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Inducible and reversible suppression of Npm1 gene expression using stably integrated small interfering RNA vector in mouse embryonic stem cells

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Abstract

The tetracycline (Tc)-inducible small interference RNA (siRNA) is a powerful tool for studying gene function in mammalian cells. However, the system is infrequently utilized in embryonic stem (ES) cells. Here, we present the first application of the Tc-inducible, stably integrated plasmid-based siRNA system in mouse ES cells to down-regulate expression of Npm1, an essential gene for embryonic development. The physiological role of Npm1 in ES cells has not been defined. Our data show that the knock-down of Npm1 expression by this siRNA system was not only highly efficient, but also Tc- dose- and induction time-dependent. Particularly, the down-regulation of Npm1 expression was reversible. Importantly, suppression of Npm1 expression in ES cells resulted in reduced cell proliferation. Taken together, this system allows for studying gene function in a highly controlled manner, otherwise difficult to achieve in ES cells. Moreover, our results demonstrate that Npm1 is essential for ES cell proliferation.

Keywords: RNA interference; Inducible; Embryonic stem cells; Nucleophosmin; Proliferation

Embryonic stem (ES) cells represent a population of undifferentiated pluripotent cells, capable of differentiating into multi-lineages and self-renewing in vitro. These features make them attractive for studying developmental processes, as well as making them a potentially unlimited source of material to develop treatments for human diseases. The molecular mechanism of regulating proliferation and differentiation in ES cells is complex, involving changes in gene expression and function. Thus, it is critical to suppress expression of genes for gaining insight into cellular proliferation and differentiation. RNA interference (RNAi), a sequence-specific, post-transcriptional gene-silencing mechanism triggered by double-stranded RNA (dsRNA), has been proven to be a powerful tool for suppressing gene expression, and is widely used in analysis of gene function in mammalian cells [1]. Initially, its application to mammalian cells was hindered as result of a presumable "interferon response" of nonspecific gene suppression when dsRNA >30 bp was introduced into cells [2], although undifferentiated ES cells were reported to lack the interferon response [3]. Further investigation shows that the nonspecific suppression in gene expression can be avoided by using synthetic small interfering RNA (siRNA) of <30 bp [4]. However, the high expense involved in synthesis along with the shortness of effect of synthesized siRNA has limited its broad utilization. Recently, RNA polymerase III has been used to transcribe the short hairpin siRNA (shRNA), single-stranded RNA molecule, which has an intramolecular dsRNA domain similar to

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siRNA, for specific suppression of gene expression in mammalian cells. The siRNA expression vectors can be stably integrated into the host genome and are relatively inexpensive to make. They have been successfully introduced into ES cells for evaluating the function of specific genes [5–7]. However, stably integrated siRNA vectors cannot be used if the target genes are essential for cell survival and proliferation. Very recently, specific suppression of gene expression by stably inducible and reversible siRNA or dsRNA vectors, using either the tetracycline (Tc) or ecdysone-responsive systems, has been developed and is in wide use in mammalian cells [8–11]. Although these systems have successfully allowed specific knock down of genes, including p53, MyoD, DNA methyltransferase 1 (DNMT1), and β-catenin/TCF4 in dose- and time-dependent manners, inducible siRNA systems are infrequently utilized in ES cells, since only a subset of polymerase II promoters function well in these cells and transfection efficiency is low. Therefore, it is very important to generate ES cell lines with a controllable siRNA system for the fine analysis of gene functions and cellular processes at the molecular level.

In this study, we modified and applied the Tc-induced H1 promoter of RNA polymerase III-driven shRNA expression vector, developed by van de Wetering et al. [8] in mouse ES cells. With this system, we specifically knocked down expression of nucleophosmin (Npm1) and studied its physiological roles in ES cells. Npm1 (also known as protein B23, numatrin, and protein NO 38) is one of the most abundant multi-functional nucleolar phosphoproteins. Its functions include ribosome biogenesis, cell proliferation, centrosome duplication, and transcription regulation [12–16]. Recent investigation shows that Npm1 is essential for embryonic development [17]. However, its physiological role in ES cells has not been defined to date. We chose Npm1 as a target, based on the facts that (1) it is more abundant in proliferating cells than in normal resting cells [12,13], and is highly expressed in undifferentiated ES cells; (2) it is haplo-insufficient in the control of centrosome duplication and cell proliferation [17]; its level of expression seems to be an important factor for its function; (3) although the physiological role of Npm1 in mammalian development has been investigated by gene knockout in mice, its function in ES cells has not yet been studied. Therefore, Npm1 is an ideal gene to study with an inducible siRNA expression vector in mouse ES cells.

Materials and methods

Cell culture and transfection. CGR8 mouse cells were cultured in Glasgow Minimum Essential Medium (GMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Hyclone), 2 mM L-glutamine (Invitrogen), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (Sigma), 1000 U/ml leukemia inhibitory factor (LIF, Chemicon), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 0.1 mM β-mercaptoethanol (Sigma). The medium was changed daily. CGR8 mouse cells were transiently transfected with LipofectAMINE^{TM} 2000 (Invitrogen) according to manufacturer's instructions. For stable cell lines, the cells were transfected with 40 μg of plasmids by electroporation, and selected by puromycin (Sigma) at 1.5 μg/ml or by zeocin (Invitrogen) at 30 μg/ml. 293 T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen), and transfected using standard calcium phosphate method.

