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Xavier Coumoul, Wenmei Li, Rui-Hong Wang, Chuxia Deng. Inducible suppression of *Fgfr2* and Survivin in ES cells using a combination of the RNA interference (RNAi) and the Cre–LoxP system. *Nucleic Acids Research*, 2004, 32 (10), pp.e85-e85. 10.1093/nar/gnh083 . hal-02173975

HAL Id: hal-02173975

<https://hal.science/hal-02173975>

Submitted on 4 Jul 2019

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Inducible suppression of *Fgfr2* and Survivin in ES cells using a combination of the RNA interference (RNAi) and the Cre–LoxP system

Xavier Coumoul, Wenmei Li, Rui-Hong Wang and Chuxia Deng*

Genetics of Development and Disease Branch, 10/9N105, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA

Received March 19, 2004; Revised April 28, 2004; Accepted May 25, 2004

ABSTRACT

RNA interference (RNAi) is a simple and powerful tool widely used for studying gene function in a number of species. Recently, inducible regulation of RNAi in mammalian cells using either tetracycline- or ecdysone-responsive systems has been developed to prevent potential lethality or non-physiological responses associated with persistent suppression of genes that are essential for cell survival or cell cycle progression. Here we show that the inducible regulation of RNAi also can be achieved by using a Cre–LoxP approach. We demonstrate that the insertion of a loxP-flanked neomycin cassette into RNA polymerase III promoter, which controls a vector-based RNAi unit, impairs the promoter activity. However, the expression of RNAi construct can be completely restored upon the removal of the neo cassette using a tamoxifen inducible Cre construct. We show that this system works with high efficiency in suppression of two endogenous genes, *Fgfr2* and Survivin, in mouse embryonic stem (ES) cells, as evidenced by the decrease of levels of gene expression, reduced cell proliferation and colony formation. This system provides a potentially important yet simple approach to establish mutant mouse strains for functional study at defined stages upon turning on the inducible switches controlled by the Cre–LoxP system.

INTRODUCTION

Recent advances in RNAi have made it possible to degrade specific mRNAs of any endogenous genes in mammals (1–3). The most common procedure consists of the introduction of a 21 nt double-stranded RNA (dsRNA) into the cells using a specific transfectant. The sequence of this dsRNA or small hairpin RNA (shRNA) has to be perfectly homologous and unique to a region of the targeted mRNA. After 1–3 days, expression of the target gene is dramatically reduced and this allows investigations on the function of the gene. Although powerful on the cell culture level, this procedure

is limited by the short period of action of the dsRNA. This limitation leads to the development of plasmid- and viral-based vectors, which can be stably integrated into host genome and produce shRNAs to suppress target genes (4–9).

Logically, several recent studies developed inducible regulation of RNAi in mammalian cells using either tetracycline- or ecdysone-responsive systems (10,11). These approaches prevent potential lethality or non-physiological responses associated with persistent suppression of genes that are essential for cell survival or cell cycle progression. As the initial applications, a number of endogenous genes, including DNA methyltransferase 1, p53 and p21, have been successfully knocked down in cultured cells (10,12).

Another inducible system is Cre–LoxP system, which has been most widely used in gene targeting field (13,14). Numerous transgenic mouse strains expressing Cre at specific tissues/organs have been developed to achieve spatial and temporal control of gene expression (15). Apparently, if the RNAi vectors can be modified to allow inducibility by the Cre–LoxP system, it will eventually allow the utilization of the large collection of Cre transgenic mice and significantly enhance our ability to use RNAi system to decipher gene function *in vivo*. Here, we demonstrate that the combination of RNAi and Cre–LoxP can be used in ES cells for inducible suppression of both an exogenous gene, GFP, and endogenous genes, *Fgfr2* and Survivin.

MATERIALS AND METHODS

Vector design and engineering

The original mouse U6 promoter (8) was remodeled to allow insertion of the neomycin cassette. First, we removed the Sall site of the polylinker, deleted 45 bp from –128 to –83 of the U6 promoter and replaced it with a Sall site by site-directed mutagenesis (Stratagene, La Jolla, CA). After restriction by Sall, a 1938 bp XhoI and Sall fragment containing the *ploxPneo* (16) was inserted. We then mutated the ApaI site of the neomycin cassette using the previous Stratagene kit (the insertion of the first oligonucleotide in the polylinker required one ApaI-restriction step). This final version of the construct can be used to perform ligation of both oligonucleotides as previously described (8) for eGFP and subsequent RNAi experiments. For FGFR2, the oligonucleotides used are 1A,

