

Establishment and Utilization of a Tetracycline-controlled Inducible RNA Interfering System to Repress Gene Expression in Chronic Myelogenous Leukemia Cells

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Abstract RNA interference (RNAi), a posttranscriptional gene silencing process mediated by small double-stranded RNA specifically complementary to the targeted transcript, has been used extensively in the development of novel therapeutic approaches against various human diseases including chronic myelogenous leukemia (CML). Here, we report the successful construction of a tetracycline-controlled siRNA in CML cell line K562. A K562 cell line stably expressing the reverse tetracycline-controlled transactivator (rtTA) was constructed. A tetracycline responsive element (TRE) was integrated into the RNA polymerase III promoter region of pBS/U6 that was used to drive specific siRNA to target the novel cytokine receptor-like factor 3 (*CRLF3*) gene. The results show that rtTA was able to recognize the TRE to prevent siRNA-mediated exogenous and endogenous *CRLF3* gene repressions. Moreover, *CRLF3*-siRNA mediated gene repression could be induced in a dose-dependent manner in the presence of doxycycline. Thus, the inducible siRNAi system in K562 cells might be useful for the study of RNAi-mediated therapeutic approaches against CML.

Key words inducible siRNA; tetracycline; reverse tetracycline-controlled transactivator; U6 promoter; *CRLF3*

RNA interference (RNAi) is a posttranscriptional gene silencing process mediated by small double-stranded RNA, 21–23 nucleotides in length, which is specifically complementary to the targeted transcript [1]. RNAi effectors can be endogenous microRNA, small hairpin RNA (shRNA) and synthetic small interfering RNA [2]. These small RNAs can intracellularly activate an RNA-induced silencing complex to turn on targeted gene repression [2]. Only six years since it was discovered, RNAi technology has been speedily developed and is likely to become an exceptional case of the shortest transfer time from basic research to application. One of the main focuses has been the

application of RNAi in leukemia research [3].

Chronic myelogenous leukemia (CML) involves a reciprocal translocation between chromosomes 9 and 22 that results in a fusion protein BCR/ABL with enhanced tyrosine kinase activity [4,5]. BCR/ABL possesses a highly oncogenic capability in transforming hematopoietic progenic cells into leukemic cells [5]. Wilda *et al.* designed a specific shRNA to target the *bcr/abl* fusion gene and found it had a potent effect on silencing targeted gene expression and inducing apoptosis in leukemic K562 cells [6]. It is believed that *bcr/abl* expression is essential for the survival of leukemic cells such as K562 because it constitutively activates an autocrine loop of intracellular mitogenic signals [7]. Thus, there are at least two aspects to the side-effects of using direct RNAi to the *bcr/abl* gene: (1) the rapid death of the targeted cells hampers the kinetic and mechanistic evaluations on the effect of BCR/ABL-RNAi *ex vivo*, such as at the cellular level; (2) it also makes

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it difficult to study the knock-down effect *in vivo* because the modified cells are unable to be further manipulated. To overcome these obstacles, an inducible *bcr/abl* expression cassette has been used in growth factor-dependent cell lines, such as BaF3 and 32D [8,9]. Although these cell lines have been used successfully for RNAi studies by repressing the expression of the integrated *bcr/abl* fusion gene [10,11], the kinetic, mechanistic and animal evaluations on wild-type leukemic cell lines such as K562 are still limited. The sophisticated device described by Ohkawa and Taira [12] for tetracycline-controlled antisense RNA synthesis can be directly applied to inducible RNAi studies [13]. By now, the inducible RNAi technology has been applied to numerous cancer-related studies, such as colon carcinoma [14,15], gastric adenocarcinoma [16], breast cancer [17] and prostate cancer [18]. However, inducible RNAi in leukemic cells is yet to be reported or tested.

In this study, we report the successful establishment of a tetracycline-controlled siRNA system in CML cell line K562. We found that this system was efficient in repressing both exogenous and endogenous cytokine receptor-like factor 3 (*CRLF3*) gene expression. This system can be further extended to study *bcr/abl* RNAi both at the cellular level and in an animal model system.