Construction of plasmids. The pPyCAG/TetR vector was generated by polymerase chain reaction (PCR) amplification of the TetR gene coding sequence in pcDNA6/TR (Invitrogen), using primers 5′-CCC CGC TCG AGA TTG ATA ATG TCT AGA TTA G-3′ and 5′-TGC AAT GCG GCC GCT CTG CTT TAA TAA GAT CTG-3′, and insertion of the PCR fragment into the cloning sites of pPyCAGIP vector (a kind gift from Ian Chambers and Austin Smith). The pcDNA4/TO/EYFP plasmid was constructed by amplification of the EYFP gene in pEYFP plasmid (Clontech), using primers 5′-CCG CTC GAG ATG GTG AGC AAG GGC GAG GAG-3′ and 5′-CTA GTC TAG ACG TCA GTT ACT TGT ACA GCT CGT CCA TG-3′, and replacement of the luciferase gene sequence in pcDNA4/TO/Luc (a kind gift from Hans Clevers) with the PCR fragment. The correct sequence of all vectors was verified by DNA sequencing.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from the cells using TRIzol (Invitrogen) and transcribed into cDNA using oligo $(dT)_{15}$ and ReverTra Ace reverse transcriptase (Toyobo). The primers used for detection of Npm1 expression were 5′-CAT GTC TGG AAA GCGATC-3′ and 5′-CCT TTG ATC TCG GTG TTG-3′, and for actin were: 5′- TTC CTT CTT GGG TAT GGA AT-3′ and 5′-GAG CAA TGA TCT TGA TCT TC-3′. PCRs were carried out with 2 μl of cDNA template, 250 nM of each primer, 200 μM dNTP mix, and 1 U of *Taq* DNA polymerase (Hua Nuo, Shanghai, China) in a volume of 20 μl. Samples were amplified in a thermocycler under the following conditions for 30 cycles: first, the denaturing step at 95 °C for 30 s, then the annealing step at 55 °C for 30 s, and finally the amplification step at 72 °C for 45 s.

RNA interference. To make siRNA vectors for Npm1, two independent 19 bp sequences (5′-GTA ACA AGG TTC CAC AGA A-3′ and 5′-GGA AGA TGC AGA GTC TGA A-3′) within the coding region of murine *Npm1* gene were selected and cloned into the pTER^{+}vectors [8]. siRNA vector for EGFP was made by selecting 19 bp sequence in the coding region of *EGFP* gene (5′-GGC TAC GTC CAG GAG CGC A-3′). The CGR8-TetR host ES cells were transfected with the pTER-siRNAs-Npm1 or pTER-siRNA-EGFP plasmids by electroporation, and selected by zeocin at 30 μg/ml. Single clones were picked and amplified.

Immunofluorescence. The cells were seeded onto glass coverslips and treated with 0 or 300 ng/ml Tc for 96 h. Then the cells were stained as described [18] with antibody against mouse Npm1 (Sigma, 1:1000) followed by TRITC-conjugated anti-mouse immunoglobulin (Jackson Immuno Research). Nuclei were stained with 0.5 μg/ml DAPI for 5 min. Fluorescence signals were detected using a Leica TCS SP2 confocal microscope.

Western blot analysis. The cells were lysed in NP-40 buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 0.5% Nonidet P-40, 1 mMNaF, and 1 mM phenylmethylsulfonyl fluoride, PMSF). The protein concentration of samples was determined using the BCA protein assay kit (Pierce). Western blot analysis was done as described [18] with antibody against Npm1 (Sigma), or α-tubulin (Sigma), or Oct-4, or Nanog. All experiments were performed for at least three times.

Luciferase reporter assays. The 293T cells were plated into 12-well plates and transiently transfected with the indicated plasmids using the calcium phosphate method. Twenty-four hours after transfection, Tc was added to a final concentration of 0–1 μg/ml, and additional 24 h later, luciferase activities in the samples were measured with a dual luciferase reporter assay system (Promega). A pRL-TK (Promega) construct containing Renilla luciferase was included in all transfections as an internal control to normalize the transfection efficiency. The experiments were performed at least three times and the data were shown as means ± SD.

MTT assay. The cells were induced by 300 ng/ml Tc for 4 days and then seeded into 24-well plates at a density of 2×10^3 cells per well. At the indicated times, 50 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)2,5-diphe-

nyltetrazolium bromide (MTT) was added, and the cells were incubated for 4 h in a CO_2 incubator. After adding 500 μl of solution containing 10% SDS, 5% iso-butanol, and 0.01 M HCl, the cells were incubated in the CO_2 incubator for additional 12 h. Optical density (OD) was measured at the absorption wavelength of 570 nm and 630 nm was used as the reference wavelength.

Soft agar assay. 2×10^3 cells were suspended in 2 ml of 0.3% Noble agar (BD)-GMEM plus 15% FBS (top layer) and then poured on top of 2 ml of solidified 0.6% Noble agar-GMEM plus 15% FBS (bottom layer) in each well of 6-well plates. One day after plating, cells were fed with medium with or without Tc at 1 μg/ml, and the medium was replaced every other day. After 14 days, the foci were counted and photographs were taken with a Nikon Eclipse TE2000-U microscope. The experiments were performed at least three times and the data were shown as means ± SD.

