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Development of a tightly regulated U6 promoter for shRNA expression

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Abstract Short hairpin RNAs (shRNAs) have been used to achieve stable target knockdown in a variety of biological systems. Here, we report the development of a tightly regulated tetracycline-responsive human U6 promoter for shRNA expression. By engineering two copies of the tet operators flanking the TATA box of the human U6 promoter, we created a U6 promoter derivative (2O2) that exhibited much lower basal transcriptional activity compared with recently reported inducible pol III dependent promoters. As a consequence of its tighter regulation, the 2O2 system greatly improved the success rate in making inducible knockdown cell lines.

Keywords: Short hairpin RNA; Small interfering RNA; Tetracycline-responsive; U6 promoter; Tight regulation

1. Introduction

The use of small interfering RNA (siRNA) is fast becoming the method of choice to study gene functions in various biological systems. The recent development of pol III dependent short hairpin RNA (shRNA) expression systems further enables stable target knockdown in cells or animals [1–5]. However, the constitutive activity of RNA polymerase III (pol III) dependent promoters imposes various restrictions on the application of these expression systems. There have been attempts to develop tetracycline responsive pol III dependent promoters. Both O1 and O2 types of tetracycline-responsive derivatives of the human U6 promoter were described in the literature [6]. Very recently, several groups reported controlled shRNA expression using the O1 or the O2 type U6 promoters, or using a human H1 promoter variant with a design similar to the O2 type U6 promoter [7–9]. Although these systems appeared to be tightly regulated in reported studies, we observed severe leakiness when we used the O1 or O2 type promoter to express an shRNA targeting luciferase. It is likely that when a potent shRNA is used, a slight leakiness of the system could lead to a significant reduction of the target protein.

To overcome the leakiness associated with the current inducible shRNA expression systems, we have developed a controlled shRNA expression system with minimal basal transcriptional activity. By engineering two tet operators into various positions of the human U6 promoter, we demonstrated that properly placing two O2 type tet operators flanking the TATA box resulted in a very tightly regulated U6 promoter variant. We further demonstrated that the tighter regulation of our expression system greatly improves the success rate in making inducible knockdown cell lines.

2. Materials and methods

2.1. Luciferase assay

Luciferase reporter constructs, pGL-3 (Promega) and pRL-TK (Promega), were transfected into cells using Lipofectamine 2000 (Invitrogen). Luciferase activity was determined using the Dual-Luciferase Assay System (Promega).

2.2. Western blot analysis

Cells were directly lysed on 6-well plates in 1× Laemmli sample buffer. Proteins were separated by SDS–PAGE, transferred to PVDF membrane, and Western blotting was performed according to standard procedures.

2.3. Cell culture

D54-MG cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). HeLa-TREx cells (Invitrogen) were grown in minimum essential medium supplemented with 10% FBS. H1299 cells were grown in RPMI1640 medium supplemented with 10% FBS. All cells were maintained at 37 °C in an environment of 5% CO_2.

2.4. Molecular cloning

The human U6 promoter was synthesized by PCR assembling of overlapping primers.

Tetracycline regulated U6 promoter variants with tet operators at various positions were all generated by PCR modification of the U6 promoter. Plasmids that use U6 promoter variants to express shRNAs were generated by PCR from respective promoter variants using a 5′ primer that hybridizes to the U6 promoter and 3′ primers that contain the hairpin sequences and hybridize to respective promoter variants. The PCR fragments were then cloned into pBluescript II (SK+).

3. Results

3.1. Construction of a tightly regulated human U6 promoter variant

We first investigated whether two tet operators can be engineered into the U6 promoter without abolishing the transcriptional activity. An O1 type tet operator was first engineered between the PSE and the TATA box to create a O1 type U6 promoter that is identical to a previously

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Abbreviations: siRNA, small interfering RNA; shRNA, short hairpin RNA; pol III, RNA polymerase III; Hif1α, hypoxia inducible factor α; tetR, tet repressor

