Development and applications of a nasopharyngeal carcinoma Tet-Off cell line

YU-PENG FENG[1*], QI-CAI LIU[4*], JUN-FENG ZHU[3], FU-KANG XIE[1] and LI LI[2]

[1]Department of Histology and Embryology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou 510080; [2]State Key Laboratory of Oncology in Southern China, Imaging Diagnosis and Interventional Center, Cancer Center; [3]Department of Pathology, The First Affiliated Hospital, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou 510060; [4]Experimental Medical Research Center, Guangzhou Medical College, Guangzhou 510182, P.R. China

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Abstract. The conditional activation and inactivation of target gene expression in a nasopharyngeal carcinoma (NPC) cell line is beneficial for the study of the roles of NPC-related genes. Based on the Tet-Off Advanced system, a NPC S18 Tet-Off cell line was developed by stable transfection of a pTet-Off Advanced vector (regulator plasmid in Tet-Off Advanced system) into NPC S18 cells. Doxycycline-dependent regulators expressed in the S18 Tet-Off cells were examined by transient and stable transfection of pTRE-Tight-Luc. The S18 Tet-Off-Luc clone selected by stable transfection of pTRE-Tight-Luc into S18 Tet-Off cells expressed firefly luciferase under tight control of doxycycline in a time- and dose-dependent manner. To test applications of the S18 Tet-Off cell line in the study of gene function, the impact of ferritin heavy chain (FTH1) gene on NPC cell growth was examined. The S18 Tet-Off-FTH1 clone was developed by stably transfecting pTRE-Tight-FTH1 (response plasmid harboring FTH1) into S18 Tet-Off cells. FTH1 levels in the S18 Tet-Off-FTH1 clone were semi-quantitatively regulated in response to varying concentrations of doxycycline. A cell proliferation assay showed that a high expression of FTH1 (cells grown in the absence of doxycycline) reduced cell growth, while moderate FTH1 overexpression (cells grown in 0.1 ng/ml doxycycline) had no adverse effect on cell growth. In conclusion, the S18 Tet-Off cell line provides a proven genetic background for convenient access to controllable gene expression in NPC.

Correspondence to: Dr Li Li, Imaging Diagnosis and Interventional Center, Cancer Center, Sun Yat-Sen University, 651 Dongfeng Road East, Guangzhou 510060, P.R. China
E-mail: li2@mail.sysu.edu.cn

Dr Fu-Kang Xie, Department of Histology and Embryology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou 510080, P.R. China
E-mail: frankxie2000@yahoo.com

*Contributed equally

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Introduction

Nasopharyngeal carcinoma (NPC) is a non-lymphomatous squamous cell carcinoma occurring in the epithelial lining of the nasopharynx. NPC occurs frequently in the populations of Southern China, Northern Africa and Alaska (1). The combination of radiotherapy and chemotherapy is currently the standard treatment strategy. However, the optimal regimens of chemotherapy and radiotherapy have yet to be determined (2). The poor survival rate and high recurrence risk require that novel therapeutic approaches be developed. The therapeutic strategy targeting specific molecules and immunotherapy may improve the of outcome of NPC (3). However, the development of novel strategies is limited due to insufficient understanding of the genes related to NPC development and the lack of conventional cell and animal models for monitoring genes affecting tumor growth and metastasis.

NPC cell line S18, a subclone that was isolated from the human NPC undifferentiated cells (CNE-2 cells) (4), exhibits easy migration and invasion *in vitro* when compared to CNE-2 cells. Moreover, the S18 xenografted cancer model has a significant metastatic potential and maintains a similar growth rate to the *in situ* cancer.

