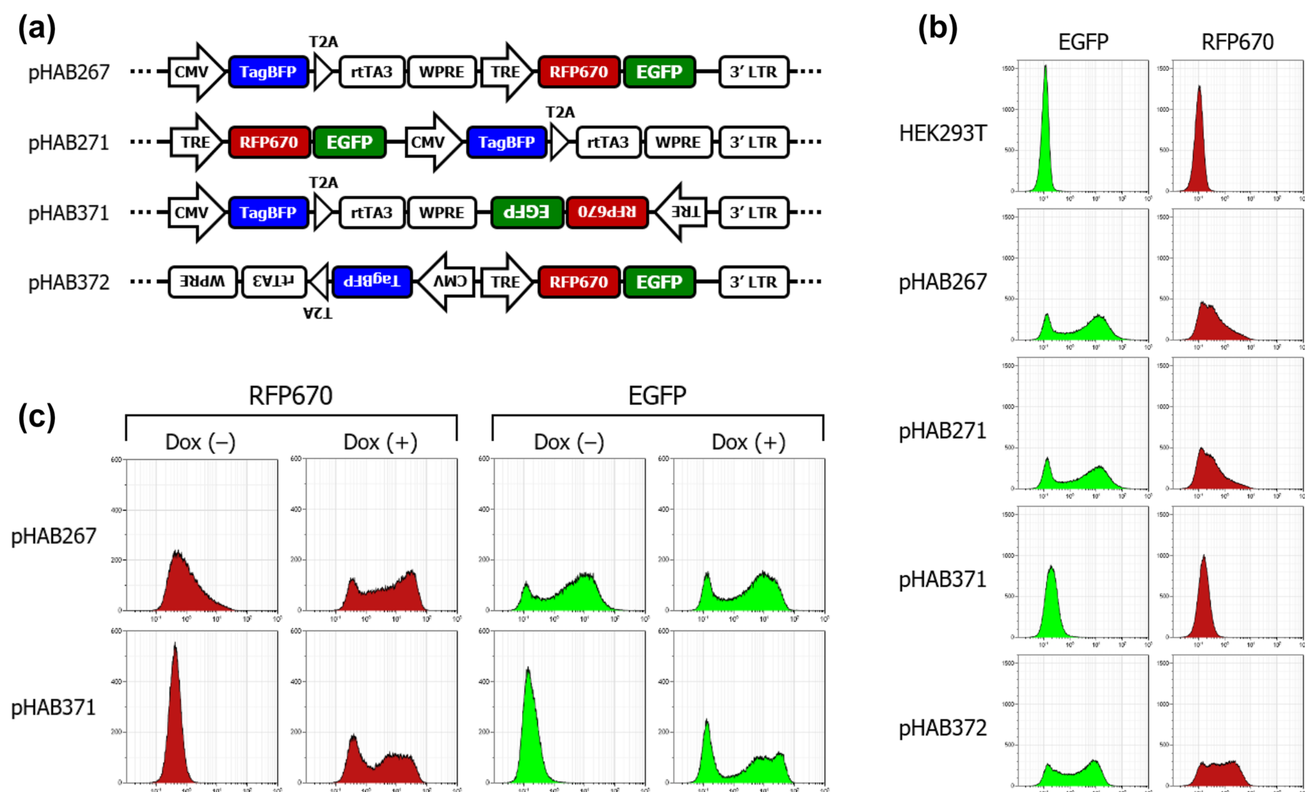

Fig. 5 Promoter cross-talk depends on the orientation and distance of promoters with respect to one another. **a** Vectors with different distances between and orientations of the CMV and TRE promoters. **b** HEK 293T cells were transfected with each vector and grown in media without doxycycline; the expression levels of RFP_{670} and EGFP were measured by flow cytometry. **c** HEK 293T cells were transfected with pHAB267 or pHAB371, and grown in the media with or without doxycycline; the expression levels of RFP_{670} and EGFP were measured by flow cytometry