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reported construct in the literature (Fig. 1, O1) [6]. A panel of modified human U6 promoters with two tet operators were then created by replacing part of the O1 type promoter with an O2 type tet operator (Fig. 1). The transcriptional activities of the modified human U6 promoters were assessed by the ability of each promoter to express an shRNA targeting luciferase and inhibit the reporter activity. Based on a dose–response experiment using U6_luc, which utilizes the wild type U6 promoter to drive the expression of a luciferase shRNA, an amount of shRNA plasmid (0.008 μg) that exhibited 80% inhibition of the reporter activity was chosen for evaluation of the transcriptional activity exhibited by the modified U6 promoters. The degree of inhibition varied in cells transfected with U6 derivatives that contain both the O1 and O2 type tet operators. A similar degree of inhibition on luciferase activity was observed in cells transfected with O1_luc, O1O2_luc3, O1O2_luc4, O1O2_luc5, and O1O2_luc6, suggesting that introducing an additional O2 type tet operator into the O1 type promoter at these positions has only a marginal effect on the transcriptional activity (Fig. 2A, O1O2_3, O1O2_4, O1O2_5, and O1O2_6).

The active U6 promoter derivatives were then examined for their response to doxycycline. Strong inhibition of luciferase activity was observed in cells transfected with O1_luc, O1O2_luc5, and O1O2_luc6 regardless of the presence or absence of doxycycline, suggesting that these promoters are very leaky under these experimental conditions (Fig. 2B, O1, O1O2_luc5, and O1O2_luc6). In contrast, cells transfected with O1O2_luc3 and O1O2_luc4 exhibited much lower luciferase activity in the presence of doxycycline than in the absence of doxycycline. However, even in the absence of doxycycline, O1O2_luc3 and O1O2_luc4 transfected cells exhibited a >50% reduction of luciferase activity compared with cells transfected with a control vector (Fig. 2B, O1O2_3, and O1O2_4), suggesting that these promoters are still quite leaky despite of improved regulation compared to the O1 type promoter.

To further improve the inducible system, the O2 type tet operator was introduced to replace the O1 type tet operator in O1O2_3 to generate a 2O2 type promoter (Fig. 1, 2O2). Because the O2 type tet operator has higher binding affinity for tet repressor (tetR) than the O1 type tet operator [10], it is likely that tetR will bind more tightly to the 2O2 type promoter than the O1O2_3 type promoter, resulting in reduced basal transcriptional activity of the promoter. In the absence of doxycycline, O1O2_luc3 caused >70% reduction of the luciferase activity as compared with the control plasmid (Fig. 2C, O1O2). Under the same condition, 2O2_luc caused no more than 30% inhibition of the luciferase activity (Fig. 2C, 2O2), indicating that the 2O2 promoter indeed has less basal activity compared with the O1O2_3 promoter. Meanwhile, O2_luc caused about 85% reduction of the luciferase activity, suggesting that single O2 type tet operator is also very leaky in the absence of doxycycline (Fig. 2C, O2). In the presence of doxycycline, both O1O2_luc3 and 2O2_luc exhibited more than 80% inhibition of the luciferase activity, suggesting that the 2O2 and O1O2_3 type promoters have similar activities upon induction (Fig. 2C). These results demonstrated that it is possible to engineer two tet operators into the U6 promoter without dramatically sacrificing the transcriptional activity. Meanwhile, with two O2 type tet operators flanking the TATA box, the resulting U6 promoter variant, 2O2, exhibited the best doxycycline response compared with U6 promoter variants with a single tet operator (O1 or O2) or a combination of O1 and O2 type tet operators (O1O2).

3.2. Inducible knockdown of endogenous proteins by stably integrated 2O2 promoter

To determine whether the 2O2 promoter retains the ability to respond to doxycycline after integrating into chromosomes, we used a commercial tetR expressing cell line, HeLa-TREx, to establish stable clones that carried the 2O2 promoter linked to an shRNA targeting human Hif1α (2O2_Hif1). Among the five clones that carried the 2O2_Hif1 cassette, two clones exhibited