Regulation of the expression of target genes in the S18 cell line, is considered to be of great significance for the investigation of target genes in NPC. The Tet-Off Advanced System is a well-developed gene regulation tool in eucaryon (5,6). The Tet-Off Advanced system comprises two key components: the doxycycline-dependent regulator, such as pTet-Off Advanced, and the response element containing the target gene, such as pTRE-Tight-X. The Tet-Off Advanced system aims to create a double-stable cell line that contains integrated copies of the regulator and response plasmids. In this cell line, transcription of the gene of interest is maintained in the 'off' state by the presence of doxycycline in the culture medium, while transcription induction may occur following the removal of doxycycline. Although this system appears to

be beneficial, more studies are required to create an inducible system. Moreover, the efficacy of doxycycline-controlled gene induction is known to be affected by cell types (7-9). To the best of our knowledge, no study has reported whether this system can confirm a high efficacy of transcriptional induction of target genes in NPC cell lines. In the present study, a NPC S18 Tet-Off cell line was developed, which effectively expressed a doxycycline-dependent regulator, and had potent transcriptional activity of genes of interest with a low background.

Materials and methods

Cell culture. Human NPC S18 cells (kindly provided by Dr Chaonan Qian) were grown in high glucose (Gibco) Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 10% Tet system-approved fetal bovine serum (FBS; Clontech). S18 Tet-Off cells were grown in complete DMEM medium containing 100 μg/ml G418 (Clontech). The S18 Tet-Off-Luc and Tet-Off-FTH1 clones were grown in complete DMEM containing 100 μg/ml G418 and 100 μg/ml hygromycin (Alexis) with or without 10 ng/ml doxycycline (Alexis). For inducible experiments, cells maintained in the 'off' state of gene expression by 10 ng/ml doxycycline were passaged. Briefly, the cells were washed twice with phosphate-buffered solution (PBS) prior to trypsinization. Following trypsinization and harvesting, the cells were washed twice with PBS, and grown in fresh medium with or without various concentrations of doxycycline.

Transfection protocol. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to modified manufacturer's instructions. For transient transfection, cells ($2x10^4$) were plated in 100 μl of complete cell growth medium on a 96-well plate and incubated for 24 h until a cell confluence of 50-60% was achieved. DNA-Lipofectamine 2000 was prepared by combining the diluted plasmid DNA [0.22 mg in 25 ml of Opti-MEM (Invitrogen)] with Lipofectamine 2000 (0.15 ml in 25 ml of Opti-MEM) followed by incubation at room temperature for 20 min. The DNA-Lipofectamine 2000 reagent complex was then directly added to each well, followed by mixing and subsequent incubation at 37˚C. After 6 h, the medium was replaced with fresh complete cell growth medium with or without 10 ng/ml doxycycline followed by incubation for 48 h. Firefly luciferase activity was then determined.

For stable transfection, $5x10^5$ cells in 500 μl of complete cell growth medium were seeded on a 24-well plate. After 16-24 h of culture, the cells were transfected with DNA-Lipofectamine 2000 reagent complex (0.8 or 0.84 μg of plasmid in 50 μl of Opti-MEM mixed with 0.5 μl of Lipofectamine 2000 in 50 μl of Opti-MEM) when cell confluence of approximately 90% was achieved. All the plasmids were digested with restriction enzyme *Sca*I. One day post-transfection, the cells were washed, trypsinized and divided equally onto two 10-cm plates. Following 24 h of incubation, either 500 μg/ml G418 or G418 plus 250 μg/ml hygromycin was added to the medium. After 16 days, antibiotic-resistance clones were selected, using cloning rings, and analyzed. Under these conditions, the transfection efficacy was >80%, with low cytotoxicity detected 48 h after transfection with pEGFP-C1 under identical transfection conditions.

Firefly luciferase activity assay. Cells were washed twice with PBS, lysed and then assayed for firefly luciferase activity with the dual-luciferase reporter assay (for the transient transfection that requires Renilla luciferase to normalize the transfection efficacy) or the luciferase assay system, according to the manufacturer's instructions (Promega, Madison, WI, USA). The firefly luciferase activity in each cell lysate was measured with the GloMax 96 Microplate Luminometer (Promega) according to the standard protocol, and the protein concentration was determined using the BCA method (Pierce, Rockford, IL, USA). Luminescence data were expressed as relative light units (measured in 10 s) per milligram of protein.