*To whom correspondence should be addressed. Tel: +1 301 402 7225; Fax: +1 301 480 1135; Email: chuxiad@bdg10.niddk.nih.gov

5'-GGCCTCAGCGTTCCTGAGCGA-3'; 1B, 5'-AGCTTCG-CTCAGGAACGCTGAGGCC-3'; 2A, 5'-AGCTTCGCTCA-GGAACGCTGAGGCCCTTTTGG-3'; 2B, 5'-AATTCAAA-AAGGGCCTCAGCGTTCCTGAGCGA-3'. For Survivin, the oligonucleotides used are 1A, 5'-GGCCTCCTAGCAG-GATCTTAA-3'; 1B, 5'-AGCTTTAAGATCCTGCTAGGAG-GCC-3'; 2A, 5'-AGCTTTAAGATCCTGCTAGGAGGCC-CTTTTGG-3'; 2B, 5'-AATTCAAAAAGGGCCTCCTAGCAG-GATCTTAA-3'.

Cell culture and treatment

eGFP-expressing transformed mammary tumor cells have been produced following transfection of W069 cells (17) with pCMS-eGFP (Clonetech, Palo Alto, CA) using lipofectamine 2000 following instructions of the manufacturer (Invitrogen, Carlsbad, CA). The eGFP-expressing cells were sorted twice at 7 and 30 days after transfection using an FACS Calibur (Becton-Dickson, San Jose, CA). Transient and stable transfections were performed on BOSC23 and W069 cells with lipofectamine 2000. Briefly, cells are seeded 1 day prior to transfection at 80% confluence in six-well plates and transfected with 100 ng of pCMS-eGFP and 3 μ g of the different pBS vectors. For stable transfections and selection of Cre-ERT2 clones, we used a ratio of 1/10 for Cre-ERT2 expressing vector on hygromycin resistance vector. ES cells are transfected by electroporation with 20 μ g of vector for two 10 cm plates. Selections with hygromycin (200 μ g/ml) and/or G418 (300 μ g/ml) are performed 36 h following transfections. Resistant colonies were picked to a 96-well plate after 8 days of selection and several clones were analyzed (PCR on genomic DNA, RT-PCR on total RNA). Treatments with 4HT (Sigma, St Louis, MO) (1 μ M) were performed one day after transfection. Growth curves are obtained by counting replicated six-wells of ES cells. Treatment by 4HT or vehicle (ethanol) was started one day after plating. Cells were counted every 24 h for monitoring proliferation. For colony formation assay, ES cells were plated into a six-well plate at a density of 300 cells/well and treatment by 4HT or vehicle (ethanol) was started 24 h later. Counting was performed on six different wells for each condition 6 days after plating. Staining was performed using Accustain (Giemsa Stain, modified from Sigma) following instructions provided by the manufacturer.

Fluorescence experiments

Fluorescent levels of eGFP-transfected cells have been monitored every day and images have been analyzed and captured using a green fluorescent microscope (Olympus IX81, IX2-UCB) and the IPLab software 3.6.3 on a Power Macintosh G4.

RNA preparation and reverse transcription (RT) experiments

Total RNAs are prepared using the Rneasy Mini kit from Qiagen, 0.5–1 μ g of RNA are used to perform RT using the kit provided by Roche Applied Science (Indianapolis, IN) following the manufacturer instructions.

PCR experiments

PCR experiments are performed on a Perkin-Elmer 9700 machine using 2.5 U of *Taq* polymerase from GeneChoice, 2.5 mM MgCl₂, 0.2 mM dNTP mix, 100 ng of each

oligonucleotide. During these experiments, 1 μ L of cDNA or 100 ng of genomic DNA are used.

Primers for Fgfr2 (RT) (T_m = 60°C): 5'-AAGGTTTACA-GCGATGCCCA-3'; 5'-ACCACCATGCAGGCGATTAA-3'.

Primers for Neo (T_m = 68°C): 5'-CGCACAGACTTGTGG-GAGAA-3'; 5'-GCTGCTAAAGCGCATGCTCC-3'.

Primers for Recomb (T_m = 60°C): 5'-CGCACA-GACTTGTGGGAGAA-3'; 5'-CACAATTACTTTACAGT-TAG-3'.