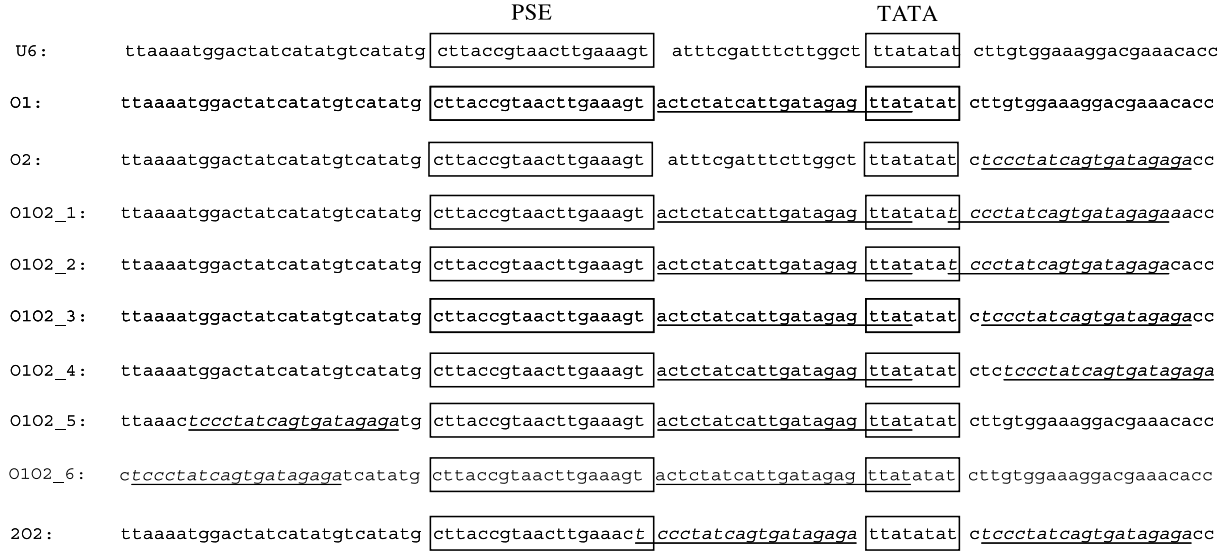

Fig. 1. Sequence alignment of U6 promoter variants. U6 is the wild type human U6 promoter. O1 or O2 is the O1 and O2 type human U6 promoter as previously described, respectively. O1O2_1, O1O2_2, O1O2_3, O1O2_4, O1O2_5, and O1O2_6 are U6 promoter variants with both O1 and O2 type tet operators. 2O2 is the U6 promoter variant with two O2 type tet operators. The underscored italic sequence represents the O2 type tet operator. The underscored non-italic sequence represents the O1 type tet operator.

