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Original article

The reverse tetracycline-controlled transactivator rtTA2^{S}-S2 is toxic in mouse embryonic stem cells

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Abstract — The efficient and reversible control of transgene expression is a powerful tool for the correct manipulation of embryonic stem cells in both cell therapy and transgenesis. The aim of this work was to investigate the possibilities of recently developed reverse tetracycline-controlled transactivator rtTA2^{S}-S2. We show that the rtTA2^{S}-S2 is useful for transient inducible expression of genes in embryonic stem cells. However, we found that it was not possible to establish mouse embryonic stem cell lines stably expressing this transactivator. Using the viral IRES sequence which couples the expression of rtTA2^{S}-S2 and neomycin phosphotransferase, we found that embryonic stem cells expressing rtTA2^{S}-S2 are not capable of growing in the presence of G418. Our results indicate that this transactivator is toxic to ES cells and raise the need for the development of other strategies for stable and inducible expression of genes in ES cells.

TetOn system / reverse tetracycline-controlled transactivator / embryonic stem cell / toxicity

1. INTRODUCTION

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the preimplantation blastocyst. The most intensively studied feature of ES cells is their ability to differentiate into a broad range of cell types (reviewed in [1]). In vitro differentiated and expanded human ES cell derivatives represent a potent source for cell therapies. However, the genetic modification of ES cells is in some cases required for the successful differentiation of specialized cell types in vitro.

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One of the most powerful strategies for the inducible expression of genes in mammalian cells involves a system based on the tetracycline (Tc)-regulatable repressor (TetR), which when fused to viral transactivation domains (e.g. an activation domain of the VP16 protein), forms a Tc controlled transactivator (tTA) [2]. Two basic forms of the Tet system are widely used: in TetOff systems, removal of Tc allows the interaction of tTA with a Tc-responsive operator (tetO) and the activation of P_{tet}, a minimal promoter fused downstream of an array of tetO sequences. In TetOn systems, a mutated form of tTA with reverse binding properties (rtTA) interacts with tetO, only in the presence of Tc [3]. In TetOn systems, the Tc analog doxycycline (Dox) is used for the induction of expression of P_{tet} driven genes because of its better activation properties.

A TetOn system would be more suitable for use in ES cells and their transplanted progeny because it does not require the presence of Dox during periods when P_{tet} is inactive. The continuous presence of Dox can affect cellular homeostasis and can be embryotoxic [4]. These obstacles can be avoided when the TetOn system is used. However, according to our knowledge, a TetOn system has not yet been succesfully applied in ES cells. Possible reasons for this may lie in the limitations of the TetOn system which seem to have been overcome by new forms of rtTA. As described by Urlinger and colleagues [5], the possibilities of a TetOn system were greatly enhanced after the discovery of new forms of rtTA, such as rtTA2^S-S2 and rtTA2^S-M2 which overcome many of the limitations of rtTA used up to now, including a high background activity of P_{tet}, a rather high instability in vivo preventing the establishment of the rtTA based system in some cell lines and finally a low Dox sensitivity which can hamper the regulation in organs with a limited accessibility of an inducer, e.g. brain.

Systems based on these novel Tc controlled transactivators should be very useful both in ES differentiation protocols which employ ectopic expression of transcription factors to drive the differentiation of ES cells and subsequently allow for the control of cell fate following transplantation. In the present study we examined the possibility of establishing ES cell lines stably expressing rtTA2^S-S2 and we partially elucidated the properties of rtTA2^S-S2 in ES cells.

2. MATERIALS AND METHODS

2.1. Plasmid construction

The coding sequence of rtTA2^S-S2 [5] was cloned into the EcoRI/BamHI site of pNeoI or pIRESNeo2 (Clontech) resulting in pNeoS2 and pTetS2-IRESNeo2, respectively. pNeoI was obtained from pEGFP-C1 (Clontech) by excision of the EGFP coding sequence by NheI and XhoI, end-filling using the Klenow enzyme and subsequent religation.