Construction of plasmids and reverse transcription-PCR (RT-PCR). The human ferritin heavy chain gene (FTH1) with Kozak sequence was amplified by RT-PCR from the total RNA of S18 cells. FTH1 gene was then subcloned into the *Sma*I site of pUC119 yielding pUC119-FTH1. Following sequencing, the fragment from pUC119-FTH1 after digestion by *Eco*RI and *Kpn*I was cloned into the same site of pTRE-Tight yielding pTRE-Tight-FTH1. For RT-PCR, the first strand cDNA was reverse transcribed with oligo (dT) primer. The full-length FTH1 with Kozak sequence was amplified using PrimeSTAR HS DNA Polymerase (Takara). The primers were forward, 5'-GAATTCGCCACCATGACGACCGCGTCCACCTC-3' and reverse, 5'-AGATCTGGTACCTTTAGCTTTCATTATCA CTGTC-3'.

Western blot analysis. Cells were lysed in RIPA buffer [50 mM Tris pH 8.0, 150 mM sodium chloride, 1.0% Triton X-100 (v/v), 0.5% sodium deoxycholate and 0.1% SDS (w/v)], agitated for 30 min at 4˚C and centrifuged at 12,000 x g for 15 min. The concentration of total proteins was determined using BCA. Total proteins (25 μg) in equal volume of 2X Laemmli buffer were then denatured and subjected to 12% SDS-PAGE. The proteins were transferred onto acetyl cellulose membranes which were subsequently blocked in 5% non-fat milk in TBST (20 mM Tris pH 7.6, 137 mM NaCL, 0.1% Tween-20). The membranes were incubated with primary antibodies overnight [rabbit anti-FTH1 1:1,000 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and rabbit anti-GAPDH 1:2,000 (GenScript, Piscataway, NJ, USA)]. Following washing, the membranes were treated with secondary antibody [HRP-conjugated goat anti-rabbit 1:5,000 (Invitrogen)] and visualized by enhanced chemiluminescence.

MTT assay. Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay. Cells were plated onto 96-well plates and maintained in 100 μl of DMEM (supplemented with different concentrations of doxycycline) for 3 days. The experiment was repeated three times for each group. After 24, 48 and 72 h of incubation, 20 μl MTT was added to each well and the incubation was maintained for an additional 4 h at 37˚C. At the end of the incubation period, 150 μl DMSO was added to each well and throroughly mixed for 10 min. Absorbance at 570 nm was then determined.

Table I. Analysis of firefly luciferase activity induction in the S18 Tet-Off clones.

Cell clone	Firefly luciferase activity (RLU/μg protein)		Regulation factor
	Dox$^+$	Dox$^-$	
A5	175±13	48,062±4,052	274
B18	397±18	93,619±8,466	236
B7	193±16	36,835±1,613	190
B4	120±11	17,623±1,489	146
A12	75±10	9,836±801	131
B15	228±12	29,571±2,763	130
A21	201±12	23,523±1,045	117
A9	74±5	3,115±188	42
B23	96±9	2,653±267	28
B19	77±6	1,766±189	23

S18 cells were stably transfected with the pTet-Off Advanced plasmid, and clones resistant to G418 were selected and transiently transfected with pTRE-Tight-Luc. Cell culture was performed in the presence (Dox$^+$) or absence (Dox$^-$) of 10 ng/ml doxycycline for 48 h, followed by harvesting and measurement of firefly luciferase activity. Data were normalized by the Renilla luciferase activity (introduced by cotransfection with pRL-TK to normalize the transfection efficacy). Of the 52 G418-resistant clones, 10 exhibited a >20-fold regulation factor. Data from four independent transfection experiments are expressed as the means ± SD.

Table II. Analysis of firefly luciferase activity induction in the S18 Tet-Off-Luc clones.