Flow cytometric analysis. Cells were plated into 35-mm dishes at a density of 10^5 and grown in the medium containing Tc at concentration of 300 ng/ml. After incubation for 4 days, the cells were fixed with 70% ethanol for 24 h at 4 °C. The cells were then incubated with RNase (100 μg/ml) for 30 min and stained with propidium iodine (Sigma; 50 μg/ml). Cell cycle analysis was performed using a BD FACSCalibur flow cytometer.

Results and discussion

Generation of mouse ES cell line stably expressing tetracycline repressor

The inducible siRNA system described by Wetering et al. [8] includes two steps. The first step is the generation of host cells carrying the tetracycline repressor (TetR), by selection of the clones that had stably integrated the TetR expression vector into their genomes with blasticidin (http://www.invitrogen.com). The stable expression of TetR in the cells is under the control of the constitutively active human cytomegalovirus (hCMV) promoter (TetR/hCMV). The second step is the introduction of a Tc-inducible form of RNA polymerase III H1 promoter-driven siRNA expression vector ($pTER^+$) into the host cells generated in the first step. The cells carrying both stably integrated TetR and siRNA expression vectors were selected in the presence of both blasticidin and zeocin. The Tc operator (TO) sequence is placed between the H1 promoter and the siRNA sequence of the target gene in the $pTER^+$ vector. Therefore, the transcription of siRNA from the H1 promoter was blocked by the binding of the TetR to the TO sequence. Thus, the addition of Tc to the culture medium inhibits binding of the TetR to the TO, leading to the transcription of siRNA and reduction in the expression of the cognate gene. It is a Tet-On-inducible siRNA expression system. This system has been widely used in the study of gene functions [8,19,20]; however, the CMV promoter used in the first step for expressing TetR in the system does not function efficiently in ES cells.

In an effort to generate a mouse ES cell line that stably expresses TetR, we constructed a TetR expression vector, pPyCAG/TetR, by subcloning the coding sequence of TetR into a pPyCAGIP expression vector, as described by Niwa and Chambers [21,22], in which TetR is transcribed under the control of a CMV/chicken β-actin (CAG) promoter, within a transcription unit linked to the puromycin resistance gene (*pac*) through an internal ribosomal entry site (IRES) (Fig. 1A). The CAG promoter has been demonstrated to be a highly efficient promoter in ES cells. We first tested the effectiveness of TetR expression by this new construct in 293T cells, as they are much more easily genetically manipulated than ES cells. We transiently transfected 293T cells with the pcDNA4/TO/Luc expression vector, which carries the luciferase reporter gene transcribed by the CMV promoter, followed by 2×TO sequence. When TetR was not present to bind the TO sequence located between the CMV promoter and luciferase coding sequence, the luciferase gene was expressed and its activity was detectable in the cell lysates (Fig. 1B, column 1). However, co-transfection of 293T cells with pcDNA6/TR (TetR expression vector with CMV promoter) significantly reduced reporter activity (Fig. 1B, column 2), as expected. Significantly, co-transfection of the cells with pPyCAG/TetR resulted in a substantial reduction in luciferase activity (Fig. 1B, column 3), indicating that TetR was efficiently transcribed by the CAG promoter, and had bound to the TO sequence in the reporter gene to block transcription of the luciferase gene. Next, we examined whether the binding of TetR, expressed from the pPyCAG/TetR expression vector, to the TO sequence in the reporter gene was responsive to Tc in the culture medium. The 293T cells were co-transfected with pPyCAG/TetR and pcDNA4/TO/Luc expression vectors, and cultured in a medium containing Tc at 0–1000 ng/ml. As shown in Fig. 1C, luciferase activity was increased in a Tc-concentration-dependent manner, suggesting that Tc in the medium inhibited binding of TetR to the TO sequence in a dose-dependent manner.

After obtaining the evidence that the pPyCAG/TetR vector functions well in 293T cells, we generated CGR8 mouse ES cell lines, stably transfected with pPyCAG/TetR through selection with puromycin. To determine expression and function of TetR in CGR8 ES cells, we constructed an EYFP reporter gene expression vector, pcDNA4/TO/EYFP, (Fig. 1D) by replacing the luciferase gene in the pcDNA4/TO/Luc reporter vector with the coding sequence of EYFP. The signal of EYFP is visible and more easily detected than that of luciferase, although both genes are driven by the CMV promoter. Afterwards, we transiently transfected the CGR8 cells that stably expressed TetR (CGR8-TetR) with the pcDNA4/TO/EYFP vector, and examined the expression of EYFP in the absence or presence of Tc, respectively. As shown in Fig. 1E, EYFP expression was not observed in CGR8-TetR cells in the absence of Tc, although expression of EYFP could be found in parental CGR8 cells (data not shown), indicating that TetR bound to TO in the pcDNA/TO/EYFP completely blocked transcription of the *EYFP* gene. However, EYFP expression was found in CGR8-TetR cells when Tc was added to the medium (Fig. 1E), although the expression of EYFP was only observed in a small percentage of the cells (about 3%). The result clearly demonstrates that