2.2. Cell culture and transfections

Quail QT6 fibroblasts were cultured and transfected as described previously [6]. ES-D3 cells [7] were maintained on gelatinized tissue culture plastic in Dulbecco's modified Eagle medium supplemented with 20% fetal calf serum, 100 mM nucleosides, 0.05 mM β-mercaptoethanol, 100 i.u·mL^{-1} penicillin, 0.1 mg·mL^{-1} streptomycin and 1000 U·mL^{-1} leukemia inhibitory factor. ES-D3 cells were cultured on mitomycin C-treated STO fibroblasts.

2.2.1. Transient transfections

Confluent ES-D3 cells on 10 cm-diameter plates were trypsinized into single cells, centrifuged and resuspended in 400 μL of complete culture medium with 10 μg of each plasmid. The mixture was incubated for 5 min, transferred into an electroporation cuvette, electroporated at 230 V and

1000 μF (ECM 600, BTX) and seeded onto culture dishes. The next day, the medium was replaced and when required Dox (1 μg·mL^{-1}) was added. The cells were harvested 48 h post-transfection for luciferase and β-galactosidase assays.

2.2.2. Stable transfection and cloning

The ES cells were electroporated as described above and then selected in the presence of G418 antibiotic (400 μg·mL^{-1}) for 10 days. Drug-resistant colonies were transferred individually onto a feeder layer in 24-well plates, expanded and analyzed for transgene expression or function.

2.3. Luciferase and β-galactosidase assays

The cells were washed twice in PBS and then lysed in a Luciferase Assay System lysis buffer (Promega). Luminiscence was detected by microplate luminometer LM-01T (Immunotech) according to the manufacturer's protocol. Fifty microliters of cell lysate were mixed with 50 μL of Luciferase Assay reagent (Promega). The light production was read immediately for a period of 180 s. Relative light units per 180 s were recorded by a Kilia software system (Immunotech) and further evaluated. The activity of β-galactosidase in lysate was determined as described previously [8].

2.4. Amidoblack assay

The cells grown on 5 cm dishes were washed twice in PBS, stained with 1 mL of amidoblack solution (0.1% amidoblack in a mixture of H_2O:acetic acid:methanol - 1:3:6) for 15 min in room temperature and destained in 2 mL of the same solution lacking amidoblack (5 min). After washing with 2 mL H_2O (5 min), the cells were lysed with 1 mL 50 mM NaOH. The absorbance measured at 620 and 405 nm corresponds to the protein content. As the blank, we used 5 cm gelatinized dishes overlayed with medium for 1 h and processed in a similar manner.

2.5. Isolation of genomic DNA and PCR

The ES cells were cultured in feeder-free culture to confluency, washed in PBS, scraped and lysed in 200 μL of PBND buffer (50 mM KCl, 10 mM Tris-Cl pH 8.3, 2.5 mM $MgCl_2$, 0.1 mg·mL^{-1}gelatine, 0.45% vol/vol NP-40, 0.45% vol/vol Tween 20) with 1 μL proteinase K (10 mg·mL^{-1}). The samples were incubated for 3 h at 55 ºC. Proteinase K was subsequently inactivated by heating to 95 ºC for 10 min. The samples were then spun down and 2 μL of the supernatant was added into 20 μL PCR reaction. PCR amplification of a 738 bp fragment corresponding to region 4–741 of the rtTA2^S-S2 coding sequence was performed using primers 5'-TCT AGA CTG GAC AAG AGC-3' and 5'-GGG GAG CAT GTC AAG GTC-3'. PCR of a p27 genomic sequence fragment was performed as previously described [9].

3. RESULTS

3.1. Construction and function of pNeoS2

The tetracycline-regulatable transcriptional activator rtTA2^S-S2 was described for the first time by Urlinger and coworkers [5] and expressed from the CMV-promoter of the pUHrT61-1 plasmid. To minimize difficulties in further work aimed at the production of stable cell lines expressing rtTA2^S-S2, we first cloned the rtTA2^S-S2 coding sequence into a pNeoI expression/selection vector (see Fig. 1). The resulting pNeoS2 is analogous to pEGFP-C1 produced by Clontech but carries rtTA2^S-S2 instead of EGFP. Subsequently, we confirmed that rtTA2^S-S2 is fully functional by transient