RESULTS

To develop an inducible approach for RNAi, we modified the polymerase III U6 promoter (18) to make a 'spatio-temporal' switch that is suitable for the Cre-LoxP system (19–21). The U6 promoter contains three major regulatory elements, which include a TATA box between –31 and –26, a proximal sequence element (PSE) between –68 and –49 and a distal sequence element (DSE) between –247 and –225 (Figure 1A). It has been shown that all three elements are required for the

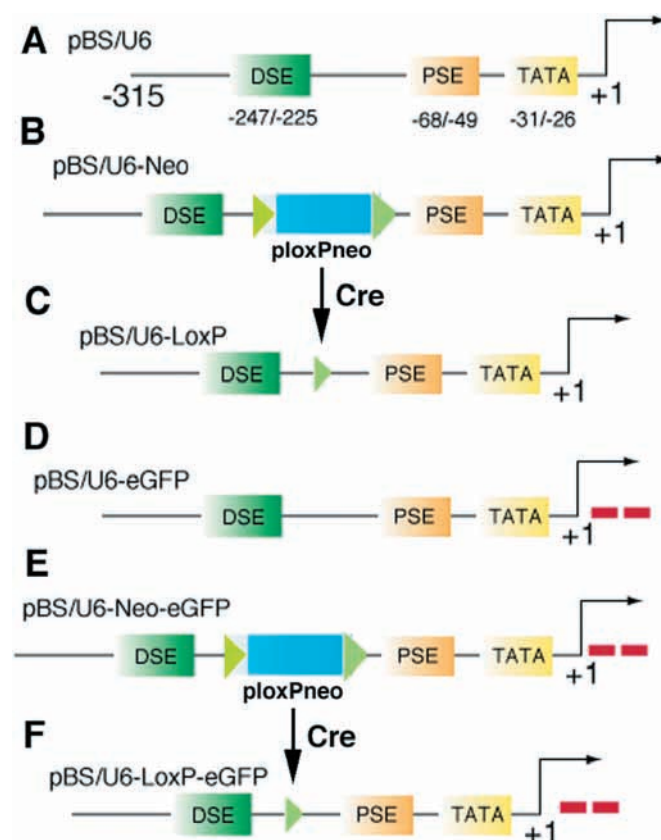


Figure 1. Vectors used for interference study. (A) Structure of the mouse U6 (mU6) promoter and of its three major regulatory elements (TATA box, TATA; Proximal Site Enhancer, PSE; Distal Site Enhancer, DSE). +1 corresponds to the transcription start site (polylinker in the empty vector). (B) The pBS/U6-Neo vector carried a *ploxPneo* cassette inserted between the PSE and the DSE inside the mU6 promoter. Expression of the neomycin cassette in the cells enables selection of positive stable clones with G418. (C) The pBS/U6-LoxP is the result of the recombination event by one Cre recombinase enzyme on the pBS/U6-Neo. (D–F) Same constructs as shown in (A–C), respectively, but with two double oligonucleotides specifically against eGFP inserted for shRNA.

cooperative binding of proteins, and the DSE is critical for the stability of the DNA–protein complex and the activation of both proximal elements (18). We assumed that the distances between these three elements are critical while the actual sequences of nucleotides are not. We therefore deleted 45 bp of sequences between the DSE and PSE, and replaced them with a neomycin cassette flanked by two LoxP sites (*ploxPneo*) (16) (Figure 1B). The insertion of the *ploxPneo* (total length: 1938 bp) dramatically increases distance between DSE and PSE and is predicted to severely disrupt the U6 promoter activity. After deleting the *ploxPneo* with Cre, the critical distance is restored due to the retention of a loxP and the flanking restriction sites (Figure 1C). We predicted that the promoter activity should be restored. If these predictions are correct, the combination of the Cre–loxP system and the RNAi technology could be used to regulate gene expression both *in vitro* and *in vivo*.

To provide a quick assay on the efficiency of our plasmids, we first targeted the eGFP mRNA in transient transfections. Oligonucleotides against eGFP were introduced in the above three constructs to generate the pBS/U6–eGFP (Figure 1D), pBS/U6–Neo–eGFP (Figure 1E) and pBS/U6–LoxP–eGFP

(Figure 1F) constructs. BOSC23 cells were used in the assay because of their high transfection efficiency. We showed that co-transfection of an eGFP-expressing vector (pCMS–eGFP) with pBS/U6 (shown in Figure 1A) produced high levels of eGFP expression (Figure 2A). The expression of eGFP was repressed by co-transfection of the pBS/U6–eGFP (shown in Figure 1D) (Figure 2B), indicating that the U6 promoter was active in BOSC23 cells. Next, we co-transfected pCMS–eGFP with pBS/U6–Neo–eGFP (shown in Figure 1E) and found that this transfection yielded eGFP levels comparable to those produced by the co-transfection of pCMS–eGFP and pBS/U6 vector (Figure 2C). This observation indicated that the presence of the *ploxPneo* between DES and PES disrupted the activity of the U6 promoter. Then, we co-transfected pCMS–eGFP with pBS/U6–LoxP–eGFP vector (shown in Figure 1F) and found that this vector could sufficiently suppress eGFP expression (Figure 2D). This observation indicated that the presence of a loxP site did not have an obvious effect on U6 promoter activity.