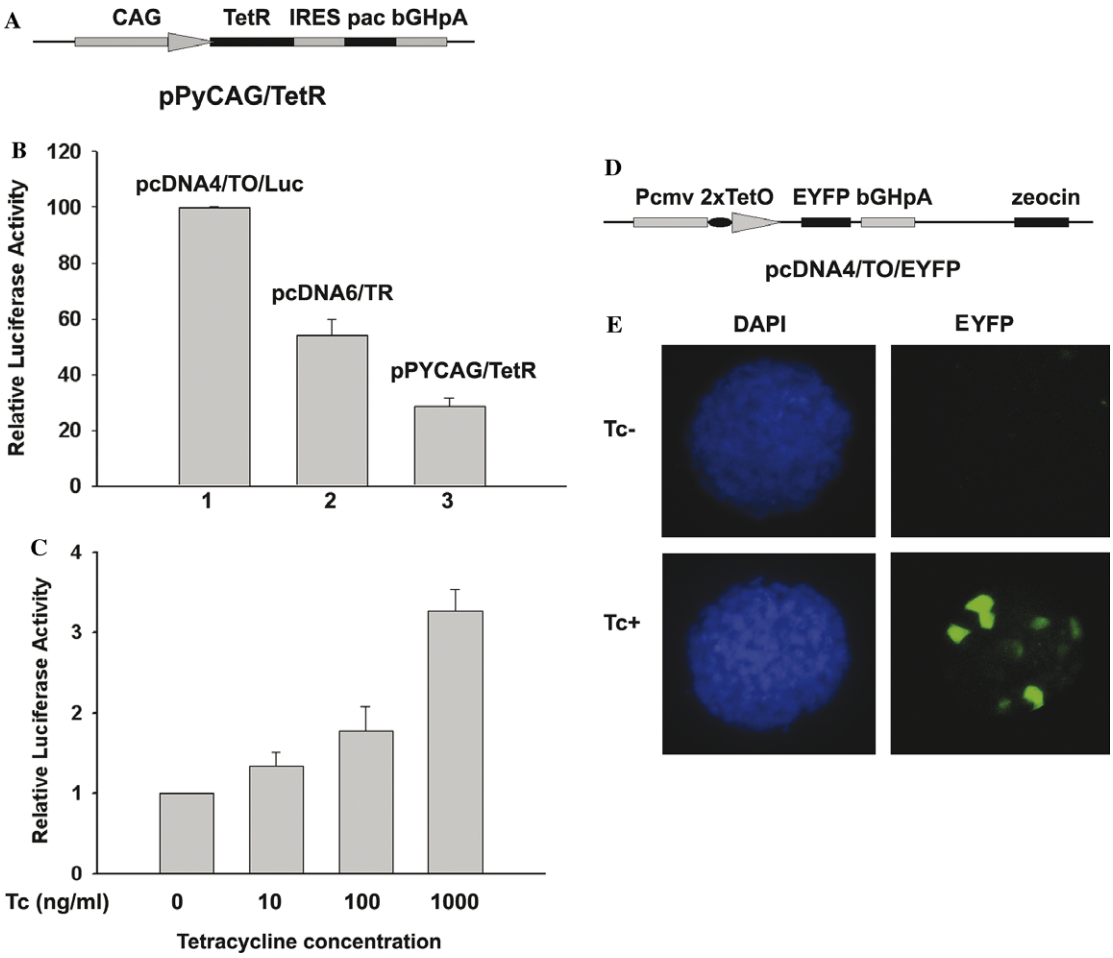

Fig. 1. Establishment of the ES cell line stably expressing TetR. (A) The schematic illustration of the pPyCAG/TetR construct. CAG, cytomegalovirus/chicken β-actin globulin promoter; TetR, tetracycline repressor; pac, puromycin-resistant gene; bGHpA, polyadenylation addition sequence from bovine growth hormone gene. (B) TetR expression and binding to the TO sequence in 293T cells. The 293T cells were transiently transfected with the luciferase reporter construct, pcDNA4/TO/Luc, alone (column 1) or in combination with the TetR expression vector, pcDNA6/TR (column 2), or pPyCAG/TetR (column 3). After 48 h of transfection, luciferase activity in the cell lysate was measured. TO, tetracycline operator. (C) Binding of the TetR to the TO is responsive to Tc added to the medium. Twenty-four hours after the co-transfection of pcDNA/TO/Luc and pPyCAG/TetR, the 293T cells were cultured in a medium containing different concentrations of Tc. After treatment with Tc for 24 h, luciferase activity was measured as in (B). Tc, tetracycline. (D) The schematic illustration of the pcDNA /TO/EYFP construct. (E) Generation of the CGR8 ES cells stably expressing TetR (CGR8-TetR). To generate the CGR8 ES cells stably expressing TetR, CGR8 cells were transfected with the pPyCAG/TetR construct and selected with puromycin at a final concentration of 1.5 μg/ml. Afterwards, the cDNA4/TO/EYFP construct was transiently introduced into the CGR8-TetR ES cells and the expression of EYFP was determined 48 h later, in the presence or absence of Tc in the medium. DAPI was used to highlight nuclei.

TetR was effectively expressed after the pPyCAG/TetR construct was stably integrated into CGR8 genome, and was bound to the TO sequence in the EYFP expression vector to prevent EYFP from being expressed in these ES cells. Furthermore, Tc could significantly induce expression of the reporter gene. Furthermore, the expression of TetR was stably maintained for at least 3 months in the presence of puromycin (data not shown). Thus, a stably integrated Tc-inducible system was successfully generated in mouse ES cells.