closest to each other (tail-to-tail orientation in pHAS372), the highest expression of RFP_{670} and EGFP from the TRE promoter occurred in the absence of doxycycline. When both promoters were in the head-to-tail orientation, the expression levels of RFP_{670} and EGFP were the same with and without doxycycline, regardless of the order of the promoters (pHAB267 vs pHAB271). From these results, we conclude that both the orientation and distance between nearby promoters affect their activity and that promoter cross-talk is significantly reduced when promoters are in a head-to-head orientation (far from each other), which is important for the differential expression of a gene from a constitutively active promoter and a gene from an inducible promoter in a single vector.

Doxycycline-inducible expression of intronic shRNAs from TRE promoter

To determine whether intronic shRNAs are expressed from the TRE promoter in the pHAB371 vector in the presence of doxycycline, a negative control and two different shRNAs (that specifically target murine p53) were first cloned in the pHAB371 vector and then transiently expressed in the CHO-K1 stable cell line expressing EGFP-p53. GFP-p53 expression was subsequently measured by flow cytometry (Fig. 6). Compared to the pHAB267 vector, which contains the CMV-TRE promoters in a head-to-tail orientation and thus expresses RFP_{670} and EGFP in a doxycycline-independent manner (Figs. 2, 6), cells transfected with pHAB371 expressed shRNAs only when doxycycline was added to the medium. Thus, shRNAs against p53 (mp53-393 and mp53-814), but not a negative control shRNA, knocked down GFP-p53 expression in the presence of doxycycline.

It is unclear how the EGFP ORF was expressed without its own promoter. A deletion of the intronic region and site-directed mutagenesis of a putative TATA element ruled out the presence of a cryptic promoter upstream of EGFP. Since the EGFP ORF is located in an intron, translation could be initiated from the 5′ end of the intron that is separated from the RFP_{670} exon by RNA splicing.