Next, we checked the efficiency of our system on the GFP reporter after it was stably integrated into the genomic DNA. We transfected a cell line, WO69, which was derived from a

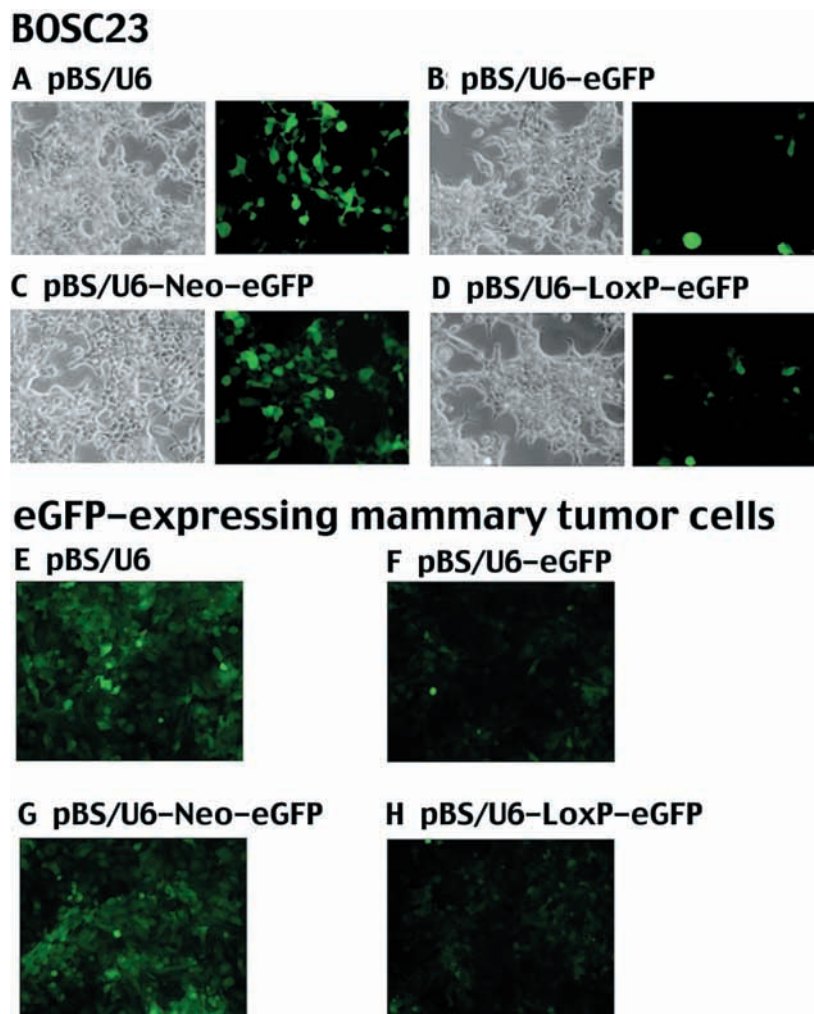


Figure 2. Suppression of eGFP in BOSC23 and WO69 cells. (A–D) Transient transfections of BOSC23 cells with pCMS–GFP and pBS/U6 (A), pBS/U6–eGFP (B), pBS/U6–Neo–eGFP (C) and pBS/U6–LoxP–eGFP (D). Left, bright field control; right, green fluorescence. (E–H) WO69 cells that are stably express eGFP are transiently transfected with constructs indicated. Pictures are taken three days after transfections using lipofectamine 2000.

mouse mammary tumor (17), with pCMS-eGFP, and enriched GFP expressing cells by sorting them twice at 7 and 30 days after transfection. We then transfected the eGFP-expressing cells with the pBS/U6 (Figure 2E), pBS/U6-eGFP (Figure 2F), pBS/U6-Neo-eGFP (Figure 2G) or pBS/U6-LoxP-eGFP (Figure 2H) vectors. We obtained similar results as shown in transient system, i.e. the presence of the *neo* blocked the U6 promoter activity and the removal of the *neo* restored the promoter function. It is important to notice that we did not modify the distance between each element of the promoter. Even in an inserted LoxP site, we deleted on purpose the corresponding number of base pairs that we add following the insertion. As a result, we did not observe any differences between the effects of the unmodified U6 and inserted LoxP-U6 promoters. These data indicated that it is possible to use the combination of RNAi and Cre-LoxP system to knock-down the expression of genes that are stably integrated in the genome in an inducible manner.