The stable and inducible siRNA-mediated suppression of Npm1 expression

We next tried to (i) develop a controllable siRNA expression system using established CGR8-TetR cells, and (ii) evaluate the usefulness of the Tc-inducible siRNA system in the suppression of *Npm1* gene expression. For the specificity of down-regulation of Npm1 expression by siRNA in the ES cells, we selected two distinct sequences from the coding region of *Npm1* gene to make Npm1 siRNA constructs. They were named as pTER-siRNA-Npm1 (1) and pTER-siRNA-Npm1 (2), respectively. As shown in Fig. 2A, the siRNA expression constructs, pTER-siRNA-Npm1(1) and (2), were generated by insertion of the double-stranded oligonucleotides that contained a mouse *Npm1*-derived sequence of 19 bp for sense and antisense strands, respectively, into pTER^{+} vector, as described by Wetering et al. [8]. In addition, a pTER-siRNA-EGFP construct was made to serve as a negative control. The CGR8-TetR ES cells were transfected with the pTER-siRNA-Npm1 or pTER-siRNA-EGFP plasmids, and

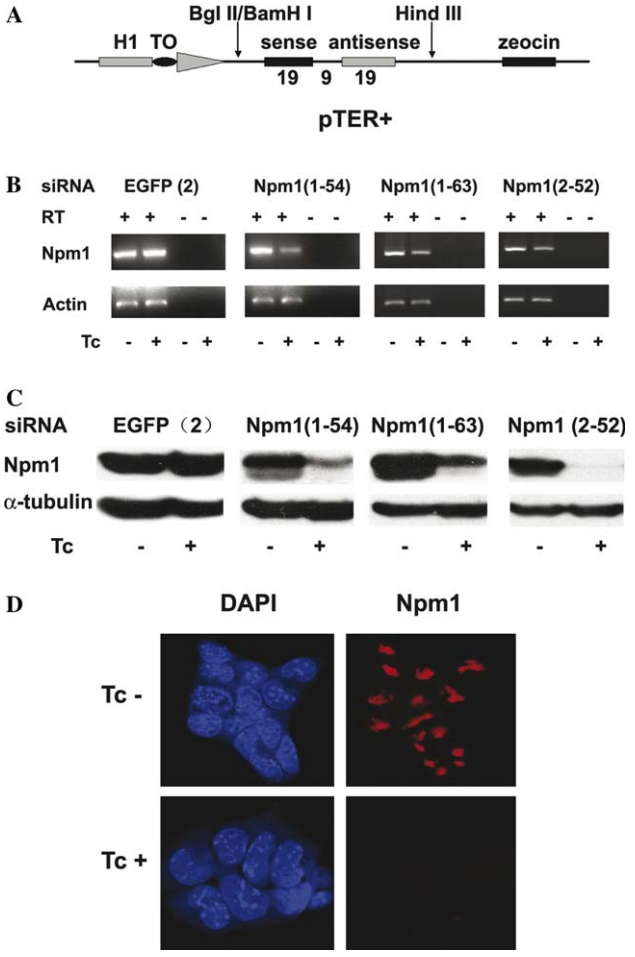

Fig. 2. Stably integrated and inducible siRNA mediated the suppression of Npm1 expression in ES cells. (A) The schematic illustration of the pTER^{+} siRNA expression construct. H1, promoter of polymerase III H1. (B) Npm1 siRNA mediated the suppression of Npm1 expression. The CGR8-TetR/siRNA-Npm1 ES cells (clones 1–54, 1–63, and 2–52) and CGR8-TetR/siRNA-EGFP ES cells (clone 2) were cultured in the absence or presence of 300 ng/ml of Tc for 96 h, and then the total RNA was extracted from the cells. RT-PCR assays were performed using the gene-specific primers. (C) Western blot analysis was performed with antibodies against Npm1 and α-tubulin, using the cell lysate from the same cells as in (B). (D) Immunofluorescence staining of Npm1 in the CGR8-TetR/siRNA-Npm1 cells from clone 1–54 before and after addition of Tc. Blue signal represents DAPI staining and red signal represents Npm1 protein. All experiments were repeated at least three times and the representative data are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

selected with zeocin. Single clones were selected and examined for their Npm1 expression before and after Tc induction. The clones 54 and 63 derived from pTER-siRNA-Npm1 (1), clone 52 from pTER-siRNA-Npm1 (2), and clone 2 from pTER-siRNA-EGFP-transfected cells were used in the following experiments. As demonstrated in Fig. 2B, the mRNA level of *Npm1* was significantly reduced in siRNA-Npm1 clones, including siRNA-Npm1 (1–54, 1–63, and 2–52), after Tc induction for 96 h. However, there was no detectable change in *Npm1* mRNA levels for siRNA-EGFP clone 2. Furthermore, the expression of siRNA for *Npm1* did not affect the expression of actin. The similar results were obtained when Npm1 protein levels were determined by Western blotting (Fig. 2C). This observation confirms the efficiency and specificity of the siRNAs that were used. To further evaluate the effect of siRNA-Npm1 at a single-cell level, we examined Npm1 expression by immunofluorescent staining. As expected, strong staining of Npm1 was found in the nucleoli of the siRNA-Npm1 ES cells (clone 1–54) before addition of Tc. However, induction with Tc resulted in a dramatic reduction in Npm1 expression (Fig. 2D). Thus, the stable and inducible siRNA expression cell lines specific for *Npm1* were established.