Materials and Methods

Target gene cloning

Total RNA of CML K562 cells (Cell Bank of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China) was isolated using RNAzol (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The *CRLF3* gene (GenBank accession No. NM_015986) was cloned from K562 cells using a one-step reverse transcription-polymerase chain reaction (RT-PCR) kit (TaKaRa, Tokyo, Japan) with forward primer 5'-tatagtcgacctggagctggagctgagct-3', and reverse primer 5'-tatactcgagctaaacactaacactttcc-3'. RT-PCR was conducted as follows: 50 °C for 45 min; 35 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 2 min; and a final extension at 72 °C for 10 min.

Plasmid construction

After *SalI/XhoI* digestion, the PCR product of *CRLF3* was subcloned into pCMV-Myc and digested with the same restriction endonucleases to generate pCMV-Myc-CRLF3. To construct the pIRESHyg2-reverse tetracycline-

controlled transactivator (rtTA) plasmid, a 1.8-kb DNA fragment containing CMV promoter and the rtTA coding sequence from pUHD172-1neo were ligated into pIRESHyg2 (Invitrogen) digested with *NruI/StuI*.

The tetracycline responsive element (TRE) was introduced into the promoter region of pBS/U6 (provided by Dr. Yang SHI, Harvard Medical School, Boston, USA) through PCR with primers 5'-ttgatagattataaatcccttgagagaaagcc-3' (P_{mU61}) and 5'-tgatagagtactttacagttagggtgagtttcttttg-3' (P_{mU62}). PCR conditions were: denaturing at 95 °C for 5 min; 35 cycles at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 5 min; and a final extension at 72 °C for 10 min. The 5' end of the PCR product was phosphorylated by T4 polynucleotide kinase (TaKaRa) to prevent self-ligation.

Four primers were synthesized as follows: O_{1a} , 5'-ggtagctgtgagcagctcgaa-3'; O_{1b} , 5'-agctttcgactgctcagactgtacc-3'; O_{2a} , 5'-agctttcgactgctcagactgtacccttttg-3'; and O_{2b} , 5'-aattcaaaaagggtacagctgtgagcagctcgaa-3'. O_{1a} and O_{1b} , O_{2a} and O_{2b} were annealed to form duplexes. The duplex products were step-wisely subcloned into pBS/U6 or pBS/U6-TRE to construct pBS/U6-siCRLF3 or pBS/U6-TRE-siCRLF3.

Cell culture, transfection and selection

For the establishment of the K562 cell line stably expressing rtTA, approximately 15 μ g pIRESHyg2-rtTA was transfected into 1×10^7 K562 cells in a 60 mm culture dish. After 24 h, transfected K562 cells were seeded at approximately 1×10^5 cells/well into a 96-well plate under the selection of 1000 μ g/ml hygromycin (Sigma, St. Louis, USA) for approximately two weeks. The hygromycin-resistant colonies were picked up and expanded in the 10 cm dishes for further analysis.

For the detection of rtTA gene expression in K562/rtTA cells, RT-PCR was performed using the ThermoScript RT-PCR system (Invitrogen). The forward primer was 5'-accatgccaagagaccag-3', and the reverse primer was 5'-tcgcgccccctaccacc-3'. RT-PCR conditions were: 50 °C for 30 min; 35 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min; and a final extension at 72 °C for 10 min.

K562/rtTA cells were cultured in 60 mm culture dishes containing RPMI 1640 medium (HyClone, Logan, USA) supplemented with 10% fetal calf serum at approximately 1×10^6 cells/ml at 37 °C with 5% CO₂. After 2 d, the cells were transfected with 10 mg of plasmid pBS/U6-TRE-siCRLF3 using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Two days posttransfection, the cells were collected and lysed for immunoblot analysis.

Western blot analysis

Cells were lysed in lysis buffer containing 1% Nonidet P-40 (NP-40), 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 200 μ M NaVO₄, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin and 1 μ M phenylmethylsulphonyl fluoride. The products were applied onto 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, then transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, USA) which was subsequently blocked in 10% non-fat milk. The membrane was first probed with either anti-Myc or anti- β -actin then with a horseradish peroxidase-conjugated secondary antibody, and visualized by enhanced chemiluminescence (ECL) kit (Santa Cruz Biotechnology, Santa Cruz, USA).