Encouraged by these results, we tested the efficiency of our system on an endogenous gene, *Fgfr2*, in mouse ES cells. We chose to study this gene for several reasons. First, FGFR2 is expressed in ES cells and we surmised that down-regulation of this protein could have an effect on the growth of those cells as demonstrated in other cell types (22–25). Second, we have previously created a mouse strain carrying *Fgfr2* mutation through gene targeting (23,26). The comparison between inducible *Fgfr2* ‘knock-down’ and *Fgfr2* ‘knock-out’ mice would allow us to assess efficiency of the RNAi system. Furthermore, mutant mice bearing inducible suppression of *Fgfr2* would be very useful after crossing them with different Cre expressing mice to assess functions of this gene in multiple biological processes during mammalian development.

To this end, we constructed two plasmids carrying oligonucleotides specific to different regions of *Fgfr2* (pBS/U6-Neo-Fgfr2). After testing the corresponding pBS/U6-LoxP-Fgfr2 constructs (obtained through recombination in Cre-positive bacteria) in transient transfections in mouse embryonic fibroblasts, we found that one of the constructs appeared to work better than the others (data not shown). We then transfected it into an ES clone (referred to D4) that we generated by stably expressing the recombinase Cre-ER^{T2} (27,28). Seven days after transfection of the pBS/U6-Neo-Fgfr2 into D4 ES cells, we picked 24 G418-resistant colonies and amplified two of them (clones 1 and 75) for further analysis. As both ES clones yielded similar results, only the data obtained from clone 1 are presented below.

First, we performed RT-PCR of *Fgfr2* following treatment with vehicle or 4OH-tamoxifen (4HT) (1 μ M) at varying time-points from 24 to 96 h. A clear reduction of *Fgfr2* is evident 24 h after 4HT treatment, with a dramatic effect after 96 h compared to vehicle treatment (Figure 3A). To determine if the decreased expression of *Fgfr2* was a direct consequence of Cre-mediated deletion of the *pLoxPneo*, we extracted the genomic DNA from the ES cells and amplified by PCR a specific region of pBS/U6-Neo-eGFP construct overlapping both distal fragment of the U6 promoter and the 5' region of the neomycine cassette. As shown in figure 3B, 24 h after the 4HT treatment, Cre action, as evidenced by the decreased intensities of PCR products, was already perceptible and continued throughout the treatment until 72 h, while the

intensities of PCR products from vehicle treated cells remained unchanged. This effect is observed even if the efficiency of the recombination event does not reach 100%. Indeed, this ‘mosaic’ effect is commonly observed in mouse studies using the Cre-LoxP system. However, this did not interfere with our following study.

Previous studies conducted in various types of cells indicated that FGF/FGFR2 signals play an essential role in cell proliferation (22–25). As our approach decreased *Fgfr2* transcripts by over 90% (Figure 3A), we were interested to know whether the acute suppression of *Fgfr2* could affect ES cell proliferation. We performed a growth curve of clone 1 ES cells in the presence or absence of 4HT. As a control, we used the parental D4 clone expressing only the Cre-ER^{T2} to ensure that an eventual effect could not be attributed to action of the recombinase. Our data revealed no obvious differences between 4HT treated, untreated and vehicle treated cells in both clone 1 and D4 cells between 24 and 96 h (Figure 3C). However, a significant effect of 4HT on the proliferation of clone 1 cells was observed at 120 h (30% of decrease) (Figure 3C). Those effects are also observed with the clone 75 (data not shown). The delayed response of cell proliferation compared with decreased *Fgfr2* transcripts may suggest that the depletion of *Fgfr2* protein to the critical levels that impairs cell proliferation requires time. Alternatively, the delay may happen somewhere in the Fgf/Fgfr2 signaling transduction pathway instead of at the receptor level.