The reversible, Tc dose- and induction time-dependent down-regulation of Npm1 expression

The uniqueness of this inducible system lies in its power for suppression of gene expression in the dose- and time-dependent manners. To show the time-dependence of inhibition of Npm1 expression, Npm1 protein levels were measured after addition of Tc for the different periods. As shown in Fig. 3A, protein level of Npm1 was gradually decreased with the increase in induction time. The maximum inhibition was reached after addition of Tc for 96 h. The phenomenon was observed in all clones tested, including siRNA-Npm1 (1–54), (1–63), and (2–52). In addition, the suppression of Npm1 expression by siRNA-Npm1 was also Tc dose-dependent. The higher the Tc-concentration in the culture medium, the more reduction in the endogenous Npm1 protein level in ES cells (Fig. 3B). Our results show that the inhibition was maximal at a final Tc-concentration of 300 ng/ml, with an induction time of 96 h. Based on the observation, Tc at 300 ng/ml and induction for 96 h were used as the standard induction condition unless otherwise indicated. We found that Tc at 1000 ng/ml could induce further reduction in the Npm1 expression, but affected the cell growth (data not shown). On the other hand, one potential advantage of the inducible siRNA expression system is its capability to reversibly suppress gene expression, thus allowing for detailed investigation of gene functions. To explore this, we induced siRNA-Npm1 expression by addition of Tc at 300 ng/ml for 96 h to the cells of siRNA-Npm1 (1–54) clone, followed by withdrawal of Tc and continued culture of the cells for 144 h. As expected, exposure of cells to Tc for 96 h produced a massive reduction in Npm1 expression. Intriguingly, Npm1 protein levels were continuously reduced after removal of Tc, implying that transcribed *Npm1* siRNA might be still present in the cell even though transcription had been blocked by TetR. Alternatively, the continuous reduction in Npm1 levels could be due to the slow synthesis of new Npm1 protein. Nevertheless, the recovery of Npm1 protein levels was observed after withdrawal of Tc for 96–144 h (Fig. 3C). Taken together, our data demonstrate that *Npm1* siRNA transcribed by a Tc-sensitive H1 promoter is highly regulated by both the inducer concentration and induction duration, and that suppression is reversible. This

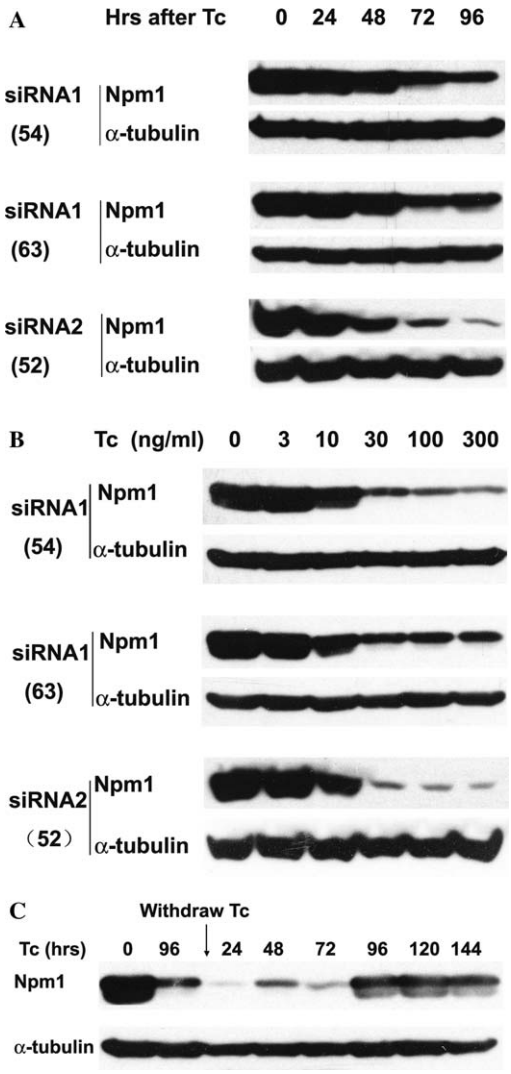

Fig. 3. Suppression of Npm1 expression by siRNA is induction time- and Tc dose-dependent and is reversible. (A) The kinetics of Npm1 protein levels revealed by Western blot analysis after addition of 300 ng/ml of Tc for different time periods in CGR8-TetR/siRNA-Npm1 cells from clones (1–54, 1–63, and 2–52). (B) Tc-concentration dependence of Npm1 protein levels in Tc-treated CGR8-TetR/siRNA-Npm1 cells, as revealed by Western blot analysis. (C) Reversibility of the suppression of Npm1 expression mediated by Npm1 siRNA. After treatment of siRNA-Npm1 ES cells from clone 1–54 with Tc at 300 ng/ml for 96 h, the cells were washed with PBS twice and continuously cultured in the medium without Tc. The cells were collected and lysed at indicated times for Western blot analysis.

system provides an ideal tool for investigating genes whose functions are dependent on their intracellular expression levels in mouse ES cells.