Cell clone	Firefly luciferase activity (RLU/μg protein)		Regulation factor
	Dox$^+$	Dox$^-$	
D7	0.89±0.17	3,281±352	3,671
D25	2.47±0.33	5,280±188	2,138
D18	29.3±2.60	44,491±1,189	1,534
D3	0.96±0.09	1,177±167	1,225

The S18 Tet-Off cell line was co-transfected with pTRE-Tight-Luc and the linear hygromycin marker. Clones resistant to G418 and hygromycin were selected and each clone was grown in the presence or absence of 10 ng/ml doxycycline for 48 h. The firefly luciferase activity was then assayed. Of the 24 clones, 4 exhibited a >1,000-fold induction. The clone with the lowest background level (D7) was termed the S18 Tet-Off-Luc cell line and subcloned for further experiments. Data from four independent experiments were expressed as the means ± SD.

Results

Generation of S18 Tet-Off clones. NPC S18 cells were transfected with the pTet-Off Advanced plasmid, the G418-resistant clones were isolated and the inducible firefly luciferase activity was detected following transient transfection of pTRE-Tight-Luc into each clone. Among the 52 clones identified, the firefly luciferase expression in 10 clones during induction was 20-fold higher than that at the non-induced state (Table I), indicating the 10 clones that expressed the functional regulator protein. Of the 10 clones, the cell clone (A9) with a 42-fold increase in the firefly luciferase expression was selected as the host cell line for the development of double-stable cells (S18 Tet-Off cell line).

Generation of clones with inducible firefly luciferase activity. To investigate whether the S18 Tet-Off cell line maintains a high induction activity of firefly luciferase and a low basal expression, clones were prepared by stable transfection with pTRE-Tight-Luc into the cell line. Of the 24 clones, 4 exhibited a >1,000-fold increase in induction (Table II). These results suggest that it is relatively simple to develop stable clones that show at least 1,000-fold inducible transcription activity based on the host cell line. The D7 clone with the lowest basal expression and the highest induction activity of firefly luciferase was termed the S18 Tet-Off-Luc cell line and subcloned for further experiments.

Time kinetics of inducible firefly luciferase expression. The time course of firefly luciferase activity of the S18 Tet-Off-Luc cell line was analyzed in the absence or presence of doxycycline. As shown in Fig. 1A, the depletion of doxycycline led to a rapid induction of firefly luciferase activity. Luciferase activity increased by 775-fold within 3 h and by 3,321-fold in 12 h; the peak level was reached within 24 h. To investigate whether residual doxycycline in culture medium inhibits the