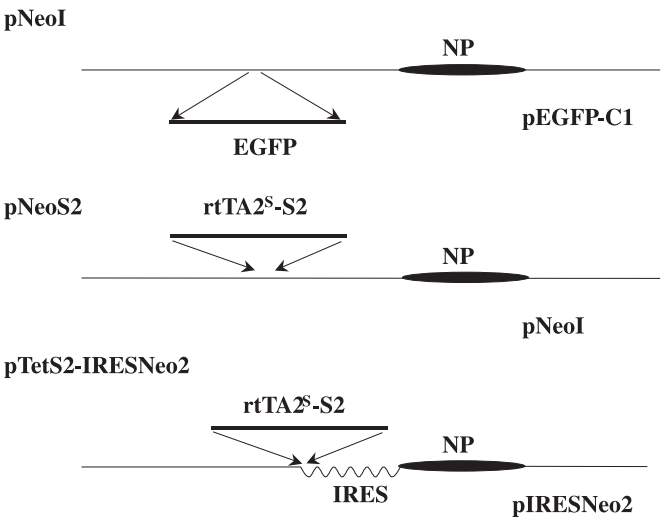

Figure 1. Structure of the vectors used in the present study. The neomycin phosphotransferase domain is indicated (NP). Transcriptional coupling of rtTA2^S-S2 with NP in the pTetS2-IRESNeo2 is indicated by IRES.

transfections in high efficiency-transfectable QT6 fibroblasts. Transcriptional activity of rtTA was monitored by the pUHC13-3 reporter plasmid carrying the luciferase coding sequence downstream of P_{tet}, the pCMVβ-Gal plasmid expressing β-galactosidase was added as an internal control. Using this system, we show that pNeoS2 can produce functional rtTA, which is tightly regulated by Dox (Fig. 2A).

In a parallel set of experiments, we transiently transfected ES-D3 cells with the same combinations of plasmids (Fig. 2B) and compared the inducibility of rtTA2^S-S2 in both cell lines. Although the intensity of luciferase activity after the addition of Dox was increased in both cell lines, the effect was more pronounced in QT6 cells in comparison to ES cells (80-fold versus 40-fold). However, in contrast to QT6 fibroblasts, the ES cells did not show any residual activity of P_{tet} in the presence of rtTA2^S-S2 and the absence of Dox (Fig. 2B).

3.2. ES cells stably transfected with pNeoS2 do not produce active rtTA2^S-S2

With a view to evaluating if transfection with vectors based on pEGFP-C1 (such as pNeoS2) can lead to the production of ES cells stably expressing the transgene, we electroporated ES-D3 cells with pEGFP-C1 and selected 13 independent clones from a G418-resistant pool. The resulting ES-EGFP clones were screened for the production of the EGFP protein and 8 of 13 (61%) clones were found to robustly express EGFP. Subsequently we transfected ES-D3 cells with pNeoS2. Thirteen independent clones of G418-resistant cells (ES-TetS2 cells) were transiently transfected with the pUHC13-3 reporter to verify the presence of functional rtTA2^S-S2. Unexpectedly, no clones showed Dox-inducible luciferase activity despite the fact that differences between individual clones varied in range by an order of magnitude (Fig. 3).