The reduction in cell proliferation mediated by the expression of siRNA-Npm1

After having established the specific down-regulation of Npm1 expression by the inducible siRNA, we next wanted to know whether Npm1 suppression had a functional consequence in ES cells. Therefore, we investigated the effect of Npm1 siRNA on mouse ES cell proliferation, an effect that has not been documented to date. The ES cells that expressed the inducible siRNA-Npm1, including clones (1–54) and (2–52), were cultured without or with Tc for 4 days, and then cell proliferation was determined for additional 7 days by the MTT assay, in which the functional mitochondrial succinate dehydrogenases in cells can convert 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) to formazan that generates a blue color. Because the extent of this conversion is proportional to the number of survival cells, MTT assay is widely used to quantify viable cells [23]. As shown in Fig. 4A, the cell proliferation rate was significantly lower in the presence of Tc than in its absence for both siRNA-Npm1 (1–54) and (2–52) clones. To confirm that the reduced cell proliferation was resulted from the down-regulation of the endogenous Npm1 expression, but not from the addition of Tc to the medium, the same experiment was performed with cells expressing siRNA-EGFP, clone 2. Results indicate that Tc at 300 ng/ml in the culture media for up to 11 days did not affect cell proliferation significantly (Fig. 4B). This observation is consistent with that of Grisendi et al. [17], who found that the proliferation rates of $Npm1^{+/-}$ mouse embryonic fibroblasts (MEFs) were lower than those of wild-type cells at early passages. Our results provide new evidence that Npm1 is essential for the normal proliferation of ES cells in vitro.

Experimental evidence demonstrates that ES cells share some similarities with cancer cells, such as anchorage-independent growth, loss of contact inhibition, and tumor formation [24]. To further characterize the effects of Npm1 knock-down on ES cell proliferation and anchorage-independent growth, the soft agar assays were performed with the ES cells of siRNA-Npm1 clone (1–54). Induction of expression of siRNA-Npm1 by addition of Tc to the medium profoundly decreased the number of foci by ~60%, although the same treatment did not reduce the number of foci in the ES cells expressing siRNA-EGFP, clone 2 (Fig. 4C). As shown in Fig. 4D, not only was the number of foci decreased, but also the size of each focus was significantly reduced in the cells expressing siRNA-Npm1 after addition of Tc. In contrast, addition of Tc affects neither number nor size of foci of the cells expressing siRNA-EGFP. This result further confirms the critical role of Npm1 in ES cell proliferation.

It has been reported that suppression of endogenous Npm1 through a short interfering RNA approach impaired Gadd45a-induced cell cycle G2/M arrest in human HCT116 colon carcinoma cells [25]. However, whether Npm1 plays a role in the control of cell cycle progression in ES cells remains unknown. Therefore, cell cycle analysis was performed with the siRNA-Npm1 ES cells (clone 1–54). The siRNA-EGFP ES cells (clone 2) were used as a control. Following addition of Tc to the culture medium for 96 h, the ES cells were collected and subjected to flow cytometric analysis. As shown in Fig. S1(supplementary data), there was no significant difference in the cell cycle profile between the ES cells from siRNA-Npm1 (1–54) and siRNA-EGFP