In addition, we have also assessed the impact of *Fgfr2* suppression upon the treatment of 4HT on colony formation in clone 1 cells in comparison with the parental D4 cells. Figure 3D and E showed a significant decrease of the number and the size of colonies in 4HT-treated clones 1 and 75, but not in vehicle-treated clone 1 cells. These effects were consistent with the experiments on cell growth. In contrast, such differences were not observed in D4 cells (Figure 3D and E).

To confirm the viability of our system, we decided to perform the same experiments with another gene: Survivin, which is a member of the inhibitor of apoptosis gene family. As indicated by its name, Survivin inhibits apoptosis *in vivo* and *in vitro* but is also implicated in cell division regulation (29,30). We then designed an RNAi construct for Survivin using the same approach for *Fgfr2* and transfected it into the parental ES clone D4. After neomycin selection of more than 75 clones, clones 1 and 35 have been chosen to perform the experiments according to their capacity to delete the neo cassette following treatment by 1 μ M of 4HT (Figure 4A). Our time course RT-PCR revealed a sharp decrease of Survivin mRNA levels after 24 h, a continuous ‘knock-down’ effect even 120 h in the presence of 4HT (Figure 4B). This observation correlates with decreased growth (almost 40% after 96 h) after several days of 4HT treatment compared to vehicle (Figure 4C). Finally, we also performed a colony formation experiment with both clones 1 and 35 and yielded reduced sizes and numbers of colonies (Figure 4D and E). The observed decreased growth is particularly correlated with the dramatic effect on colony sizes (Figure 4D). These results confirmed that combination of the RNAi and the Cre-LoxP system works efficiently in ES cells bearing the shRNA conditional constructs.