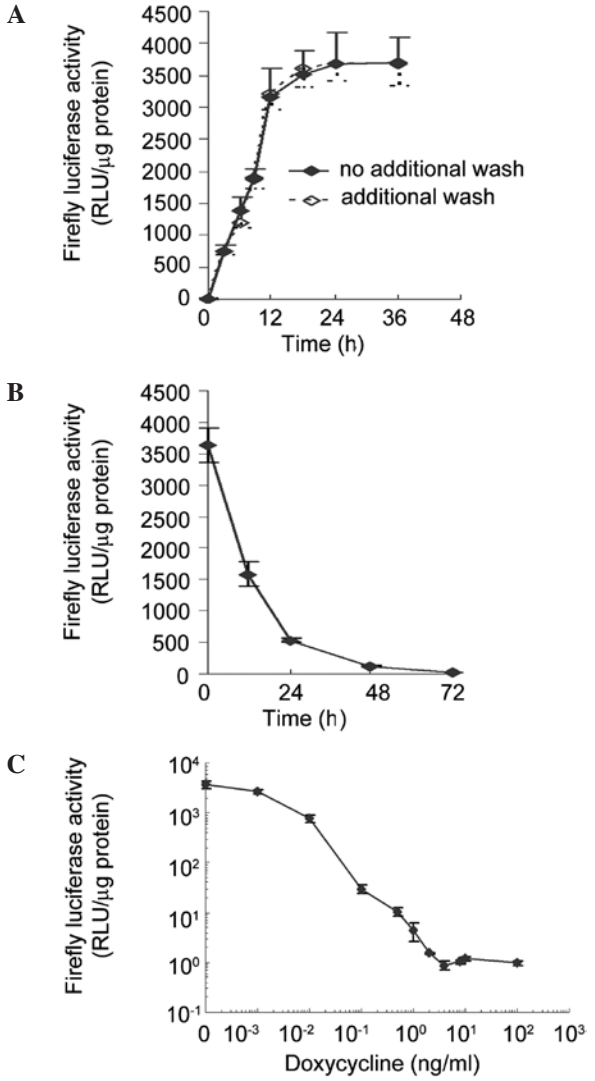

Figure 1. Time- and dose-dependent firefly luciferase activity of the S18 Tet-Off-Luc cell line. (A) Cells were grown in the medium containing 10 ng/ml doxycycline for at least 5 days. The cells were then transferred into doxycycline-free medium with or without an additional wash after 3 h of the initial removal of doxycycline. The cell extracts were harvested at the designated time points and examined for luciferase activity. The two time courses almost overlapped each other, leading to a rapid induction of firefly luciferase activity with or without an additional wash following the depletion of doxycycline, indicating that residual doxycycline did not inhibit maximum transcription activity. (B) Cells were grown in the medium without doxycycline for 5 days. The cells were then transferred to a medium containing 10 ng/ml doxycycline. The cells were harvested at the designated time points and examined for luciferase activity. A rapid reduction of firefly luciferase activity was observed in the presence of doxycycline. (C) Cells were grown in the medium containing 10 ng/ml doxcycline for 5 days and then in fresh medium with various concentrations of doxycycline. Incubation was performed for 48 h and cells were harvested and examined for firefly luciferase activity. The induction of luciferase activity by doxycycline in S18 Tet-Off-Luc cells occurred in a dose-dependent manner. The doxycycline concentration ranged between 0 and 4 ng/ml, within which the expression of firefly luciferase was the most sensitive to doxycycline induction. Data from three independent experiments are expressed as the means ± SD.

full gene expression (maximum transcription activity), the cells were washed once with PBS 3 h after initial removal of doxycycline, and the luciferase activity was analyzed. As shown in Fig. 1A, the two time courses almost overlapped. As shown in Fig. 1B, a similar rapid reduction of firefly luciferase

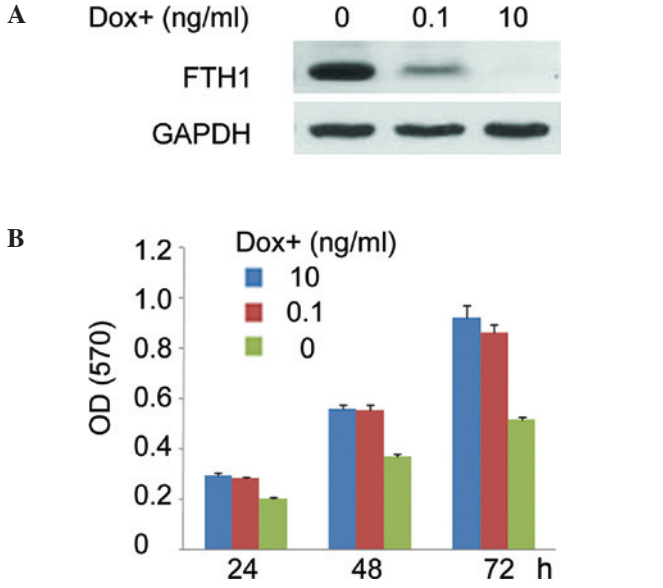

Figure 2. FTH1 gene levels in the S18 Tet-Off-FTH1 clone regulated by various concentrations of doxycycline and its effect on cell growth. (A) FTH1 levels induced by different concentrations of doxycycline were detected by Western blotting. In the S18 Tet-Off-FTH1 clone, the expression of FTH1 was semi-quantitatively controlled by doxycycline. (B) Effect of various levels of FTH1 on the growth of cells. A high expression of FTH1 at 0 ng/ml doxycycline reduced cell growth, while no obvious growth changes were observed by a slight overexpression of FTH1 (0.1 ng/ml). The growth rate of cells at 10 ng/ml doxycycline was used as the control. Values given are the means ± SD (n=3; P<0.05, two-tailed unpaired t-test).

activity was observed in the presence of doxycycline. The luciferase activity dropped to 43.7% of its initial level within 12 h and to <15% in 24 h.