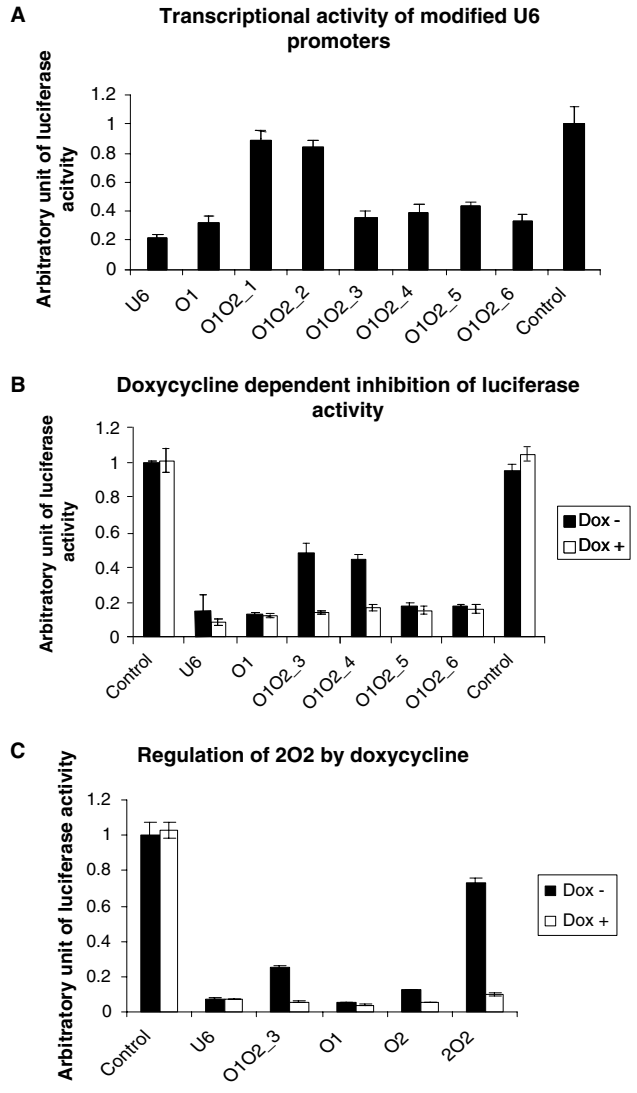

Fig. 2. Transcriptional activity and tetracycline response of U6 promoter variants. (A) The plasmids that use each U6 promoter variants to express shRNAs are designated as U6_luc, O1_luc, O1O2_luc1, O1O2_luc2, O1O2_luc3, O1O2_luc4, O1O2_luc5, and O1O2_luc6. 0.008 μg each of these plasmids or a control vector was cotransfected with 1 μg pGL3-control and 0.5 μg pRL-TK. Luciferase activities in transfected cells were determined 72 h post transfection. (B) 0.2 μg each of a control plasmid, U6_luc, O1_luc, and O1O2_3–6 was contransfected with 1 μg pGL3-control, 0.5 μg pRL-TK and 1 μg of pcDNA6/TR. (C) 0.2 μg each of a control plasmid, U6_luc, O1O2_3, O1_luc, O2_luc, or 2O2_luc was contransfected with 1 μg pGL3-control, 0.5 μg pRL-TK and 1 μg of pcDNA6/TR. For doxycycline treatment, cells were changed to culture medium containing 1 μg/ml of doxycycline 24 h post transfection. Luciferase activities were determined 48 h after induction by doxycycline.

a more than 90% reduction of HIF1α protein upon induction (Fig. 3A, Hif1-6 and Hif1-7). These results demonstrated that the 2O2 promoter retains its doxycycline responsive property after integrating into a chromosome.

Using the best-regulated 2O2_Hif1 clone (Hif1-7), we further characterized the time and dose dependency of doxycycline induction of the 2O2 expression system. A significant reduction of hypoxia inducible factor α (Hif1α) protein was observed as early as 12 h after induction and more than 90% inhibition of Hif-1 protein was observed 24 h after doxycycline

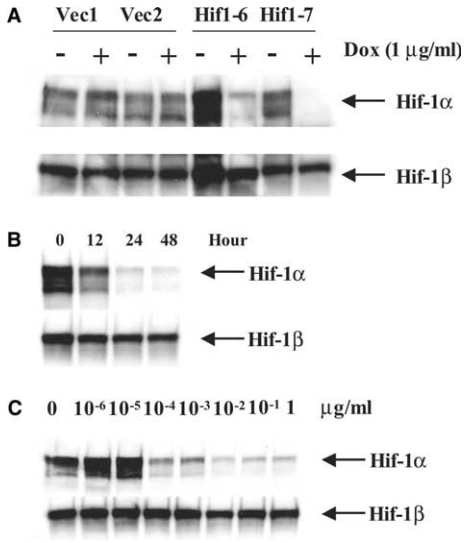

Fig. 3. Tetracycline dependent target knockdown in stable cell lines using the 2O2 expression system. (A) Hela-TREx stable cell lines with integrated 2O2 vector (vec1, vec2) or 2O2_Hif1(Hif1-6, Hif1-7) were treated with 1 μg/ml doxycycline for 24 h followed by 6 hour treatment with 100 μM DFO. (B) Hif1-7 cells were treated with 1 μg/ml of doxycycline for indicated time before being treated with 100 μM DFO for 6 h. (C) Hif1-7 cells were treated with different concentrations of doxycycline for 48 h followed by 6 h treatment with 100 μM DFO. After treatment, cells were lysed and analyzed by Western blotting using an antibody against Hif1α or Hif1β.

treatment. Longer induction did not lead to more complete inhibition of Hif1α protein (Fig. 3B). The doxycycline concentration that is required for maximal induction of the 2O2 system was determined in a dose–response experiment. A more than 90% inhibition of Hif-1 protein was observed in the presence of 0.1 ng/ml of doxycycline and the maximal inhibition of Hif1α protein was reached in the presence of 10 ng/ml of doxycycline (Fig. 3C). These results highlight the fast response and extreme sensitivity of the 2O2 system to doxycycline induction.