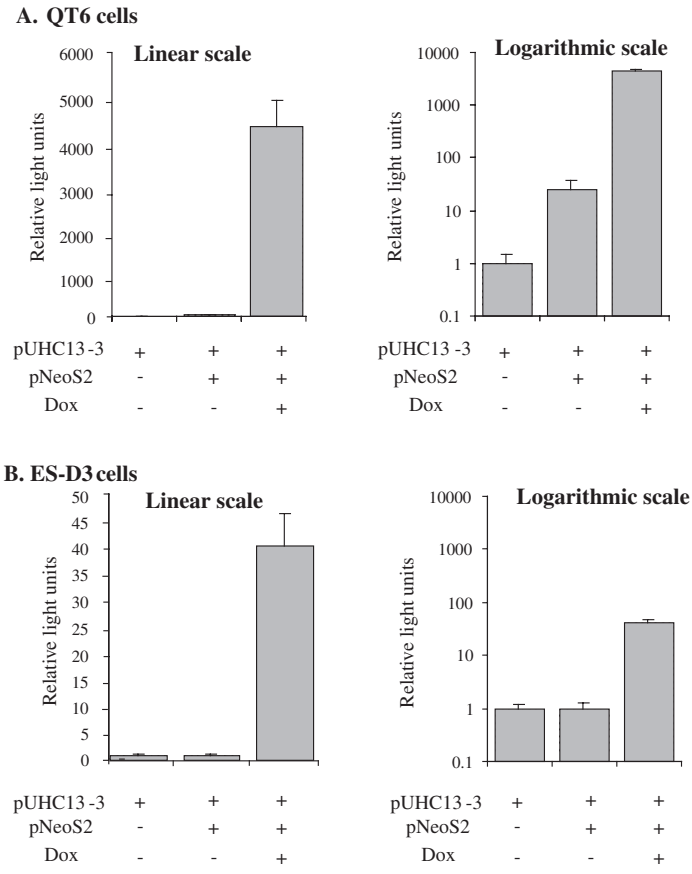

Figure 2. Characterization of rtTA2^S-S2 in QT6 fibroblasts (**A**) and ES cells (**B**) which were transiently transfected with the indicated combinations of plasmids and pCMVβ-Gal. Luciferase activity, measured 48 h post-transfection, was normalized to β-galactosidase activity and the values obtained from lysates transfected only by the reporter were assigned a value of 1. Data represent the average ± standard deviation of three independent experiments. The results are shown in both linear and logarithmic scales.

3.3. Production of ES-TetS2 cells is hampered due to the absence of integration of rtTA2^S-S2 into the genome of ES cells

In the pNeoS2 plasmid, different promoters drive genes encoding rtTA2^S-S2 and neomycin phosphotransferase. This raises the possibility that ES-TetS2 cells that are G418-resistant may have integrated the neomycin phosphotransferase fragment of the pNeoS2 plasmid, but not the full rtTA2^S-S2 fragment. This preferential disruption of the rtTA2^S-S2 coding sequence may underlie its functional defects. Thus, we tested 6 randomly selected ES-TetS2

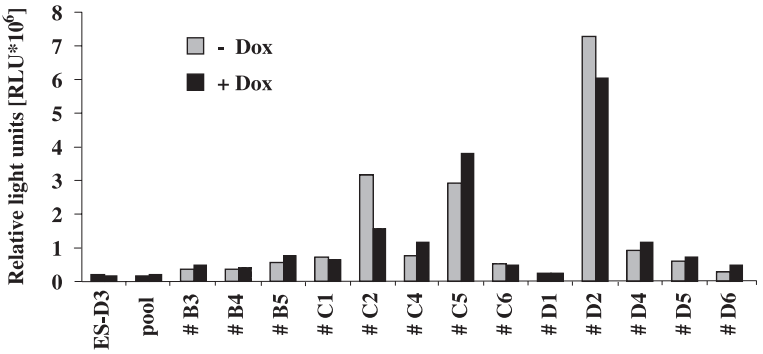

Figure 3. Characterization of ES-TetS2 clones. ES-TetS2 cells were screened by transient transfection with pCMVβ-Gal and pUHC13-3 reporters. Luciferase activity was normalized to β-galactosidase activity. ES-D3 cells were used as a negative control.

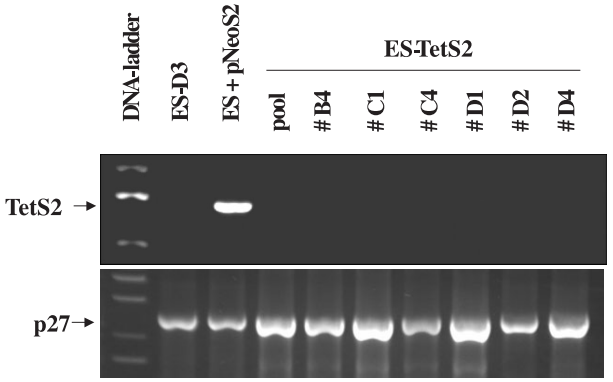

Figure 4. Detection of the rtTA2^S-S2 coding sequence in ES-TetS2 cells. Genomic DNA of ES-TetS2 cells was subjected to PCR to amplify a 738 bp fragment of the rtTA2^S-S2 coding sequence. The quality of DNA samples was confirmed by amplification of p27 genomic DNA sequence.