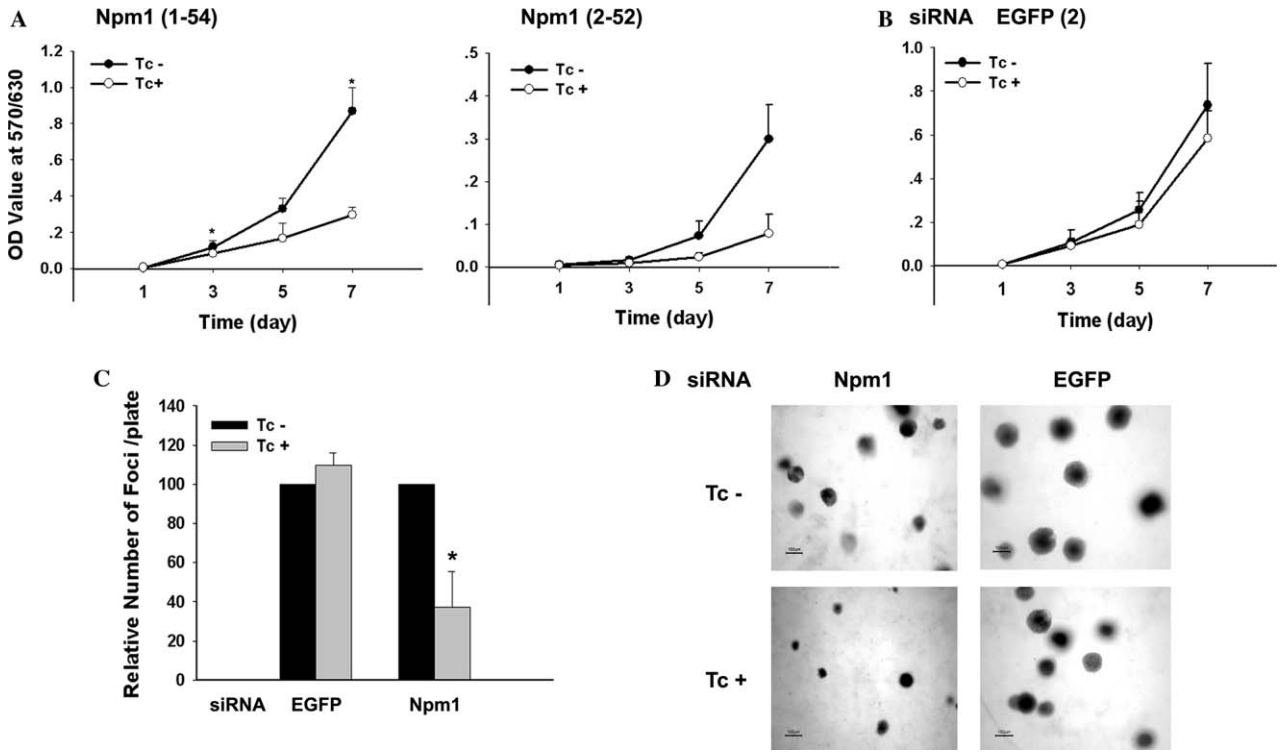

Fig. 4. Npm1 siRNA decreases ES cell proliferation rate without affecting their cell cycle profile. (A) Growth curves of siRNA-Npm1 ES cells (clones 1–54 and 2–52) in the absence and presence of Tc. MTT assay for monitoring cell growth was performed for three times, and a paired *t*-test was used for statistical analysis. $*p < 0.05$. (B) Growth curves of siRNA-EGFP ES cells (clone 2) in the presence and absence of Tc. MTT assay was performed and analyzed as in (A). (C) The comparison of foci formation before and after addition of Tc in the soft agar assay for the siRNA-Npm1 ES cells (clone 54) and siRNA-EGFP ES cells (clone 2). A paired *t*-test was used for statistical analysis. $*p < 0.05$. (D) Representative view of foci in the soft agar assay.

(2) clones, although Npm1 protein level was significantly down-regulated by Tc induction in the cells from clone (1–54) (data not shown). The cells from both groups displayed a typically normal cell cycle profile of mouse ES cells. Recently, Grisendi et al. reported that *Npm1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) underwent premature senescence and growth arrest, with accumulation of cells having a 4*n* DNA content and polyploidy. In addition, *Npm1*$^{-/-}$ cells had high rates of aberrant mitotic figures with multiple centrosomes and multi-polar spindles [17]. We did not detect the accumulation of polyploidy cells after the down-regulation of Npm1 expression in ES cells. This could be attributed to the relatively short period, during which the Npm1 expression was suppressed, in the current study, or different cell types used in the experiments. The reduced proliferation with normal cell cycle profile observed in the siRNA-Npm1 ES cells implies that the suppression of endogenous Npm1 expression led to prolonged cell doubling time, without affecting the length of individual cell phase. The siRNA system established in the study will facilitate elucidation the molecular mechanisms for Npm1 to regulate proliferation in ES cells.

Transcription factors, Oct-4 and Nanog, have been shown to be important regulators of proliferation and self-renewal in ES cells. We tested whether induction of siRNA-Npm1 expression had any effect on intracellular protein levels of these two factors. As shown in Fig. S2 (supplementary data), treatment of CGR8-TetR/siRNA-Npm1 cells from clone 1–54 with Tc at 300 ng/ml for 96 h did not alter protein levels of either Oct-4 or Nanog, although the treatment substantially reduced Npm1 protein levels. These results indicate that inhibition of cell proliferation in ES cells by siRNA-Npm1 could not be attributed to its effect on expression of Oct-4 or Nanog. On the other hand, these findings suggest that normal levels of Oct-4 and Nanog are not sufficient to maintain proliferation of ES cells. Meanwhile, normal Oct-4 and Nanog protein levels in the presence of Tc, compared to substantial decrease in Npm1 protein levels, further prove the specificity of siRNA-Npm1 in our study. Notably, we did not observe any increase in cell death or differentiated morphological changes in the cells expressing siRNA-Npm1 (data not shown), compared with siRNA-EGFP-expressing cells, although the proliferation rates of the former were much slower. This observation suggests that Npm1 might not be essential for ES cell survival, at least for the short period. This notion is supported by experimental evidence that *Npm1* knock-out results in embryonic lethality between E11.5 and E12.5, and that heterozygous mice are viable [17]; instead, Npm1 is required for cell proliferation, a role perhaps related to its function in ribosome biogenesis.

ES cells are attractive for both basic research and regenerative medicine studies as the result of their developmental pluripotency and self-renewal capabilities. However, it is

difficult to manipulate ES cells genetically. Coumoul et al. recently reported inducible suppression of Fgfr2 and survive in mouse ES cells using a combination of the RNAi and Cre-LoxP systems, which allows inducible expression of RNAi by treatment of the ES cells with 4HT [26]. Although induction is time-dependent, it is not concentration-dependent for the inducer and is not reversible. While preparing this manuscript, Szulc et al. [27] reported lentiviral vector-based polymerase III promoter-controlled sequences encoding small inhibitory hRNAs in embryonic or hematopoietic stem cells. However, the involvement of a lentivirus would limit its application in certain situations. In contrast, our plasmid-based siRNA system is safer and simpler to use.

In conclusion, we report the establishment of a stably integrated, plasmid-based, Tc-inducible, and reversible siRNA expression system in mouse ES cells. Using Npm1 as an example, we demonstrated the feasibility and high efficiency of the system. Induction is not only time- and inducer concentration-controllable, but also reversible. We have used this system to knock-down expression of genes other than Npm1 in ES cells (data not shown). Development of a similar siRNA expression system in human ES cells is in the process in our laboratory. This system adds additional option for investigating gene functions in a more defined way, and will greatly accelerate identification of important factors in ES cell proliferation and differentiation. In addition, our study indicates that Npm1 is an essential gene for ES cell proliferation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.07.020.

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