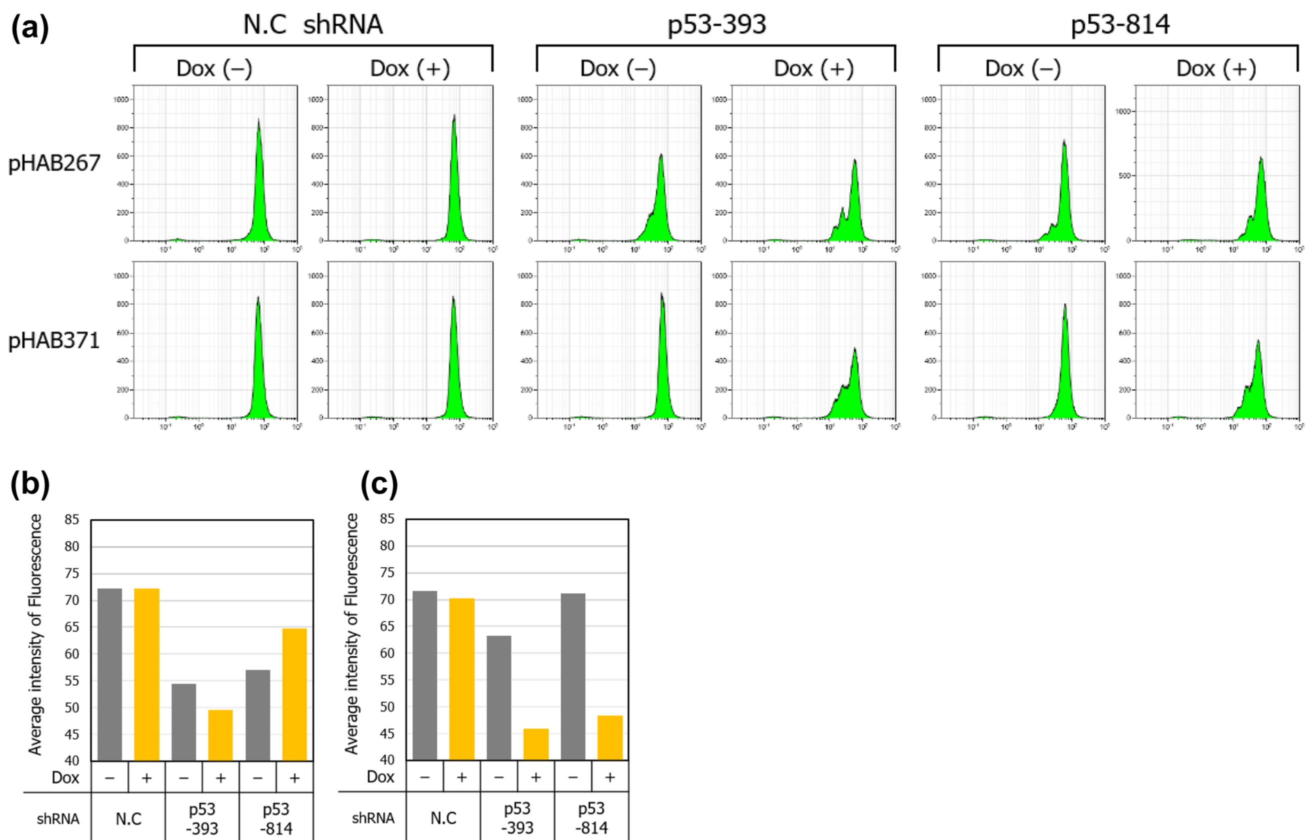

Fig. 6 Inhibition of EGFP-p53 expression by the doxycycline-inducible expression of intronic shRNAs from the TRE promoter. **a** CHO-K1 cells stably expressing EGFP-p53 were transfected with pHAB267 or pHAB371 vectors containing negative control shRNA, p53-393 shRNA, or p53-814 shRNA. Cells were grown in media with or without doxycycline, and GFP levels were measured by flow cytometry. **b, c** The EGFP intensity in each cell was divided by the number of cells analyzed to obtain the average EGFP signal and EGFP-p53 level

Conclusions

In this study, we showed that promoter cross-talk depends on the relative position and orientation of the promoters involved. The head-to-head orientation of promoters results in minimal effects on promoter activity, compared to other orientations (tail-to-tail and head-to-tail). Based on this information, we constructed a vector that could inducibly express intronic shRNAs from the TRE promoter, allowing the performance of controlled experiments of temporally regulated shRNA expression in the presence or absence of doxycycline.

Acknowledgements This work was supported by the Basic Science Research Program through the NRF by the Ministry of Education (Grant No. NRF-2015R1D1A3A01015641) and a grant from the National R&D Program for Cancer Control, Ministry of Health & Welfare, Republic of Korea (Grant No. HA17C0035) to B.J. Hwang, and both the SGER Program through the NRF by the Ministry of Education (Grant No. NRF-2015R1D1A1A02060346) and the 2015 Research Grant from Kangwon National University to Y. Kee.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Borchert GM, Lanier W, Davidson BL (2006) RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol 13:1097–1101

Czauderna F, Santel A, Hinz M et al (2003) Inducible shRNA expression for application in a prostate cancer mouse model. Nucl Acids Res 31:e127

Fire A, Xu S, Montgomery MK et al (1998) Potent and specific genetic interference by double-stranded RNA in caenorhabditis elegans. Nature 391:806–811

Giering JC, Grimm D, Storm TA, Kay MA (2008) Expression of shRNA from a tissue-specific pol II promoter is an effective and safe RNAi therapeutic. Mol Ther 16:1630–1636

Hammond SM, Bernstein E, Beach D, Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature 404:293–296

Kordower JH, Emborg ME, Bloch J et al (2000) Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. Science 290:767–773

Lee Y, Kim M, Han J et al (2004) MicroRNA genes are transcribed by RNA polymerase II. EMBO J 23:4051–4060