clones for the presence of rtTA2^S-S2 cDNA in genomic DNA to verify the integration of TetS2 into the ES cell genome. As a most suitable positive control we used ~100 molecules of pNeoS2 diluted in a sample of ES cell genomic DNA in a volume ratio of 1:10 to simulate sequence competition occuring in the PCR of genomic DNA. Genomic DNA from ES-D3 cells was used as a negative control. PCR amplification of a 738 bp fragment of the rtTA2^S-S2 coding sequence revealed that the intact sequence was not present in any of the tested clones (Fig. 4). The integrity of the DNA of all samples was confirmed by the amplification of p27 genomic DNA (Fig. 4).

3.4. rtTA2^S-S2 is toxic for mouse ES cells

The absence of clones containing rtTA2^S-S2 could stem from selection against them due to specific toxicity of the transactivator and the preferential survival of clones with the rtTA sequence disrupted

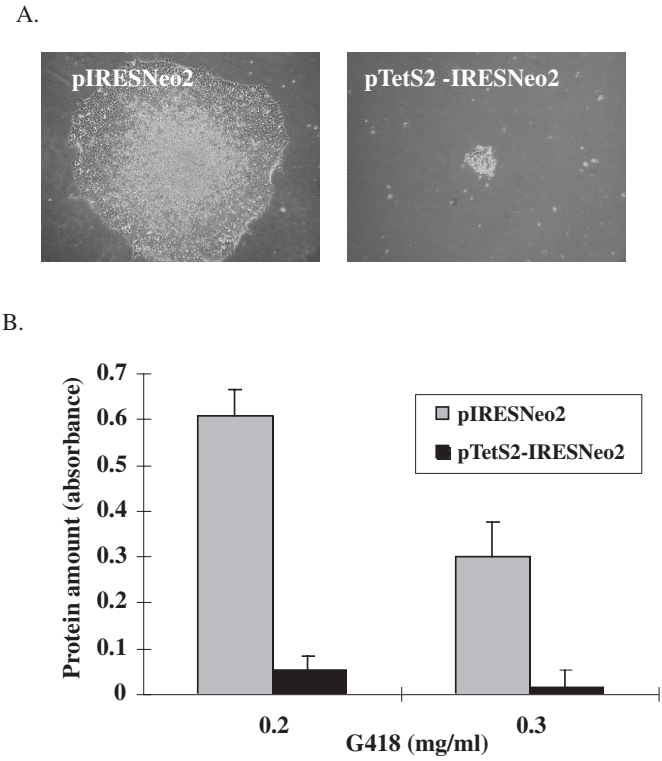

Figure 5. The toxic effect of rtTA2^S-S2 on mouse ES cells. (**A**) The morphology of G418-resistant colonies was determined by phase contrast microscopy. Typical colonies resulting from 12-day cultivation in the presence of 200 μg·mL^{-1} G418 are shown. (**B**) Protein content per dish after 12 days of cultivation was determined using amidoblack assay. The data represent the average ± standard deviation of two independent experiments.

during integration into the chromatin. In order to test the toxicity of rtTA2^S-S2 directly, we cloned the rtTA2^S-S2 coding sequence into the pIRESNeo2 vector (see Fig. 1) which permits the translation of two open reading frames from one messenger RNA in eukaryotic cells. When the resulting pTetS2-IRESNeo2 plasmid is transfected into eukaryotic cells, only cells which express rtTA2^S-S2 are able to grow in the presence of G418 because of coupling of the transgene to neomycin phosphotransferase expression. pTetS2-IRESNeo2 was found to produce functional rtTA2^S-S2 when transiently cotransfected with pUHC13-3 in QT6 cells in the presence or absence of Dox (data not shown).