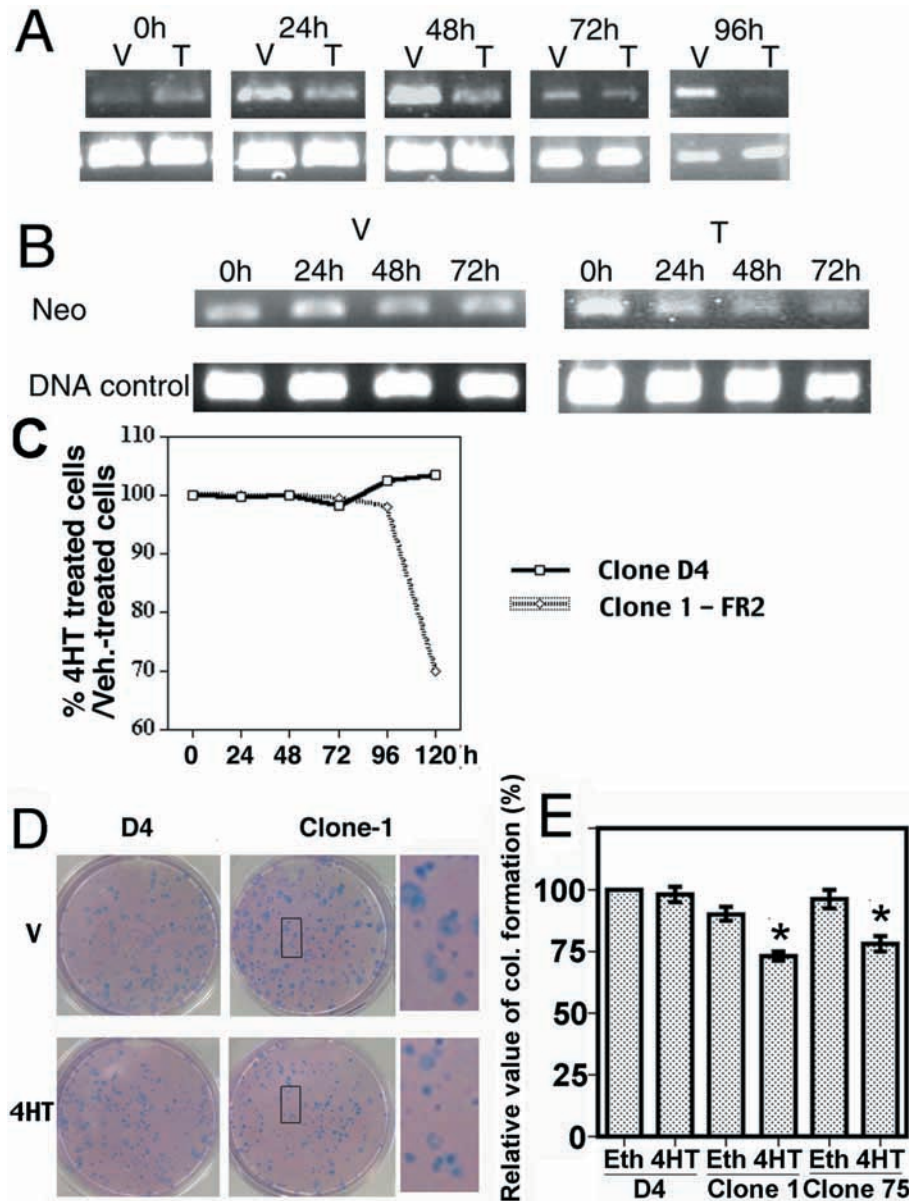


Figure 3. Suppression of *Fgfr2* in ES cells. (A) Kinetics of *Fgfr2* levels revealed by RT-PCR after vehicle (V) or 4HT (T) treatment in Cre-ER^{T2} expressing ES cells (Clone-1) that bear the pBS/U6-Neo-Fgfr2 construct. *Gapdh* is used as control. (B) Kinetics of removal of the neomycine cassette (Neo) evaluated by PCR on genomic DNA isolated from ES cells after treatment by vehicle or 4HT (DNA control = *Gapdh*). (C) Growth curves of Cre-ERT2 expressing ES clones that bear (Clone-1) or do not bear (parental D4) the pBS/U6-Neo-FGFR2 construct expressed by percentage ratio of the number cells after 4OH-Tamoxifen treatment (1 μ M) on the number of cells treated with ethanol (vehicle). (D and E) Colony formation of Clone D4 (parental), Clone-1 and Clone-75 ES (U6-Neo-FR2) cells. (D) The cells are seeded one day before beginning of treatment by vehicle (ethanol) or 4HT. Pictures are taken 6 days later after staining by Accustain. (E) Relative values of colony formation in D4 and Clone-1 ES cells. Number of colonies obtained from vehicle-treated are set as 100%, and those of 4HT-treated are calculated as following: nb. of colonies from 4HT treatment/nb. of colonies from vehicle treatment \times 100/100. Six independent counts have been performed. Astral indicates significant difference as assayed by Student's *t*-test ($p < 0.01$).

DISCUSSION

In summary, we have demonstrated the feasibility of a novel technique that allows the inducible production of shRNA using the U6 promoter and the Cre-LoxP system upon 4HT treatment. At the cellular level, this system is applicable for establishing stable cell lines to decipher functions of genes, which are essential for cell growth and viability. Indeed, even with an uncompleted efficiency of recombination, we have observed significant effects after down-regulation of *Fgfr2* and *Survivin*. Similarly, this system should be very useful *in vivo* for

functional analysis of genes in which the targeted disruption is not compatible for embryonic or early postnatal development. The techniques from ES cells to mouse germline transmission have been well established (14,31); we believe that the generation of mice via blastocyst injection of modified ES cells, although time consuming, should not be a major concern. To this end, successful germline transmission of ES cells carrying non-inducible RNAi constructs have been achieved recently (32). It has also been demonstrated that embryos generated by tetraploid aggregation from ES cells carrying an RNAi construct against *Rasal* recapitulated the null