Dose-dependent expression of firefly luciferase by doxycycline. S18 Tet-Off-Luc cells were grown in medium containing 10 ng/ml doxycycline for 5 days and then in medium containing various concentrations of doxycycline for 48 h. The cell extracts were then analyzed for firefly luciferase activity. As shown in Fig. 1C, the firefly luciferase activity was completely inhibited at 4 ng/ml doxycycline, an event that was also observed at 100 ng/ml. A stepwise reduction in the concentration of doxycycline gradually increases the activity of firefly luciferase from complete inhibition to an increase of 3,600-fold. This result showed that induction of the luciferase expression by doxycycline in S18 Tet-Off-Luc cells occurred in a dose-dependent manner. The doxycycline concentration ranged between 0 and 4 ng/ml, within which the expression of firefly luciferase was the most sensitive to doxycycline induction.

Expression of human ferritin heavy chain controlled by doxycycline. For further applications of the S18 Tet-Off cell line, the clone inducibly expressing FTH1 was developed. The construct of pTRE-Tight-FTH1 was stably transfected into S18 Tet-Off cells. FTH1 levels in clones were determined by Western blotting. One of these clones expressing the highest levels of FTH1 in the absence of doxycycline with a low background level in the presence of 10 ng/ml doxycycline was selected and termed S18 Tet-Off-FTH1 clone. By adjusting the concentrations of doxycycline, the semi-quantitative expression of FTH1 was achieved in the S18 Tet-Off-FTH1

clone. Fig. 2A shows that in the S18-Off-FTH1 cell line, FTH1 expression was maximally inhibited at a concentration of 10 ng/ml doxycycline and increased to a moderate level at 0.1 ng/ml, and then completely depressed when doxycycline was removed.

Effect of human ferritin heavy chain overexpression on NPC S18 cell proliferation. The S18-Off-FTH1 clone is maintained in culture in the absence of doxycycline for up to 2 months without evident signs of toxicity. However, it was observed that the clone achieved confluence more rapidly in the presence of doxycycline than in the absence thereof. Doxycycline at 10 ng/ml had no evident effects on cell growth. To assess the impact of FTH1 on cell proliferation, a MTT assay that measures mitochondrial activity was used (Fig. 2B). The results showed a correlation between the effect on cell viability and the FTH1 levels. When cells were grown in the absence of doxycycline, a significantly high overexpression of FTH1 caused at least a 30% decrease in cell growth rate at 48 h. When cells were grown at 0.1 ng/ml doxycycline, the moderate overexpression of FTH1 did not affect cell growth.

Discussion

Among head and neck cancers, NPC is highly susceptible to lymph node metastasis. Undifferentiated NPCs have a higher rate of local control after treatment, but a higher incidence of distant metastasis than differentiated NPCs (10,11). The undifferentiated NPC S18 cell line, not only showed characteristics of advanced cancers, but also had a high potential of lymph node metastasis. Therefore, S18 cells serve as an option for the development of a cell model in which the S18 target gene is conditionally overexpressed.

In this study, following the stable transfection of pTet-Off Advanced plasmid into S18 cells, G418-resistant clones were selected and analyzed using the transient luciferase expression assay. In the investigation of 10 clones with an increase of luciferase activity of more than 20-fold, the expression levels in the absence of doxycycline varied by 53-fold, whereas the expression levels at 10 ng/ml doxycycline varied by less than 6-fold.