We transfected ES-D3 cells growing in the absence of feeder fibroblasts with pIRESNeo2 or pTetS2-IRESNeo2 and cultured them for up to 12 days in the presence of G418 at 200 and 300 μg·mL^{-1}. As shown in Figure 5A, the cells transfected with pIRESNeo2 developed large colonies of thousands of cells by the 12th day, whereas cells transfected with pTetS2-IRESNeo2 formed colonies composed of only a few cells unable to grow exponentially and expand. To quantify the differences between cells expressing rtTA2^S-S2 and controls, we

measured the protein content on dishes with the amidoblack assay. We show that after 12 days of selection, the protein content is more than 10 times lower in dishes transfected with pTetS2-IRESNeo2 cultured in 200 $\mu g \cdot mL^{-1}$ of G418 and the difference is further increased in higher concentrations of G418 which corresponds to a higher level of expressed rtTA2^S-S2 (Fig. 5B). It should be noted that the amount of protein in dishes transfected with pTetS2-IRESNeo2 is probably overestimated, because the extracellular matrix and some dead cells still remained attached to the tissue culture plastic.

4. DISCUSSION

The inducible expression of transgenes is often required for the correct manipulation of ES cells in vitro and for the control of their transplanted derivatives in vivo. Great possibilities are offered by inducible tetracycline-regulatable expression systems. The TetOff system has already been succesfully used in ES cells to control the transcription of the Oct3/4 [10] and Bcr-abl [11] transgenes. However, in many applications, TetOn seems to be a better choice and that is why we tested the possibilities of the TetOn system based on the novel rtTA2^s-S2 transactivator [5] in ES cells.

First we show that rtTA2^s-S2 is suitable for the application in transient transfections of ES cells because no background activity of P_{tet} was observed in the absence of the tetracycline analogue Dox. We were not, however, successful in preparing clones of ES cells stably expressing functional rtTA2^s-S2. The main disadvantage of the TetOn system resulting from the instability of TetR mRNA [12] and rtTA protein in some types of mammalian cells was eliminated by base-pair substitutions in rtTA [13, 14] as well as in rtTA2^s-S2 [5]. It has been shown previously that in some cases, problems in producing rtTA expressing clones can be overcome when the rtTA coding sequence is driven by the EF-1α promoter instead of the CMV promoter [15]. It is, however, unlikely that the use of the CMV promoter in ES cells represents a major problem in establishing stable ES cell clones since ES-EGFP positive cells produced with high efficiency and expression of EGFP from the CMV promoter, were clearly detectable over more than 20 passages (end of experiment) and also in ES-EGFP differentiated progeny (not shown).

The establishment of an ES-TetOn system is hindered by the apparent toxicity of rtTA (see Fig. 5). The toxicity of tTA/rtTA was also observed in other cell types but never to the extent that we observed in ES cells. Gallila and Khalili [16] showed that tTA when overexpressed produces adverse effects including morphological cellular changes, a decrease in proliferation rate and alterations in cell cycle distribution. It has previously been postulated that the toxicity of tTA/rtTA stems from the presence of the viral VP16 transactivation domain [2, 17]. Consequently, Baron and colleagues [17] reduced the VP16 moiety of tTA to 12 amino acids to eliminate the potential targets for interaction with cellular transcription factors. Resulting tTA based on these minimal activation domains are tolerated by cells in higher concentrations and were used in our study. However, the toxicity of tTA or rtTA in ES cells can be observed regardless of the presence of a complete VP16 transactivation domain (H.Niwa, personal communication) or the presence of three minimal activation domains of VP16 only (O.Witte, personal communication and this study).

Our results indicate that ES cells, derived from 3.5-day-old blastocysts are very sensitive to the presence of rtTA2^S-S2. It is, however, also likely that this is the case in vivo, in rtTA transgenic animals, since the earliest expression of β-galactosidase controlled by Ptet was detected at about embryonal day 11 (E 11) [18, 19]. Thus, it is possible that both embryonic cells before E 11 as well as ES cells expressing rtTA have lower

viability due to rtTA toxicity. Such selective pressure can lead to the survival of animals, which express the transactivator after E 11. The transgene thus needs to be integrated into the chromatin region, which is silent during early development. This fact can be responsible for the necessity to screen many transgenic animals when looking for good Tet inducible lines [20].

The data presented here would provide a mechanism for any embryotoxicity of rtTA observed in vivo and further raise the need for the development of other strategies for stable and inducible expression of genes in ES cells.

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