Results

Introduction of one copy of TRE into the promoter region of pBS/U6

We employed a PCR-directed cloning approach to integrate one copy of bacterial tetracycline operon TRE into the promoter region of pBS/U6. PCR was conducted to amplify the whole plasmid sequence of pBS/U6 using primers P_{mU61} and P_{mU62} containing the TATA box and the proximal sequence element (PSE), respectively [Fig. 1(A)]. Then the linear pBS/U6 DNA with modification sequences at both ends was produced. After self-ligation, the tetracycline-controlled pBS/U6-TRE vector was constructed. Successful cloning will render an extra *SacI* site at the junction position between PSE and TRE. After *SacI* digestion, pBS/U6-TRE was produced with two expected bands (1.9 kb and 1.3 kb), whereas pBS/U6 had only one band [Fig. 1(B)]. The pBS/U6-TRE vector was further used for the construction of pBS/U6-TRE-siCRLF3 (data not shown).

Establishment of K562 cell line stably expressing rtTA

In the Tet-on system, the binding of rtTA to TRE can repress downstream gene expression, but the high affinity interaction between the substrate doxycycline (Dox) and rtTA will release the inhibition to turn on gene expression [19,20]. In order to establish the Tet-on system, we built a K562 cell line that can stably express rtTA protein. Plasmid pIRESHyg2-rtTA was transfected into K562 cells. After a 2-week selection, 15 hygromycin-resistant colonies were isolated and expanded. Eight colonies were selected for RT-PCR analysis and two colonies were positive for

rtTA gene expression (Fig. 2). The positive colonies were named K562/rtTA and used for inducible RNAi analysis.

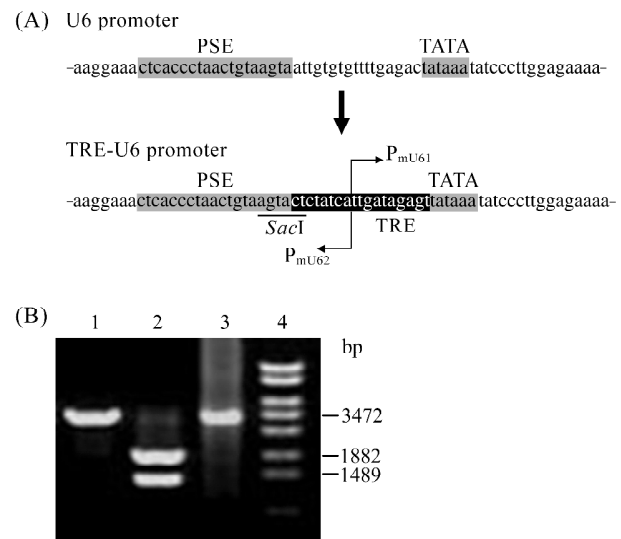


Fig. 1 Construction of tetracycline-inducible siRNA vector pBS/U6-tetracycline responsive element (TRE)

(A) Schematic diagram of promoter modification. The upper panel shows the proximal sequence element (PSE) and TATA elements of mouse U6 promoter. The lower panel shows the modified mouse U6 promoter by polymerase chain reaction (PCR)-directed cloning. Grey boxes represent the PSE and TATA elements. The black box indicates the TRE sequence. The forward primer (P_{mU61}) and the reverse primer (P_{mU62}) used in PCR were located. An extra *SacI* site created by this cloning strategy is underlined. (B) Diagnostic analysis on pBS/U6-TRE vector. 1, the linearized products of pBS/U6 after *SacI* digestion; 2, the two DNA fragments (1.9 kb and 1.3 kb) produced from pBS/U6-TRE after *SacI* digestion; 3, the PCR products amplified from pBS/U6-TRE using primers P_{mU61} and P_{mU62}; 4, λ ECOT14I-digested DNA marker.

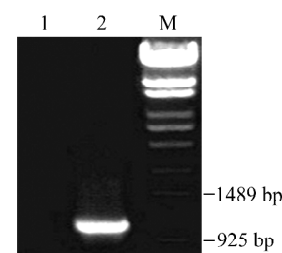


Fig. 2 Detection of reverse tetracycline-controlled transactivator (rtTA) expression in established K562/rtTA stable cells

Total RNA was isolated from either wild-type K562 cells or K562/rtTA stable cells. Approximately 2 μ g of RNA was subjected to one-step reverse transcription-polymerase chain reaction (RT-PCR) analysis using rtTA specific primers. 1, rtTA expression in wild-type K562 cells; 2, the 1044 bp PCR product of rtTA expression in K562/rtTA stable cells; M, λ ECOT14I-digested DNA marker.