This clonal variation is most likely contributed to the variation in the concentration of regulator proteins in each clone (12). According to the manufacturer's instructions of the Tet-Off Advanced system (Clontech), only clones exhibiting 20- to 50-fold induction were selected to develop double-stable cell lines. We selected the clone with a 42-fold induction as the host cell line. Following stable transfection with pTRE-Tight-Luc into this clone, clones with more than 1,000-fold induction of transcriptional activity were developed. The basal expression level (RLU=0.89, n=4, SD=0.17) of the double-stable cell line (S18 Tet-Off-Luc) in the presence of 4 ng/ml doxycycline was comparable to that of the background level. This extremely low background level is similar to that of well-established double-stable Tet controlled cell lines, such as HeLa (clone X1) and CHO AA8 (clone 19) (5,12). As shown in the majority of studies, if a low level of background expression is achieved, a regulation factor of between 50- and approximately 1,000-fold is sufficient (13). Therefore, the 42-fold clone was termed S18 Tet-Off cell line.

Rennel and Gerwins previously reported that residual doxycycline binding to cells or extracellular matrix prevents full gene induction in the Tet-Off system. Moreover, a robust and rapid transgene expression is induced in certain cell lines if doxycycline is removed by washing 3 h after the initial removal of doxycycline (14). We found that the additional wash was not required for the double-stable cell lines based on the S18 Tet-Off cell line, making the operation more convenient.

As shown in Fig. 1C, the transcription activity was quantitatively regulated by doxycycline. The doxycycline at between 0 and 4 ng/ml allowed adjustment of promoter activity within a range of three orders of magnitude. This may allow assessment of quantitative parameters of gene function.

To further test the application of the S18 Tet-Off cell line, another clone that inducibly expressed candidate MRI reporter gene FTH1 (15,16) was successfully created. As an MRI candidate reporter, evidence of its safety warrants further investigation of its practical applications. Thus, in the present study, the impact of FTH1 overexpression on NPC S18 cell growth was addressed. The highest expression of FTH1 (grown in the absence of doxycycline) reduced cell growth. However, a moderate overexpression of FTH1 (grown in 0.1 ng/ml doxycycline) in NPC S18 cells did not reduce cell growth. These results were in accordance with those of Cozzi *et al* (18). Findings of that study showed that the overexpression of FTH1 in HeLa cells induced an iron-deficient phenotype with significantly reduced cell growth. In a previous study, overexpression of FTH1 in C6 glioma cell lines (19), A549 cells (16) and stem cells (20) did not reduce cell growth. However, Cheng *et al* (21) found that a significant reduction in growth rate was observed in C6 glioma cell lines under markedly high FTH1 transgene expression (>500% of the level in mouse embryonic stem cells described in their study). On the other hand, in other S18 double-stable clones, those with low FTH1 levels did not reduce cell growth (data not shown). It was hypothesized that a significantly high expression of FTH1 decreases cell growth in certain types of cells, whereas a moderate level of FTH1 expression may be safe.

To investigate the role of genes relative to NPC, a NPC cell line stably expressing the genes is a powerful tool. Routinely, target genes under the general and non-controlled promoter, including CMV promoter, are transfected into host cells, and then clones with high expression levels are selected to investigate the involvement of these clones in NPC. However, this process leads to certain issues: i) To develop stable clones with a high expression of level of target genes is impossible if the target genes are toxic to cells. ii) If the gene of interest has no evident cell cytotoxity, a high expression of target genes continues for the whole clone-selected process. Chronic alterations may exist, however, and these changes are often not easy to detect. For example, a high expression of FTH1 reduces NPC S18 cell growth, and does not form clones in the early clone-selected days. Clones with low expression levels are usually selected. If this selection occurs, it is difficult to judge the impact of FTH1 on cell growth. iii) The expression levels vary largely as a result of different vector integration sites. In order to study the biological properties on different levels of the target gene, more cell clones should be selected and investigated. However, this process is time-consuming

and not cost-effective. A gene-controllable system would be beneficial. Therefore, the NPC S18 Tet-Off cell line is an effective tool that provides a proven genetic background for gene regulation. Using the NPC S18 Tet-Off cell line, only one-step transfection is required to produce a cell model in which the target genes can be specifically and tightly regulated. The characteristically low level of basal activity is beneficial for studies on novel genes that may disrupt the cell cycle, induce apoptosis or exhibit cytotoxicity, in the case that experiments depend on the absolute control of the background of the gene expression level.

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