3.3. Comparison of the O1 and 2O2 systems in making cell lines

During the course of our study, three groups reported pol III dependent inducible expression systems for regulated target knockdown [7–9]. In contrast to these reported observations, we observed severe leakiness of the O1 promoter in our initial studies (Fig. 2B, O1). To determine whether the observed leakiness of the O1 system will have a negative impact on the ability of using the O1 system to create stable cell lines, we directly compared the success rate of making inducible cell lines using both the O1 and the 2O2 systems. A D54MG cell line with high level of tetR expression was first established, and plasmids that utilize the O1 or 2O2 promoters to drive the expression of shRNAs targeting luciferase (O1_luc and 2O2_luc) were transfected with a hygromycine resistant gene into this cell line. The drug resistant clones were selected and analyzed by PCR to identify clones that carry the inducible shRNA expression cassette. We obtained four clones with stably integrated O1_luc and seven clones with stably integrated 2O2_luc cassette as analyzed by PCR (data not shown). All the clones displayed similar level of tetR expression (Fig. 4B). These clones were examined for their response to

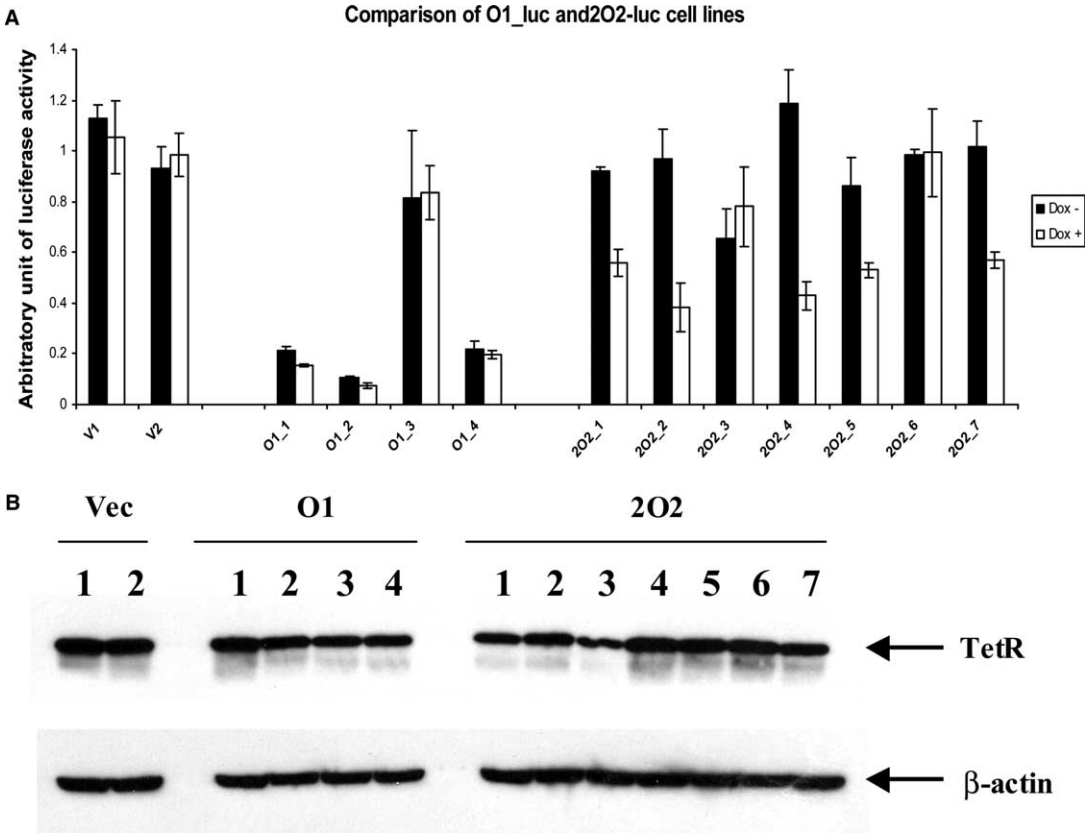

Fig. 4. Comparison of the O1 and 2O2 expression system in making stable cell lines. (A) D54MG-tetR cells with stably integrated O1_luc (O1_luc1...O1_luc4), 2O2_luc (2O2_luc1...2O2_luc7) or the 2O2 vector (control) were transfected with 1 μg pGL3-control and 0.5 μg pRL-TK. For doxycycline treatment, cells were changed into medium containing 1 μg/ml doxycycline 24 h post transfection. Luciferase activities were determined 48 h after induction by doxycycline. (B) The cells in (A) were lysed after treatment with 1 μg/ml doxycycline for 48 h and analyzed by Western blotting using an anti-tetR antibody. The same blot was stripped and immunoblotted with an anti-actin antibody to show the equal loading of sample in each lane.