Inducible RNAi-mediated *CRLF3* gene repression in K562/rtTA cells

To evaluate the tetracycline-controlled RNAi system in K562/rtTA cells, *CRLF3*, a newly cloned cytokine-like factor 3 gene with unknown function, was chosen as a targeted gene. pCMV-Myc-CRLF3 was co-transfected into K562/rtTA cells with pBS/U6-TRE-siCRLF3 or pBS/U6-siCRLF3. pBS/U6-siCRLF3 served as both the Dox-unresponsive negative control and the *CRLF3* shRNA-mediated RNAi positive control. **Fig. 3** shows that pBS/U6-siCRLF3 significantly repressed *CRLF3* gene expression independent of the addition of Dox. In the absence of Dox, pBS/U6-TRE-siCRLF3 was unable to affect the expression level of *CRLF3* (**Fig. 3**), indicating that rtTA was able to fully recognize TRE to prevent RNA polymerase III (Pol III)-mediated siCRLF3 transcription. In contrast, as the dose of Dox increased to 1 $\mu\text{g/ml}$ or 5 $\mu\text{g/ml}$, TRE-siCRLF3 significantly repressed *CRLF3* gene expression, respectively (**Fig. 3**), indicating that Dox was able to release rtTA-mediated Pol III promoter inactivation to induce specific *CRLF3* shRNA transcription, and in turn activate *CRLF3*-RNAi-mediated gene repression.

Inducible *CRLF3*-RNAi efficiently inhibited endogenous *CRLF3* gene expression

The next question is whether the tetracycline-controlled

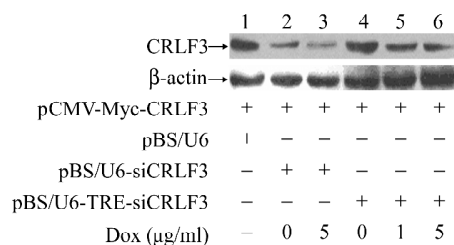


Fig. 3 Cytokine receptor-like factor 3 (*CRLF3*) gene expression was significantly repressed by an inducible RNA interference system in K562/reverse tetracycline-controlled transactivator (rtTA) stable cells

K562/rtTA cells transfected with various combinations of expression plasmids were subjected to Western blot analysis. Approximately 0.5 μg of pCMV-Myc-CRLF3 was co-transfected with 1.5 μg different plasmids into K562/rtTA cells in the absence or presence of doxycycline (Dox) stimulation. 1, pBS/U6; 2 and 3, pBS/U6-siCRLF3; 4–6, pBS/U6-tetracycline responsive element (TRE)-siCRLF3. After resolved onto 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, the reaction products were probed with either anti-Myc or anti- β -actin primary antibodies, then with a horseradish peroxidase-conjugated secondary antibody, and visualized with an enhanced chemiluminescence (ECL) detection kit.

RNAi system could effectively inhibit endogenous *CRLF3* gene expression. Our preliminary study indicated that *CRLF3* has a high level of gene expression in numerous human cell lines including HEK293 (data not shown). To directly address the utility of this inducible RNAi system for the inhibition of endogenous *CRLF3* expression, pIRESHyg2-rtTA was co-transfected with pBS/U6, pBS/U6-siCRLF3 and pBS/U6-TRE-siCRLF3 into HEK293 cells. Consistent with the results shown in **Fig. 3**, pBS/U6-siCRLF3 markedly inhibited endogenous *CRLF3* gene expression regardless of Dox (**Fig. 4**). Moreover, rtTA could recognize TRE to prevent pBS/U6-TRE-siCRLF3-mediated endogenous *CRLF3* gene expression (**Fig. 4**). Differently from pBS/U6-siCRLF3, pBS/U6-TRE-siCRLF3 was able to induce Dox-dependent *CRLF3* gene repression when Dox concentration reached 5 $\mu\text{g/ml}$ (**Fig. 4**). Thus, the result demonstrates that the Dox-inducible RNAi system can effectively inhibit endogenous gene expression.