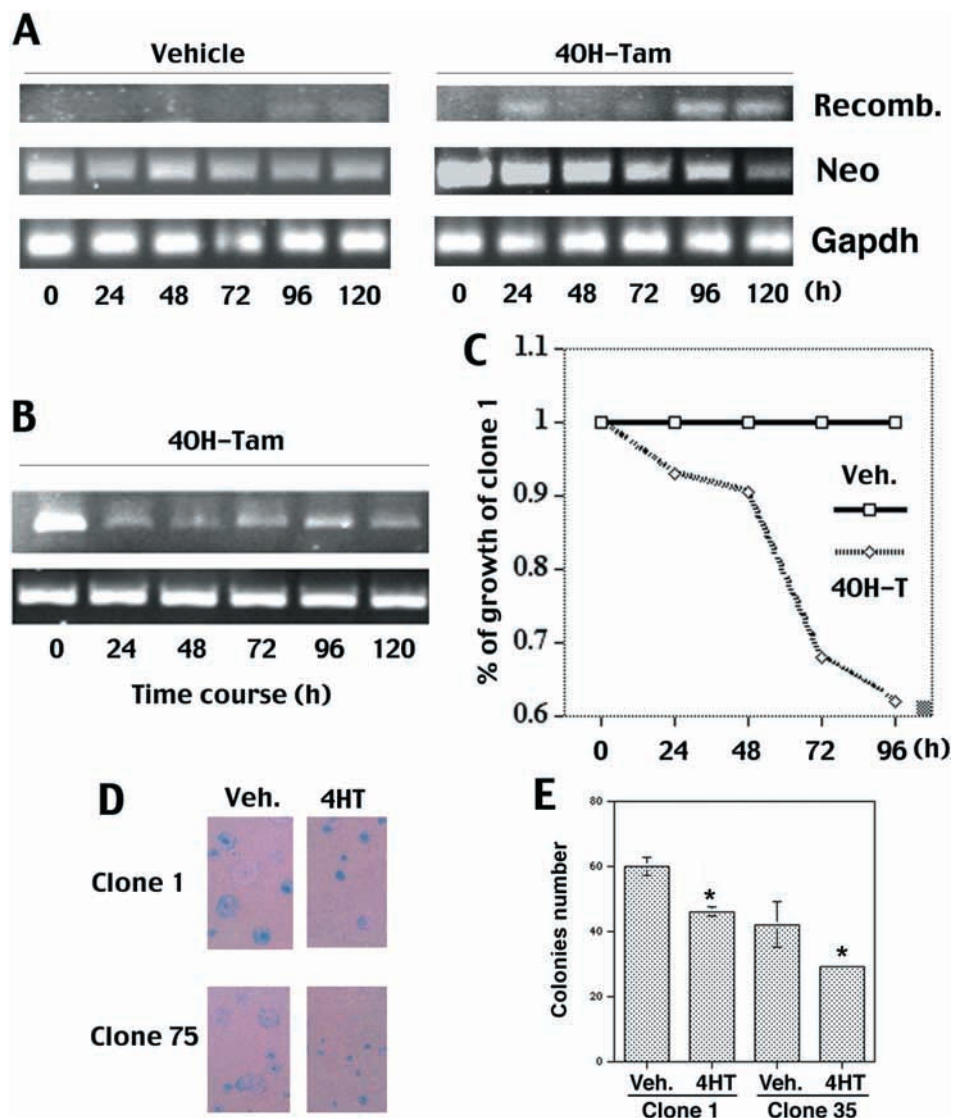


Figure 4. Suppression of Survivin in ES cells. (A) Kinetics of removal of the neomycin cassette and recombination event evaluated by PCR on genomic DNA isolated from ES cells after treatment by vehicle or 4HT. (B) Kinetics of Survivin levels revealed by RT-PCR after 4HT (T) treatment in Cre-ERT² expressing ES cells (Clone-1) that bear the pBS/U6-Neo-Survivin construct. Gapdh is used as control. (C) Growth curves of Cre-ERT²-expressing ES clone 1 that bear the pBS/U6-Neo-Survivin construct after 4OH-Tamoxifen or ethanol (vehicle) treatment expressed by ratio versus ethanol-treated cells. (D and E) Colony formation of D4, Clone-1 and Clone-35 ES cells. (D) The cells are seeded one day before beginning of treatment by vehicle (ethanol) or 4HT. Pictures are taken 6 days later after staining by Accustain. (E) Real values of colony formation in Clone-1 and Clone-35 ES cells. Astral indicates significant difference as assayed by Student's *t*-test ($p < 0.01$).

phenotype (33). Our method aims at generation of mice carrying inducible RNAi constructs, which would allow the utilization of large collection of Cre-transgenic mice to study the consequence of decreased levels of gene expression *in vivo* in animal models in a spatio-temporal manner.

Recent investigations pointed out the fact that RNA interference could target other non-specific genes (34,35) while others indicated that the repression by RNAi is highly specific (36,37). Because the non-specific effect appears to be primarily sequence-dependent, careful screening of RNAi sequences is recommended to avoid potential problems. Furthermore, majority of the studies used siRNA transiently transfected at high levels, the observed non-specific response could be due to the transient transfection of large amounts of siRNA. In this case, we predict that a stable integrated vector strategy combined with an inducible system could attenuate this

problem. Nonetheless, like all other newly developed technologies, RNAi system is not perfect at its current stand and needs to be consistently improved in such a way that high specific gene suppression at any given stages *in vivo* can be achieved.

ACKNOWLEDGEMENTS

We thank Dr Y. Shi for providing the vector pBS/U6, P. P. Chambon for ERT2 plasmids, and S. Lee and members of Deng laboratory for critically reading and commenting on the manuscript.

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