doxycycline induction. None of the four O1_luc clones exhibited significant doxycycline dependent reduction of luciferase activity (Fig. 4A, O1_luc1 to O1_luc4). Interestingly, three out of the four O1_luc clones exhibited constitutive inhibition of the luciferase activity regardless of the presence or absence of doxycycline, indicating severe leakiness of the O1 system (Fig. 4A, O1_luc1, O1_luc2, and O1_luc4). In contrast, among the seven 2O2_luc clones, two clones exhibited strong doxycycline dependent inhibition of luciferase activity (Fig. 4A, 2O2_luc2, 2O2_luc4) and three clones displayed modest degree of doxycycline dependent inhibition of luciferase activity (Fig. 4A, 2O2_luc1, 2O2_luc5, and 2O2_luc7). The shRNA expression cassette for clones O1_3, 2O2_3 and 2O2_6 could be inserted into transcriptional inactive site in a chromosome, resulting in no inhibition of luciferase activity regardless of the presence or absence of doxycycline.

4. Discussion

We have developed a tightly regulated tetracycline responsive U6 promoter for expressing shRNA. Although the originally described O1 system was successfully used for inhibition of DNMT in a tetracycline-dependent manner [6], the O1 system was extremely leaky when used to express shRNA targeting luciferase both in transient assays and in stable cell lines. There are multiple factors that could contribute to these different observations. For example, it is well known that different siRNAs function at vastly different concentrations. For potent siRNAs, a 90% knockdown of targets can be achieved by transfecting sub-nanomolar amounts of siRNA; while for others, 100 nM may be required to achieve the same degree of target knockdown. This difference in functional concentration of siRNA could result in a different tolerance of leakiness of the shRNA expression system. For less potent siRNAs, slight leakiness of the system will not generate a dramatic effect on the target protein level. However, for potent siRNAs such as the luciferase shRNA used in our study, a slight leakiness of the system will produce enough siRNA to significantly knockdown the target.

Another factor that will affect the regulation of the O1 system is the tetR expression level. In our experience, although the O1 system appeared to be constitutively active when the O1_luc and a plasmid for tetR expression were transfected at a 1:5 ratio, the system did exhibit a small degree of tetracycline responsiveness when the two plasmids were transfected at about a 1:300 ratio (data not shown). The tetR expression cell line that was used to demonstrate inducible knockdown of DNMT might express an extremely high level of tetR, because the induced target knockdown is only apparent when 10 μg/ml of doxycycline was used as compared to 0.1 ng/ml of doxycycline in our study. The requirement for high tetR expression

level could limit the use of the inducible system in several ways. First, a significant amount of effort will need to be spent on identifying tetR expressing cell lines or tetR expressing mice with a high enough level of tetR expression for complete inhibition of the basal activity of the O1 system. Second, the high dose of doxycycline required to induce the shRNA expression might generate undesired side effects. This is especially problematic if the inducible system is used in vivo.

Two other regulated pol III dependent promoters with designs similar to the O2 type U6 promoter have also been used to express shRNA [7,9]. Although we did not compare O2 system directly to 2O2 system in making stable cell lines, we did observe severe leakiness of O2 systems in transient assays (Fig. 2C). The direct comparison of 2O2 system with the inducible H1 promoter is difficult because the optimal shRNA sequence that can be used in the H1 promoter system and the U6 promoter system is different. Because the inducible H1 promoter is similar in design to the O2 type U6 promoter, it is likely that the regulation of the two systems is similar.

Although it is possible to obtain an inducible knockdown of targets in cell lines or in animals using a single tet operator inducible system, provided a shRNA with proper potency is used and sufficient number of clones are screened, using a much tightly regulated two tet operator system such as the 2O2 system could markedly improve the success rate in generating stable cell lines. The high success rate is particularly important for making inducible knockdown mice, because of the difficulty and high cost associated with screening for large number of ES clones or founder mice for desired regulation of targets. The ease of generating stable cell lines using 2O2 coupled with the prompt response to very low dose of doxycycline, etc., makes it a useful system for studying loss of function phenotype in cell lines and in animals or applying RNAi to gene therapy.

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