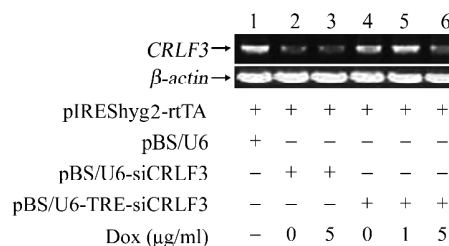


Fig. 4 Inducible cytokine receptor-like factor 3 (*CRLF3*)-RNA interference significantly inhibited endogenous *CRLF3* gene expression

Approximately 0.5 μg pIRESHyg2-reverse tetracycline-controlled transactivator (rtTA) was co-transfected with 1.5 μg different plasmids into HEK293 cells in the absence or presence of doxycycline (Dox) stimulation. 1, pBS/U6; 2 and 3, pBS/U6-siCRLF3; 4–6, pBS/U6-tetracycline responsive element (TRE)-siCRLF3. Twenty-four hours after transfection, total RNA was isolated from each transfection. Approximately 2 μg of RNA was subjected to one-step reverse transcription-polymerase chain reaction (RT-PCR) analysis using specific primers. Arrows indicate the expected RT-PCR products of *CRLF3* and β -actin.

Discussion

Conditional expression of gene-specific RNAi has become an important tool for the study of temporally- and spatially-regulated gene functions. It has many advantages and great potential for therapeutic purposes [21,22]. In this study, we successfully established a tetracycline-controlled inducible siRNA system in CML K562 cells. K562 cells stably expressing rtTA protein were able to fully

recognize the integrated TRE from the modified U6 promoter. Moreover, inducible siRNA was induced to repress both exogenous and endogenous *CRLF3* gene expression. This system may be useful for further biological and therapeutic studies on K562 cells.

Pol III promoter usually contains three sequence elements important for its promoter activity: the TATA box, PSE, and the distal sequence element (DSE) [23]. Study on the Pol III promoter of the *H1* gene has indicated that eliminating the sequence element immediately upstream of transcriptional start site does not significantly affect promoter activity [24]. Thus, the tetracycline operator TRE can be placed between the TATA box and the transcription initiation site to functionally mediate RNAi responses with no harm to Pol III function [18,25]. We showed that integration of the TRE element between PSE and the TATA box of mouse U6 promoter was also effective for Dox-mediated gene repression (Figs. 1 and 3). Similar results have been documented in the inducible-RNAi studies of human U6 promoter [12,26]. However, the substitution of the sequence between PSE and TATA by TRE has been shown to significantly impair H1 promoter activity [24]. This discrepancy might be due to the structural differences between H1 and U6 promoters. In H1 promoter, DSE is compact and closely adjacent to PSE [24], but in U6 promoter, DSE and PSE are separated by a 148 bp spacer element [23]. Thus, placing TRE between PSE and TATA in H1 promoter might have a more direct impact on the binding of transcriptional activators to DSE than that in U6 promoter.

Like conditional knock-out technology, the inducible RNAi system offers an alternative method for biological researchers to study essential gene functions *in vivo* and evaluate therapeutic potentials when the essential gene is selected as an RNAi-targeted gene. Negeri *et al.* employed this technology to study the functions of *Bx42* during *Drosophila* development and found that *Bx42* is essential for the development of many tissues through its interaction with Notch signaling [27]. In addition, researchers have been trying to develop an effective RNAi-mediated therapeutic approach against CML since the beginning of RNAi technology. Direct RNAi targeting at *BCR/ABL* was effective in killing the leukemia cells [6]. The mechanisms underlying this process are difficult to elucidate, but an understanding of them is particularly important for the application of efficient RNAi drugs against this type of disease. Another critical point is to control the viability of the RNAi-targeted cells when the manipulated cells are needed for animal study. Therefore, the establishment of an inducible RNAi system in leukemia cells will be in

immediate demand for the therapeutic study of RNAi in CML.

Our results show that the tetracycline-inducible siRNA in K562 cells established in this study was sensitive to inducing the degradation of exogenous gene expression, and also effective in the knockdown of endogenous gene expression. K562 cells are usually detached, possess suspension cell characteristics, and are difficult to modify. Therefore, the successful establishment of an inducible RNAi system in K562 cells might provide an *in vivo* platform for mechanistic studies of CML pathogenesis. Future work will focus on the design of an inducible RNAi to specifically target the *BCR/ABL* fusion gene in K562 cells.

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