Levine BL, Humeau LM, Boyer J et al (2006) Gene transfer in humans using a conditionally replicating lentiviral vector. Proc Natl Acad Sci 103:17372–17377

Lin SL, Miller JD, Ying SY (2006) Intronic microRNA (miRNA). J. Biomed. Biotechnol. 2006:1–13

Lois C, Hong EJ, Pease S et al (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science 295:868–872

Manjunath N, Wu H, Subramanya S, Shankar P (2009) Lentiviral delivery of short hairpin RNAs. Adv Drug Deliv Rev 61:732–745

Matsushita N, Matsushita S, Hirakawa S, Higashiyama S (2013) Doxycycline-dependent inducible and reversible rna interference mediated by a single lentivirus vector. Biosci Biotechnol Biochem 77:776–781

MD PSP, MD JMG, PhD GSR et al (2014) Long-term safety and tolerability of ProSavin, a lentiviral vector-based gene therapy for Parkinson's disease: a dose escalation, open-label, phase 1/2 trial. Lancet 1–9

Montini E, Cesana D, Schmidt M et al (2006) Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. Nat Biotechnol 24:687–696

Moore CB, Guthrie EH, Huang MTH, Taxman DJ (2010) Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown. Methods Mol Biol 629:141–158

Naldini L, Blömer U, Gallay P et al (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272:263–267

Noguchi K, Ishitu Y, Miyano-Kurosaki N, Takaku H (2007) Expression of shRNA using intron splicing. Nucleic Acids Symp Ser 51:409–410

Noguchi K, Ishitu Y, Takaku H (2011) Evaluating target silencing by short hairpin RNA mediated by the group I intron in cultured mammalian cells. BMC Biotechnol 11:79

Paddison PJ, Caudy AA, Bernstein E et al (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev 16:948–958

Pan X, Chen Z, Ni Q et al (2006) Application of PCR-based tRNAval Pol III-shRNA expression cassettes in screening of efficient siRNA for inhibiting hepatitis B virus. Zhejiang Da Xue Xue Bao Yi Xue Ban 35:154–160

Pluta K, Kacprzak MM (2009) Use of HIV as a gene transfer vector. Acta Biochim Pol 56:531–595

Rangasamy D, Tremethick DJ, Greaves IK (2008) Gene knockdown by ecdysone-based inducible RNAi in stable mammalian cell lines. Nat Protoc 3:79–88

Silva JM, Li MZ, Chang K et al (2005) Second-generation shRNA libraries covering the mouse and human genomes. Nat Genet 37:1281–1288

Strebhardt, Matthess Y, Kaufmann M (2007) Silencing of mammalian genes by tetracycline- inducible shrna expression. Nat Protoc 2:3257–3269

Wiederschain D, Wee S, Chen L et al (2009) Single-vector inducible lentiviral RNAi system for oncology target validation. Cell Cycle 8:498–504

Xia XG, Zhou H, Ding H et al (2003) An enhanced U6 promoter for synthesis of short hairpin RNA. Nucl Acids Res 31:e100

Ying S-Y, Lin S-L (2009) Intron-{Mediated} {RNA} {Interference} and {microRNA} {Biogenesis}. Methods Mol Biol 487:387–413

Yuan J, Wang X, Zhang Y et al (2006a) Mammalian Pol III promoter H1 can transcribe shRNA inducing RNAi in chicken cells. Mol Biol Rep 33:33–41

Yuan J, Wang X, Zhang Y et al (2006b) shRNA transcribed by RNA Pol II promoter induce RNA interference in mammalian cell. Mol Biol Rep 33:43–49

Zhou H, Xia XG, Xu Z (2005) An RNA polymerase II construct synthesizes short-hairpin RNA with a quantitative indicator and mediates highly efficient RNAi. Nucl Acids Res 33:1–8

Zou GM, Wu W, Chen J, Rowley JD (2003) Duplexes of 21-nucleotide RNAs mediate RNA interference in differentiated mouse ES cells. Biol Cell 95:365–371

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.